Characterization of the Role of the FluG Protein in Asexual Development of *Aspergillus nidulans*

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ABSTRACT

We showed previously that a Δ*fluG* mutation results in a block in *Aspergillus nidulans* asexual sporulation and that overexpression of *fluG* activates sporulation in liquid-submerged culture, a condition that does not normally support sporulation of wild-type strains. Here we demonstrate that the entire N-terminal region of FluG (~400 amino acids) can be deleted without affecting sporulation, indicating that FluG activity resides in the C-terminal half of the protein, which bears significant similarity with GSI-type glutamine synthetases. While FluG has no apparent role in glutamine biosynthesis, we propose that it has an enzymatic role in sporulation factor production. We also describe the isolation of **d**ominant **s**uppressors of Δ*flu**G***(*dsg*) that should identify components acting downstream of FluG and thereby define the function of FluG in sporulation. The *dsgA1* mutation also suppresses the developmental defects resulting from Δ*flbA* and dominant activating *fadA* mutations, which both cause constitutive induction of the mycelial proliferation pathway. However, *dsgA1* does not suppress the negative influence of these mutations on production of the aflatoxin precursor, sterigmatocystin, indicating that *dsgA1* is specific for asexual development. Taken together, our studies define *dsgA* as a novel component of the asexual sporulation pathway.

FORMATION of organs in multicellular eukaryotes is a complex process involving a multitude of sensory pathways that function in perceiving signals from the extracellular environment. Cells are then directed to form higher order structures that perform specific functions. The genetically tractable filamentous fungus *Aspergillus nidulans* serves as a model organism for the study of complex multicellular development in fungi and other eukaryotes. The asexual phase in the life cycle of this fungus involves the formation of multicellular structures called conidiophores that produce chains of uninucleate spores called conidia (Timberlake 1990). Initiation of conidiation is a programmed event that is genetically determined and occurs at a precisely scheduled time in the *A. nidulans* life cycle (Axelrod 1972). The primary activator of conidiation-specific genes is BrlA, a C_2H_2 zinc-finger DNA-binding protein (Adams *et al.* 1988; Adams and Timberlake 1990; Chang and Timberlake 1992). The isolation of conidiation mutants has facilitated the identification of several genes required for *brlA* expression and the programmed induction of conidiophore development, namely *flbA*, *fluG*, *flbB*, *flbC*, *flbD*, and *flbE* (Lee and Adams 1994a; Wieser *et al.* 1994; Figure 4). These mutants have a predominantly fluffy phenotype with conidiation being impaired to varying extents.

The aconidial phenotype of *fluG* mutants can be partially rescued in at least two ways. First, *fluG* mutants can be induced to sporulate by growth on the surface of nutrient-limited media, suggesting that in the absence of *fluG* conidiation can occur in response to nutritional stress (Adams *et al.* 1992). Such stress-induced conidiation may also occur in wild-type strains but is masked by the overwhelming *fluG*-dependent response. Second, *fluG* mutants can also be induced to conidiate when grown in proximity to a wild-type strain or strains with mutations in different sporulation genes (Lee and Adams 1994a). This result has led to the proposal that *fluG* is required for the production of an extracellular factor(s) needed to initiate the developmental pathway leading to conidiation. The mechanism by which FluG activates extracellular factor production remains largely unknown. FluG shares significant sequence similarity with type I glutamine synthetases found in prokaryotes (Lee and Adams 1994a), although FluG-like genes have also recently been found in higher plants (Mathis *et al.* 1999, 2000).

Overproduction of FluG can drive conidiation in liquid-submerged culture, a condition that is normally repressive for wild-type conidiation (Lee and Adams 1995). This ability of FluG to initiate conidiation is dependent on the function of FlbA (Lee and Adams 1994b, 1995), an RGS (regulator of G-protein signaling)

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domain protein (De Vries *et al.* 1995; Druey *et al.* 1996; Koelle and Horvitz 1996; Yu *et al.* 1996). RGS proteins stimulate the intrinsic GTPase activity of specific heterotrimeric G-protein α-subunits, converting them to the inactive GDP-bound state (Berman *et al.* 1996; Watson *et al.* 1996), and have been 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.* 1996). FlbA is proposed to inactivate the α-subunit of a heterotrimeric G-protein that negatively regulates conidiation, encoded by the *fadA* (*f*luffy *a*utolytic *d*ominant) gene (Yu *et al.* 1996). Dominant activating mutations in *fadA* that are predicted to eliminate GTPase activity and lock the protein in a GTP-bound state, like *flbA* loss-of-function mutations, result in an aconidial phenotype that is characterized by proliferative mycelial growth followed by colony autolysis. A null mutation in *fadA* is able to suppress the need for *flbA* in sporulation but does not bypass the need for *fluG*, indicating that FluG has a specific function in development that is distinct from inhibition of the FadA signaling pathway and presumably involves stimulating products of the *flbB*, *flbC*, *flbD*, *flbE*, and *brlA* genes (Lee and Adams 1995).

Here we describe two approaches to more precisely define the role of FluG in asexual development. First, we have delimited the region of FluG that is required for its activity and show that the glutamine synthetase I (GSI)-like C-terminal half of FluG is sufficient to induce conidiation in this fungus. Second, we describe a genetic screen for **d**ominant **s**uppressors of a Δ*flu**G*** mutation (*dsg*) aimed at identifying components of the conidiation signaling pathway that act downstream of FluG. We reasoned that use of a diploid for the mutant screen would preclude isolation of recessive mutations that cause conidiation as a result of growth inhibition, thereby facilitating the isolation of dominant mutations that would specifically identify activators of the conidiation pathway. Indeed, our results indicate that the mutation *dsgA1* may represent a component that is specifically involved in *A. nidulans* conidiophore development.

MATERIALS AND METHODS

***A. nidulans* strains, growth conditions, and genetic analysis:** The strains used in this study are listed in Table 1. Standard *A. nidulans* genetic (Pontecorvo *et al.* 1953; Clutterbuck 1974) and transformation protocols (Yelton *et al.* 1984; Miller *et al.* 1985) were used. The *alcA(p)::fluG* transformant strains were generated by integration of plasmid sequences at the *trpC* locus as described previously (Lee and Adams 1995). Presence of constructs in these transformants was verified by Southern blot analysis and positive transformants were crossed with the Δ*fluG* strain RBN119 (Wieser and Adams 1995) to generate Δ*fluG*; *alcA(p)::fluG* strains.

Supplemented minimal media for growth of *A. nidulans* was prepared as described (Kafer 1977). Complete medium is minimal medium supplemented with yeast extract at 5.0 g/liter. Threonine shift time course experiments with *alcA(p):: fluG* strains were carried out as described (Lee and Adams 1995). The effect of different nitrogen sources on conidiation of *dsgA1* mutant strains was determined by inoculating 5×10^5 conidia/ml into 100 ml of minimal medium containing different sources of nitrogen equivalent to the amount present at 2.0 g/liter of yeast extract. The nitrogen sources tested were glutamine (6.3 g/liter), ammonium tartarate (8.0 g/liter), bactopeptone (Difco; 7.8 g/liter), casein hydrolysate [Sigma (St. Louis); 9.2 g/liter], L-arginine (4.5 g/liter), L-asparagine (6.5 g/liter), and L-glutamic acid (14.6 g/liter). Cultures were examined microscopically for conidiation following 16 hr of growth at 37°. The degree of conidiation observed in the Δ*fluG; dsgA1* strain varied, but was always either diminished or reduced relative to the *dsgA1* strain under the same conditions, indicating some level of inhibition. Sterigmatocystin (ST) production (shown in Table 3) and *stcU* mRNA levels (Figure 3) in strains grown in liquid complete medium was determined from 1- to 4-day-old stationary cultures as described earlier (Hicks *et al.* 1997).

The *dsgA1* mutant was isolated by 4-nitroquinoline-1-oxide (NQO) mutagenesis of the homozygous Δ*fluG* strain DCD1 using a protocol described earlier (Wieser *et al.* 1994). Spores were mutagenized to a survival rate of 42% and mutants were screened on complete medium with 0.004% Triton-X-100. The fluffy phenotype of a Δ*fluG* strain is not rescued on complete medium as on minimal medium, facilitating a visual screen for conidiating mutants. Strains Δ*fluG; dsgA1* (HMDCD1.4), *fluG*$^+$*; dsg*A1 (HDCD3.8), and Δ*flbA; dsg*A1 (HDCD4.26) were obtained by haploidization of the diploids MDCD1, DCD3, and DCD4, respectively, by treatment with the microtubule destabilizing agent benomyl (Hastie 1970). Presence of *fluG* or *flbA* alleles was checked by Southern analysis. The *fadA*^{Q204L}*; dsgA1* strain was constructed by transforming an *argB; dsgA1* strain (HDCD9.1) with plasmid pJYPK26 (Yu *et al.* 1999) bearing the *fadA*^{Q204L} allele and *argB* as a selectable marker. To assess growth rates of *dsgA1* mutants, the radii of three different colonies of each strain were determined from the center of the colony to two different margins ($n = 6$) over a period of 5 days of growth on minimal medium. We report the mean values for radial growth rates and standard deviation calculated at 95% confidence levels.

Mitotic mapping of *dsgA1* was carried out by constructing diploids between a Δ*fluG; dsgA1* mutant and various strains with scorable markers on different chromosomes as shown in Table 2. Cosegregation of the Δ*fluG* phenotype with the wild-type allele of a given mapping marker served as an indicator of the absence of *dsgA1* on the corresponding chromosome, thereby facilitating mapping of *dsgA1* by a process of elimination. Haploid segregants of diploid strains were isolated following treatment with benomyl and assortment of the *dsgA1* mutation with mapping markers was followed. Phenotypes scored include FlbA (fluffy autolytic), W (white spores), FluG (fluffy aconidial), Met (methionine requirement), Lys (Lysine requirement), S (inorganic sulfate requirement), His (histidine requirement) and Fwa (fawn spores). From data in Table 2, it can be seen that among haploid segregants of benomyl-treated diploids DCD13 and DCD14, *dsgA1* does not show free recombination with the *sB3* and *lysA1* markers on chromosome VI, indicating that *dsgA1* is located on chromosome VI.

Nucleic acid isolation and manipulation: Samples collected for RNA analysis were frozen in liquid nitrogen, lyophilized, pulverized, and extracted with TRIzol reagent as specified by the manufacturer (GIBCO BRL, Gaithersburg, MD). Fifteen micrograms of RNA/lane were separated by electrophoresis on formaldehyde-agarose gels, transferred directly onto nylon membrane (Hybond-N; Amersham, Arlington Heights, IL), and hybridized to ^{32}P-labeled random probes. A 2.5-kbp *Xho*I fragment from the plasmid pFM1 (Adams *et al.* 1992) was used as a *fluG*-specific probe.

TABLE 1

***A. nidulans* strains**

Strain	Genotype	Source
DCD1	+/*pabaA1*, +/*yA2*, *biA1*/+; Δ*fluG*/Δ*fluG*, *argB2*/+; *methG1*/+	This study
DCD2	+/*pabaA1*, +/*yA2*, *biA1*/+; Δ*fluG*/Δ*fluG*, *argB2*/+; *methG1*/+; +/*dsgA1*	This study
DCD3	+/*pabaA1*, +/*yA2*, *biA1*/+; +/Δ*fluG*; *pyroA4*/+; +/*dsgA1*	This study
DCD4	+/*pabaA1*, +/*yA2*, *biA1*/+, Δ*flbA*/+; +/Δ*fluG*, *argB2*/+; *methG1*/+; +/*dsgA1*	This study
DCD5	+/*pabaA1*, +/*yA2*, *biA1*/+; +/Δ*fluG*, *argB2*/+; *methG1*/+; +/*dsgA1*	This study
DCD9	+/*pabaA1*, +/*yA2*, *biA1*/+; +/Δ*fluG*, *argB2*/+; *methG1*/+; +/*dsgA1*	This study
DCD10	*pabaA1*/*pabaA1*, *yA2*/*yA2*; *wA3*/+; +/Δ*fluG*; +/*methG1*; +/*dsgA1*; *trpC801*/+	This study
DCD12	+/*pabaA1*, +/*yA2*; +/Δ*fluG*; *lysB5*/+; +/*dsgA1*; *sD85*/+, *facC102*/+, *fwa2*/+	This study
DCD13	+/*pabaA1*, +/*yA2*, *biA1*/+, *lysF51*/+; +/Δ*fluG*; +/*dsgA1*, *sB3*/+	This study
DCD14	+/*pabaA1*, *yA2*/*yA2*; +/*wA3*; +/Δ*fluG*; +/*dsgA1*, *lacA1*/+, *bwA1*/+, *lysA1*/+, *sB3*/+, *sbA3*/+	This study
DCD15	+/*pabaA1*, +/*yA2*, *biA1*/+; +/Δ*fluG*; +/*dsgA1*, *sB3*/+; *hisJ122*/+	This study
FGSC33	*biA1*; *pyroA4*	FGSC[a]
FGSC237	*pabaA1*, *yA2*; *trpC801*	FGSC
FGSC297	*biA1*, *lysF51*; *sB3*	FGSC
FGSC299	*biA1*; *sB3*; *hisJ122*	FGSC
FGSC484	*lysB5*; *facC102*, *fwa2*, *sD85*	FGSC
FGSC795	*yA2*; *lacA1*, *bwA1*, *lysA1(AbVI)*, *sB3*, *sbA3*	FGSC
HMDCD1.4	*pabaA1*, *yA2*; Δ*fluG*; *dsgA1*	This study
HDCD2.1	*pabaA1*, *yA2*; Δ*fluG*; *methG1*; *dsgA1*	This study
HDCD3.8	*pabaA1*; *yA2*, *dsgA1*	This study
HDCD4.26	*biA1*, Δ*flbA::argB*; *methG1*; *dsgA1*	This study
HDCD9.1	*biA1*; *argB2*; *dsgA1*	This study
HDCD10.2	*pabaA1*, *yA2*; *wA3*; Δ*fluG*; *dsgA1*	This study
MDCD1	+/*pabaA1*, +/*yA2*, *biA1*/+; Δ*fluG*/Δ*fluG*, *argB2*/+; *methG1*/+; *dsgA1*/+	This study
PW1	*biA1*; *argB2*; *methG1*	P. Weglenski
RBN119	*biA1*; Δ*fluG*, *argB2*; *methG1*	Wieser and Adams (1995)
RCD45	*biA1*; Δ*fluG*; *trpC::alcA(p)::fluG(1–865)*	This study
RCD47.18	*pabaA1*, *biA1*; Δ*fluG*; *trpC::alcA(p)::fluG(387–865)*	This study
RM16	*pabaA1*, *yA2*; *wA3*; *trpC801*	J. Marhoul (unpublished data)
TBN39.5	*biA1*, Δ*flbA::argB2; methG1*	Lee and Adams (1994a)
TBN57.8	*pabaA1*, *yA2*; *trpC::alcA(p)::fluG(1–865)*	Lee and Adams (1995b)
TBN68.22	*pabaA1*, *yA2*; *trpC::alcA(p)::fluG(387–865)*	This study
TCD13	*biA1*; *methG1*; Δ*fluG*, *argB::fluG(p)::fluG(387–865)*	This study
TCD18	*biA1*; *methG1*; Δ*fluG*, *argB::fluG(p)::fluG(1–865)*	This study
TTA11	*pabaA1*, *yA2*	Han *et al.* (1993)
TTA127.4	*pabaA1*, *yA2*; Δ*fluG*	Lee and Adams (1994a)
TJYPK26.15	*biA1*; *argB2::*pJYPK26; *dsgA1*	This study

All strains are *veA1*.

[a] Fungal Genetic Stocks Center.

TABLE 2

Linkage analysis of *dsgA1*

Linkage group	Diploid used	Phenotype scored	No. of haploid segregants	
			dsgA$^+$	*dsgA1*
I	DCD4	FlbA$^+$	6	10
		FlbA$^-$	3	2
II	DCD10	W$^+$	28	73
		W$^-$	ND[d]	ND[d]
III	DCD3	FluG$^+$	14	2
		FluG$^-$[a]	ND[d]	ND[d]
IV	DCD4	Met$^+$	7	8
		Met$^-$	19	13
V	DCD12	Lys$^+$	47	64
		Lys$^-$	16	11
VI	DCD13	S$^+$	2	77
		S$^-$[b]	181	1
	DCD14	Lys$^+$[c]	0	75
		Lys$^-$	60	2
VII	DCD15	His$^+$	115	21
		His$^-$	55	20
VIII	DCD12	Fwa$^+$	83	37
		Fwa$^-$	ND	ND

[a] For chromosome III Δ*fluG*-like segregants were observed but were not scored.

[b] Since the *sB3* mutant shows leaky growth on inorganic sulphate media, presence of *sB3* was also checked by resistance to 5 mM K_2CrO_4 (Kafer 1977).

[c] Linkage to chromosome VI was confirmed by assessing assortment with *lysA1*.

[d] Not determined.

Oligonucleotides used in this study were engineered to include restriction enzyme sites (underlined) and are listed below:

G1: 5′-CAG AAT GGG GAT CCT ACC ATT GA-3′
BN9: 5′-AGG AGA AAG CTT AGA CTC-3′
BN16: 5′-GAG AGA GTG GGG ATC CCG ATG AAC CAG-3′
BN17: 5′-GTC GAG CTC GAC GCT G-3′
OL1: 5′-CCA GAC GGA TCC CCG TAT CTC GTC-3′
OL2: 5′-CAA TAC CTC TCG AGA AGC CAC TTC CTG-3′.

To overproduce the C-terminal GSI FluG domain a 1.5-kbp *fluG*(387–865) fragment, synthesized by PCR using oligonucleotides G1 and BN9, was inserted into a *Bam*HI-*Hin*dIII-digested pBN55 vector (Lee and Adams 1995). This resulted in placement of *fluG*(387–865) under *alcA* promoter control in the plasmids pBN67 and pBN68. Since the *alcA::fluG*(387–865) construct complemented Δ*fluG* and resulted in the overproduction of FluG protein of the expected size (detected by Western blot) we did not sequence the PCR-generated *fluG*(387–865) allele.

To construct the vector for production and purification of FluG(387–865) from *Escherichia coli*, a 1.4-kbp region corresponding to the FluG-coding sequence was PCR amplified using a cDNA clone as a template and oligonucleotides OL1 and OL2 as PCR primers. The resulting product was digested with *Bam*HI and *Xho*I and inserted into the plasmid pET-27b(+) (Novagen) to create pCD2. To delete the N-terminal region of *fluG* and place the C-terminal region under the control of its own promoter, plasmid pCD9 was constructed as follows: A 1.1-kbp *Sac*I-*Bam*HI fragment, bearing sequence upstream of the FluG open reading frame, was PCR amplified using oligonucleotides BN16 and BN17. This fragment was inserted into pBN67 (see above) upstream of 1.5-kbp of the C-terminal *fluG* region, to produce pCD9. Plasmid pCD18 bearing the full-length *fluG* gene under the control of its own promoter was constructed by first inserting the 2.6-kbp *Sac*I-*Hin*dIII fragment of pCD9 into pET-27b(+) (Novagen) from which a similar sized *Xba*I-*Xho*I fragment was inserted into pPK1 (provided by Pat Kennedy and Dr. Lawrence Yager, Temple University, Philadelphia), a vector bearing the *argB* gene. The resulting construct was called pCD13. An internal 1.0-kbp *Pst*I fragment of pCD13 encompassing the N-terminal deletion was replaced by a 2.3-kbp *Pst*I fragment from the genomic *fluG* clone pFM1 (Adams *et al.* 1992) to produce pCD18.

Microscopy and thin layer chromatography (TLC) analysis: Photomicrographs of hyphal development were taken using an Olympus BH2 compound microscope and differential interference contrast optics. ST was extracted from stationary cultures and subjected to TLC chromatography and detection as described earlier (Hicks *et al.* 1997).

RESULTS

The N terminus of FluG is dispensable for conidiophore development: Our earlier work demonstrated that *fluG* was required for wild-type sporulation and that *fluG* overexpression could cause conidiation in submerged culture, a condition that normally represses development. Several *fluG* mutations that produce a fluffy aconidial phenotype were found to reside in the C-terminal GSI-like domain (amino acids 387–865) of FluG (Lee and Adams 1994a) and therefore we tested the hypothesis that this region would be sufficient to direct development. Strain RCD47.18 was constructed lacking the wild-type *fluG* gene but bearing a portion of the *fluG* gene encoding the C-terminal GSI domain under the control of the inducible *alcA* promoter. The aim was to test whether FluG(387–865) could function like full-length FluG in causing conidiophore development when overproduced in submerged vegetative hyphae. Overexpression of *fluG*(387–865) caused submerged conidiation similar to that observed following overexpression of the full-length *fluG* gene (Figure 1A). Reduced conidiophores with spores at the ends of hyphal tips were seen as early as 9 hr postinduction (not shown), and fully developed conidiophores bearing vesicles and sterigmata were present by 24 hr after *alcA* induction. Western blot analysis using anti-FluG(387–865) antibody verified that a 53-kD C-terminal polypeptide was indeed overproduced in the Δ*fluG; alcA::fluG* (387–865) strain.

Gene overexpression can produce gain-of-function mutations that provide insight into function. However, it is important to recognize that overexpression mutations may result in phenotypes that have little to do with protein function under normal conditions. To address this issue we tested whether the C-terminal half of FluG was also sufficient to cause conidiation when expressed from the native *fluG* promoter under growth conditions

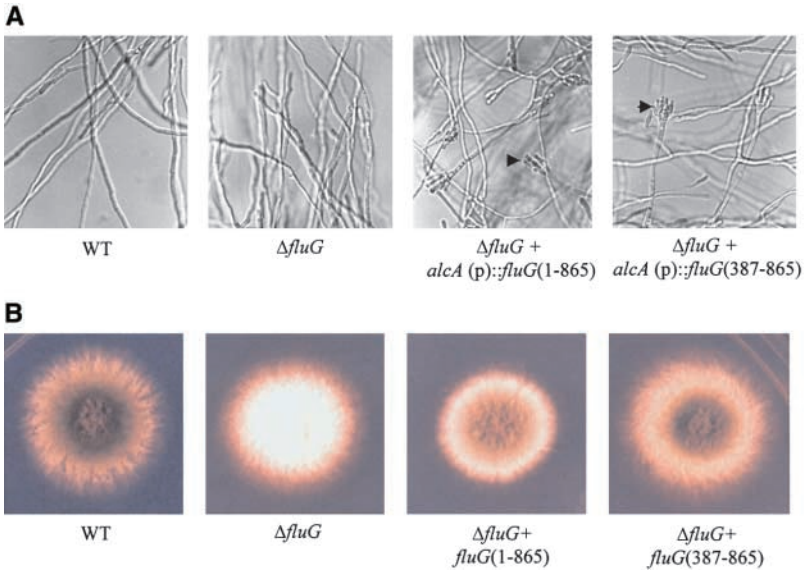

FIGURE 1.—(A) Development in Δ*fluG* strains bearing *alcA*(p)::*fluG* fusions. Wild-type (TTA11), Δ*fluG* (TTA127.4), and Δ*fluG* strains bearing *alcA*(p) expression constructs with different portions of the *fluG* gene (indicated in parentheses) were grown in *alcA*(p)-repressing liquid minimal medium (glucose) for 14 hr at 37° and then shifted to *alcA* (p)-inducing liquid minimal medium (threonine). Micrographs were taken 24 hr after *alcA* (p) induction. Conidiophore development (indicated by arrowheads) is observed only in strains overexpressing the entire *fluG*(1–865; RCD45) and the C-terminal *fluG*(387–865; RCD47.18) indicating that FluG(387–865) is necessary and sufficient for FluG function. The wild-type strain and Δ*fluG* strains do not sporulate under these conditions. (B) Complementation of the Δ*fluG* phenotype in strains bearing plasmids with different portions of *fluG* under the control of its own promoter introduced at the *argB* locus. Sporulation (seen as green color of spores in the center of the colony) is observed in Δ*fluG* + *fluG*(387–865) strain (TCD13) on solid minimal medium with supplements. Also shown are the Δ*fluG* and Δ*fluG* + *fluG*(1–865; TCD18) strains. Complementation of Δ*fluG* by *fluG*(387–865) further confirms the sufficiency of *fluG*(387–865) for FluG function.

that support wild-type conidiation. To this end, we constructed the strain Δ*fluG* + *fluG*(387–865) bearing a copy of the *fluG* gene from which codons 1–386 were specifically deleted but containing its own upstream regulatory sequences, integrated at the *argB* locus. As shown in Figure 1B, the Δ*fluG* + *fluG*(387–865) strain conidiated similarly as a Δ*fluG* strain complemented with a wild-type *fluG*(1–865) allele, indicating that the N-terminal region of FluG is not required for normal conidiophore development.

***dsgA1* dominantly suppresses the conidiation defect of a Δ*fluG* mutant:** To better understand how FluG functions in activating conidiation, we isolated Δ*fluG* suppressor mutations to identify genes that act downstream of *fluG*. Given that Δ*fluG* mutations could be partially suppressed by nutritionally limiting growth conditions, we expected that many types of recessive, growth-retarding mutations could result in suppression due to complex nutritional effects. To avoid this, we set out to isolate dominant Δ*fluG* suppressor mutants that would identify activators of the FluG signaling pathway. Colonies of NQO mutagenized, diploid homozygous Δ*fluG* mutant cells (DCD1) were screened for the ability of cells to conidiate on complete medium. One strong suppressor mutant (MDCD1) and 67 relatively weaker suppressors were found among 116,000 surviving colonies. The weak suppressors were set aside for later study while the strong suppressor mutation was designated as *dsgA1* and characterization of this locus is described below.

Treatment of the diploid *dsgA1* mutant with the microtubule destabilizing agent benomyl (HASTIE 1970) resulted in formation of both fluffy and conidial haploid segregants, as expected if the suppressor was dominant. To further test dominance we constructed a diploid between a conidial haploid Δ*fluG; dsgA1* segregant (HMDCD1.4; Figure 2A, left) and a Δ*fluG* mutant strain. The resulting diploid strain was conidial, thereby confirming dominance of *dsgA1* (not shown). All strains were examined by Southern blot analysis to confirm the absence of a wild-type *fluG* gene (data not shown). *dsgA1* was mapped to chromosome VI as described in MATERIALS AND METHODS.

Close examination of the Δ*fluG; dsgA1* strains indicated numerous differences from the *fluG*$^+$ wild-type strain. The Δ*fluG; dsgA1* strains displayed a hyperconidial phenotype in which conidiophores not only arose from specialized aerial mycelium but also formed on the basal mycelium on the surface of the agar (not shown). Through observation of the *dsgA1* mutant and wild-type colonies originating from single spores and growing on an agar surface, we found that *dsgA1* mutants produced recognizable conidiophores within 12 hr of placing a spore on media while wild-type strains required 22 hr to reach a similar developmental point. This rapid conidiophore development by *dsgA1* mutants was not limited to colonies growing on agar surfaces but also occurred with similar timing when mutants were grown in liquid shake culture (Figure 2B).

Additionally, we found that Δ*fluG; dsgA1* mutants had other defects relative to a *fluG*$^+$ strain. The Δ*fluG; dsgA1* mutant (HMDCD1.4) exhibited reduced radial growth rate (4.41 $\pm$ 0.33 mM/day) in comparison to the *fluG*$^+$ strain (TTA11; 6.64 $\pm$ 0.196 mM/day) and the Δ*fluG* strain (TTA127.4; 6.35 $\pm$ 0.34 mM/day). The *dsgA1* strains also displayed a dominant defect in sexual development. Although Δ*fluG; dsgA1* mutants readily formed heterokaryons with developmentally wild-type strains,

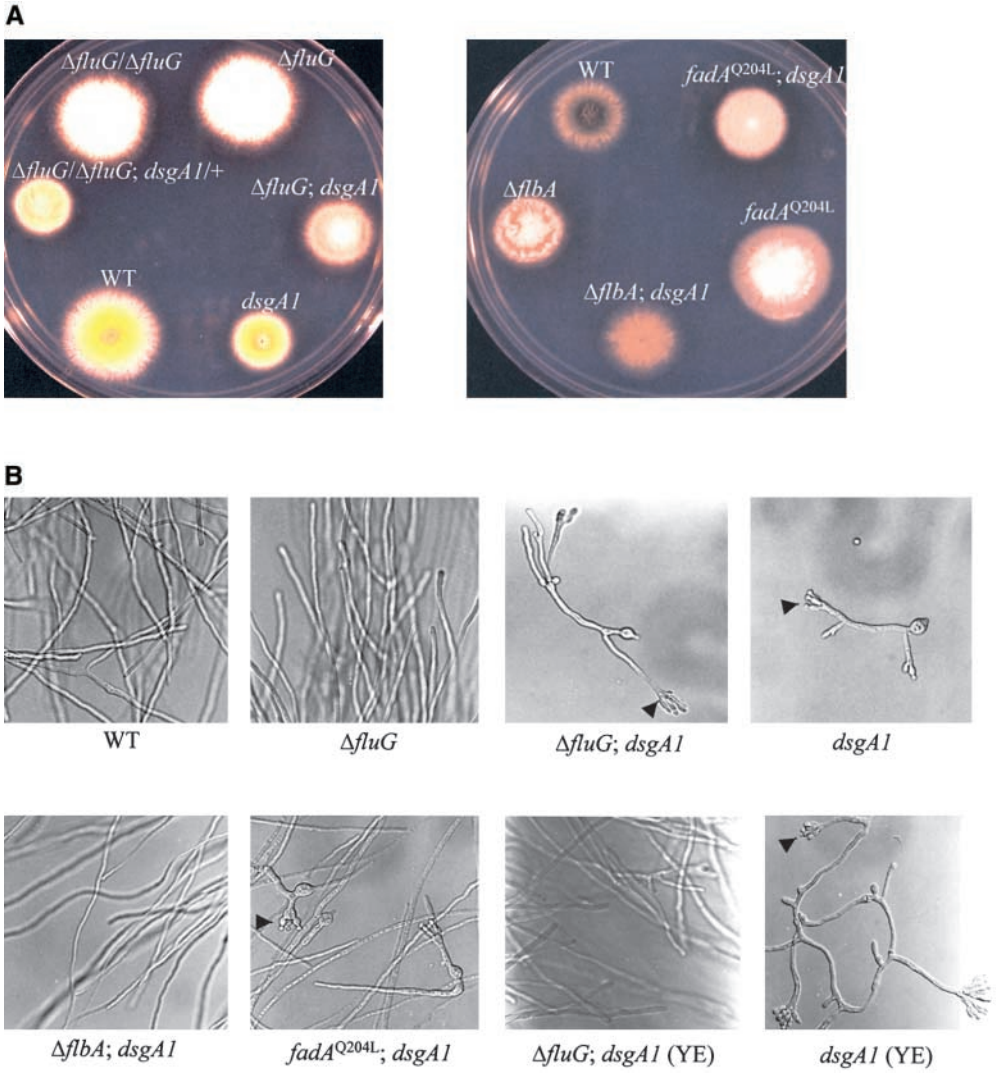

FIGURE 2.—(A) Morphology of *dsgA1* mutant strains and ability of the *dsgA1* mutation to suppress conidiation defects of Δ*fluG*, Δ*flbA*, and *fadA*^{Q204L} mutations. Strains grown on supplemented minimal agar medium for 2 days at 37°. On the left is a plate indicating suppression of Δ*fluG* by the *dsgA1* mutation as seen from conidiation of the strains shown (indicated by the yellow or green color of spores in the colony). The Δ*fluG*/Δ*fluG; dsgA1*/+ isolated by NQO mutagenesis of the corresponding homozygous Δ*fluG* strain (fluffy aconidial) can be seen to sporulate under these conditions, indicating dominant suppression of the Δ*fluG* mutation. The plate on the right demonstrates the ability of *dsgA1* to suppress the Δ*flbA* and *fadA*^{Q204L} fluffy autolysis phenotypes. (B) Conidiophore formation in *dsgA1* mutant strains grown in liquid shake culture. A total of 5×10^5 conidia/ml from wild type (TTA11), Δ*fluG* (TTA127.4), Δ*fluG; dsgA1* (HMDCD1.4), *dsgA1* (HDCD3.8), Δ*flbA; dsgA1* (HMDCD4.26), and *fadA*^{Q204L}*; dsgA1* (TJYPK26.15) were inoculated into 100 ml of supplemented liquid minimal medium (KAFER 1977) incubated at 37° in a shaker set at 300 rpm. Strains Δ*fluG; dsgA1* and *dsgA1* were also grown in liquid minimal medium supplemented with 2.0 g/liter of yeast extract. Following 16 hr of growth micrographs were taken of hyphae to observe formation of conidiophores (indicated by arrowheads).

these heterokaryons did not produce cleistothecia under growth conditions that support wild-type sexual reproduction. This reproductive defect is conditional, however, in that *dsgA1* strains can cross with wild-type strains when grown as heterokaryons on minimal plates with 20 mM glycine as the sole source of nitrogen and 2% glucose as the carbon source.

***dsgA1* causes hyperconidiation independently of *fluG*:** To examine interactions between the *dsgA1* mutation and *fluG*, a *fluG*$^+$*; dsgA1* mutant strain was constructed (HDCD3.8; Figure 2A, left). The *fluG*$^+$*; dsgA1* strain also displayed reduced radial growth rates (3.95 ± 0.09 mM/day) relative to the *fluG*$^+$ strain (TTA11; 6.64 ± 0.196 mM/day) and had a hyperconidial phenotype that was even more pronounced than that seen for Δ*fluG; dsgA1* mutants. We concluded that under these conditions the *dsgA1* phenotype prevails independently of *fluG* function, supporting the hypothesis that *dsgA1* is a gain-of-function mutation in a gene that has a positive influence on conidiation.

Observation of submerged conidiation by *dsgA1* mutants allowed us to detect an interesting difference between Δ*fluG; dsgA1* mutants and *fluG*$^+$*; dsgA1* strains. While both strains sporulated between 9 and 11 hr after inoculation in defined minimal medium (Figure 2B), only the *fluG*$^+$*; dsgA1* strain sporulated during growth in minimal medium supplemented with yeast extract (Figure 2B), bactopeptone, or other high quality nitrogen sources such as glutamine or ammonium (not shown). This inhibitory effect was not observed when strains were grown exposed to air.

***dsgA1* suppresses Δ*flbA* and *fadA* activating mutations:** One role we have proposed for FluG is to inactivate FadA, a Gα-subunit of a heterotrimeric G-protein that stimulates vegetative growth (YU *et al.* 1996) by inducing the RGS activity of FlbA. Both loss-of-function *flbA* mutations and dominant activating *fadA* mutations cause a fluffy autolytic phenotype due to constitutive and uncoordinated induction of hyphal growth (LEE and ADAMS 1994b; YU *et al.* 1996). We wanted to determine if *dsgA1*

bypasses the need to inactivate FadA-mediated signaling (*i.e.*, Do *dsgA1* mutants activate sporulation even when FadA is activated constitutively?). To address this question, Δ*flbA; dsgA1* and *fadA*^{Q204L}*; dsgA1* double mutant strains were constructed. Both Δ*flbA; dsgA1* (HDCD4.26) and *fadA*^{Q204L}*; dsgA1* (TJYPK26.15) were now able to conidiate, indicating that the *dsgA1* mutation is able to overcome the requirement for inactivation of FadA in stimulating conidiophore development (Figure 2A, right). However, in liquid minimal medium conidiation was observed in the *fadA*^{Q204L}*; dsgA1* strain but not in the Δ*flbA; dsgA1* strain (Figure 2B), possibly because FlbA has a FadA-independent activity in stimulating conidiation under these conditions (Yu *et al.* 1996; Hicks *et al.* 1997).

Δ*fluG* and Δ*flbA* sterigmatocystin biosynthesis defects are not suppressed by *dsgA1*: Several Aspergillus species produce the carcinogenic polyketides, aflatoxin and ST, that are detrimental to both health and the economy. The genes required for synthesis of the aflatoxin precursor ST in *A. nidulans* have been found to reside within a 60-kbp gene cluster (*stc*; Brown *et al.* 1996). Recent data have revealed that *fluG* and *flbA* genes are required for ST production, and dominant activating mutations in *fadA* disrupt ST biosynthesis (Hicks *et al.* 1997). It has been hypothesized that ST production is dependent on the inactivation of FadA by FlbA, which in turn is stimulated by FluG activity. Given the ability of *dsgA1* to suppress the aconidial phenotypes conferred by Δ*fluG*, Δ*flbA*, and dominant activating *fadA* mutations, we were interested in determining the effect of *dsgA1* on ST production in these mutant backgrounds. As indicated in Table 3, *dsgA1* strains produce ST in a *fluG*- and *flbA*-dependent manner. Thus, while the *dsgA1* mutation suppresses defects in conidiation of the *fluG* and *flbA* null mutants, the *dsgA1* mutation does not suppress their requirements for ST production. Additionally, it does not alleviate the inhibition imposed by *fadA* dominant activating mutations on the ST production pathway. Examination of the expression of *stcU* (one of the genes in the *stc* cluster) by Northern analysis (Figure 3) further indicated that the *dsgA1* mutation does not bypass the need for *fluG* in *stc* gene expression. These results support the conclusion that *dsgA1* is exclusively involved in the asexual conidiation pathway.

TABLE 3

Effect of *dsgA1* mutation on ST production and conidiation in *A. nidulans*

Strain	ST production	Conidiation	
		Aerial	Submerged
Wild type (FGSC26)	+	−	−
Δ*fluG* (TTA127.4)	−	−	−
Δ*fluG*/Δ*fluG* (DCD1)	−	−	−
Δ*fluG*/Δ*fluG*; *dsgA1*/+ (MDCD1)	−	+	+
Δ*fluG*; *dsgA1* (HMDCD1.4)	−	+	+
dsgA1(HDCD3.8)	+	+	+
Δ*flbA*; *dsgA1* (HDCD4.49)	−	+	−
fadA^{Q204L}; *dsgA1* (TJYPK26.15)	−	+	+

DISCUSSION

The *A. nidulans fluG* gene plays a pivotal role in the switch from vegetative growth to initiation of conidiation and is hypothesized to cause this by inducing the production of an extracellular factor(s) (Adams *et al.* 1992; Wieser *et al.* 1994). While FluG is a bipartite protein, conidiation-inducing activity is confined to the GSI-like C-terminal half of the protein. We have shown that overexpression of the C-terminal half of FluG is sufficient to cause development in submerged culture. Moreover, expression of a C-terminal derivative *fluG*(387–865) under the control of its own promoter was sufficient to complement a Δ*fluG* mutant. These results demonstrate that the C-terminal region of FluG has a distinct function in development even at wild-type levels of expression.

Although the critical part of FluG is related to glutamine synthetase I, FluG appears to have no role in glutamine biosynthesis because *fluG* mutants are not glutamine auxotrophs (Lee and Adams 1994a) and an endogenous glutamine synthetase exists in *A. nidulans* (MacDonald 1982; Cornwell and MacDonald 1984). We propose that the GSI region of FluG has a unique role in extracellular factor biosynthesis, perhaps catalyzing an enzymatic reaction very similar to that carried out by glutamine synthetases (GS). Alternatively, FluG may promote conidiation by degrading a conidiation inhibitory factor. In support of this hypothesis, recent BLAST search (Altschul *et al.* 1990) results revealed significant matches of the C-terminal FluG region to a *Pseudomonas putida* amino-group transfer protein TdnQ (*e* value = $1e^{-12}$; Fukumori and Saint 1997) and a GS-like component of the aniline dioxygenase complex from *Acinetobacter* sp (*e* value = $3e^{-12}$; Fujii *et al.* 1997; Takeo *et al.* 1998), both of which are involved in degradation of aromatic amines like aniline to catechol. From these comparisons, it also seems possible that FluG may catalyze a reaction(s) similar to an amino-group transfer.

Although the N terminus of FluG is not required for development, two protein sequences were found in the database that have significant similarity to this region of FluG. These include nodulin 6 from the plant *Medicago truncatula* (28% identity, 49% similarity), associated with the infection of its roots with *Sinorhizobium meliloti* (Mathis *et al.* 1999), and a nodulin-like protein of unknown function from *Arabidopsis thaliana* (29% identity, 47% similarity). It is noteworthy that similarity with the Arabidopsis protein extends to the full-length FluG protein, suggesting the presence of a FluG homolog in plants.

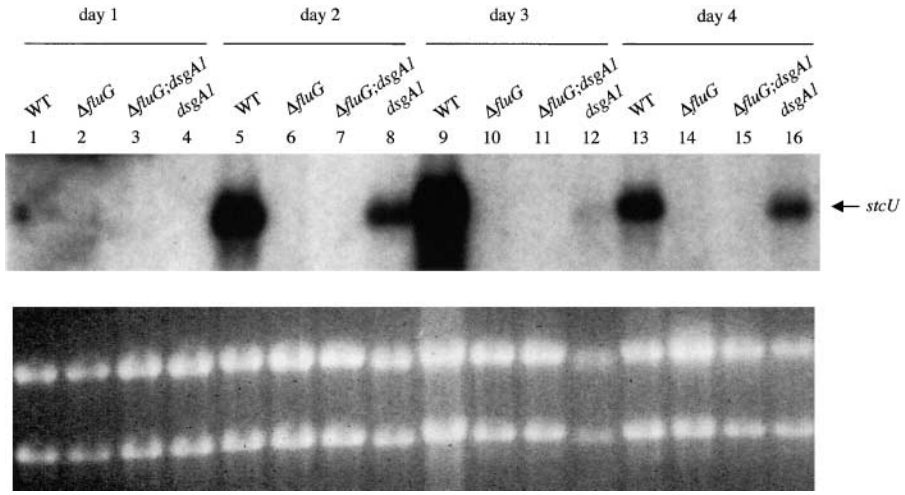

FIGURE 3.—*dsgA1* does not bypass the requirement of *fluG* in *stcU* gene expression. Total RNA was isolated from 1- to 4-day-old liquid stationary cultures of WT (TTA11), Δ*fluG* (TTA127.4), Δ*fluG*; *dsgA1* (HMDCD1.4), and *dsgA1* (HDCD3.8). Equal loading of the RNA was determined by ethidium bromide staining of the gel (bottom). The RNA was hybridized to a *stcU*-specific radiolabeled probe that detected a 0.9-kbp transcript. No signal was observed for the Δ*fluG* and Δ*fluG*; *dsgA1* strains, indicating that *dsgA1* does not bypass the requirement for *fluG* in *stcU* gene expression.

The significance of these similarities is not readily apparent but suggests that the N terminus of FluG may have some unknown function that is not essential for asexual development. Identification of the function of this *A. thaliana* protein might provide some useful insights into the function of FluG.

Regardless of the specific mechanism of FluG action, its function is required for both initiating conidial development and inhibiting mycelial growth. Initiation of asexual development in *A. nidulans* is dependent on the balance of two interacting pathways that respectively signal vegetative growth and conidiation (Figure 4). There is an increasing body of evidence to indicate that cross-talk between these pathways shifts the balance in favor of either growth or sporulation (ADAMS and TIMBERLAKE 1990; ADAMS *et al.* 1992; LEE and ADAMS 1994a, 1995; WIESER and ADAMS 1995). Besides *fluG*, the asexual development pathway is also regulated by the early conidiation genes *flbA*, *flbB*, *flbC*, *flbD*, and *flbE* that are all required for the expression of *brlA*, encoding the primary activator of conidiation-specific genes. On the other hand, the vegetative growth pathway is in part regulated by the heterotrimeric G-protein α-subunit FadA along with the Gβγ- subunit (ROSEN *et al.* 1999). FluG is hypothesized to inhibit vegetative growth by signaling activation of FlbA, which in turn interferes with the FadA-mediated growth-signaling pathway to promote conidiation (YU *et al.* 1996). Dominant or recessive loss-of-function *fadA* mutations suppress the requirement of *flbA* but not *fluG* in conidiation, indicating that FluG has a FadA-independent, development-specific role in addition to its proposed role in downregulation of growth. Initiation of development may therefore depend on both functions of FluG.

To gain further insight into the role of FluG in development, we also performed a genetic screen for dominant suppressors of *fluG* with the aim of isolating development-specific mutations that would help identify components acting downstream of FluG in the asexual development pathway. We identified *dsgA1* as a dominant suppressor of the *fluG* fluffy aconidial phenotype. Observations of *dsgA1* strains grown in submerged cultures indicate that it not only bypasses the need for FluG but also the requirement for air, the only known extrinsic factor that induces the genetically programmed, FluG-dependent pathway. A plausible mechanism of *fluG* suppression by *dsgA1* would be the constitutive activation of a conidiation pathway component acting downstream of FluG, possibly a receptor of the extracellular factor or a signal transduction component such as a conidiation pathway specific G-protein. Alternately, the *dsgA1* mutation could identify a component of a third, as yet uncharacterized, pathway that activates sporulation in a FluG-independent manner.

In addition to suppressing the *fluG* deletion mutation, we were surprised to learn that the *dsgA1* mutation was able to suppress the loss-of-conidiation phenotypes observed in dominant activating *fadA* and *flbA* deletion mutant strains. This result raises the possibility that DsgA functions as part of the FadA growth-signaling pathway rather than having a direct role in sporulation. However, other mutations that are known to inactivate FadA signaling, such as Δ*fadA* (YU *et al.* 1996) and Δ*sfaD* (ROSEN *et al.* 1999) that otherwise suppress Δ*flbA* mutants, are unable to suppress Δ*fluG* mutants. Therefore, we propose that DsgA functions directly in activating sporulation and that the *dsgA1* mutation stimulates such a strong activation of the sporulation pathway that conidiation takes place while FadA-mediated growth signaling remains active. In keeping with this hypothesis, we found that *dsgA1*-directed sporulation is enhanced by the presence of the wild-type *fluG* gene, possibly reflecting a DsgA-independent role for FluG in promoting sporulation by inhibiting FadA-mediated growth signaling. This requirement for FluG may also be reflected in the observation that conidiation of a *dsgA1* mutant in liquid complete medium and in media containing rich nitrogen sources is *fluG* dependent.

In addition to the mycelial proliferation pathway, the FadA Gα-protein also regulates biosynthesis of ST, the

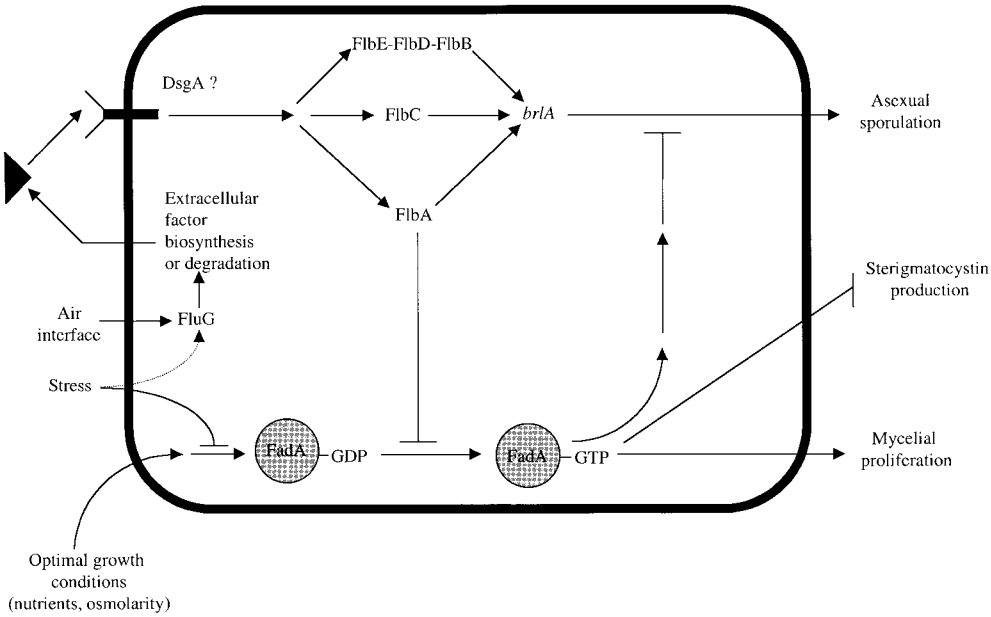

FIGURE 4.—Model for the role of DsgA in the asexual sporulation pathway. On solid medium, irrespective of nutrient status or osmolarity, the presence of air induces the FluG-dependent asexual sporulation pathway, which is in competition with the vegetative growth pathway promoted by FadA. FadA also negatively regulates the ST biosynthesis pathway. FluG stimulates FlbA to inactivate FadA but also has a specific role in the asexual sporulation pathway, acting either directly or via FlbA. A role for DsgA as a receptor of the FluG-generated signal or an early component of the signaling pathway leading to activation of *brlA* is proposed. In the absence of FluG, sporulation then becomes dependent on nutrient status. In liquid media with optimal growth conditions, the growth pathway prevails as the FluG signal cannot compete with the FadA signal. Under stressful conditions (nutrient limitation, osmolarity), FadA signaling is repressed as the FluG pathway is stimulated.

penultimate precursor of the fungal metabolite aflatoxin, in that FadA inhibits *stc* gene expression and ST production (YU *et al.* 1996; HICKS *et al.* 1997; ADAMS *et al.* 1998). The early developmental regulators FluG and FlbA are also required for ST production by negatively influencing FadA activity in a similar way as in asexual sporulation. FluG is believed to promote the production of this secondary metabolite via inactivation of FadA signaling. Although the *dsgA1* mutation bypasses the need for *fluG* and *flbA* in conidiation, and circumvents the inhibition of the dominant activating $fadA^{Q204L}$ allele on this process, *dsgA1* fails to restore ST biosynthesis in these mutant strains. Thus, the *dsgA1* mutation specifically influences asexual development, and as indicated in the model shown in Figure 4, characterization of *dsgA1* mutants has further clarified the function of interacting signaling pathways in regulating conidiation under different environmental conditions in *A. nidulans* and the influence of FluG on this regulation. How DsgA fits into this model awaits the isolation and characterization of the *dsgA* gene.

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