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Overexpression of *flbA*, an early regulator of *Aspergillus* asexual sporulation, leads to activation of *brlA* and premature initiation of development

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Summary

***Aspergillus nidulans* reproduces asexually by forming thousands of mitotically derived spores atop highly specialized multicellular organs termed conidiophores. We have identified a gene called *flbA* (for fluffy low *brlA* expression) that is required for initiation of *A. nidulans* conidiophore development. *flbA* mutants form abnormal colonies that have a distinct fluffy phenotype characterized by tightly interwoven aerial hyphae that autolyse as the colony matures. The requirement for *flbA* in conidiophore development precedes activation of *brlA*, a primary regulator of conidiophore development. The wild-type *flbA* gene was isolated and found to encode a 3.0 kb mRNA that is expressed throughout the *A. nidulans* asexual life cycle. Overexpression of *flbA* using an inducible promoter resulted in misscheduled expression of *brlA* in vegetative cells and caused hyphal tips to differentiate into spore-producing structures. Sequence analysis of a nearly full-length *flbA* cDNA clone showed that *flbA* is predicted to encode a 717-amino-acid polypeptide with 30% identity to the *Saccharomyces cerevisiae* SST2 protein. *SST2* is required by yeast cells for resuming growth following prolonged exposure to yeast mating pheromone and for mating partner discrimination. We propose that *flbA* plays a related role in a signalling pathway for *Aspergillus* conidiophore development.**

Introduction

The asexual cycle of the filamentous fungus *Aspergillus nidulans* involves formation of an organized mycelial colony that produces specialized spore-bearing structures termed conidiophores (for review see Timberlake, 1990). Mycelial colony development has been described for numerous fungi, but the mechanisms regulating hyphal growth and colony formation remain uncharacterized (Trinci, 1974; Prosser and Tough, 1991). Generally, spore germination leads to formation of tubular structures, called hyphae, that grow through apical extension and branching to form additional primary and secondary hyphae. In this way, a mycelial network is produced, forming a radially symmetric colony that expands at a constant rate until growth becomes limited. Several hours after *A. nidulans* spore germination, aerial hyphal branches arise from cells within the centre of the developing colony. During the next few hours, some of the aerial hyphae differentiate to form conidiophores that yield long chains of spores called conidia. In general, the process of conidiophore development from the initiation of aerial growth to the production of the first conidium takes 6–8 h (Champe and Simon, 1992). Conidiophore development continues from the centre of the colony towards the outer edge, so that a cross-section through a growing *A. nidulans* colony reveals the time course of development with the youngest growing hyphae at the margin and the oldest conidiophores towards the centre.

A. nidulans conidiophore development primarily initiates as a programmed event in the life cycle rather than as a response to specific environmental cues (Pastushok and Axelrod, 1976; Champe *et al.*, 1981; Adams *et al.*, 1992). The switch from vegetative growth to conidiophore development takes place at a set time following spore germination, regardless of nutritional conditions, and involves the controlled activation of specific sets of genes (Axelrod, 1972; Boylan *et al.*, 1987). The best-characterized developmental regulatory gene in *A. nidulans* likely to be involved in controlling the switch from growth to development is *brlA* (Clutterbuck, 1969; Boylan *et al.*, 1987; Adams *et al.*, 1988; Han *et al.*, 1993; Prade and Timberlake, 1993). *brlA* mRNA is present at undetectable levels in hyphae and begins to accumulate during development at about the time conidiophore vesicles appear. *brlA* null mutants differentiate conidiophore stalks that grow somewhat indeterminately rather than producing additional conidiophore-specific cell types, including spores (Clutterbuck, 1969; Boylan *et al.*, 1987). By contrast, forced expression of *brlA* at inappropriate times causes a cessation of apical hyphal growth and initiates development of reduced conidiophores that form viable

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spores (Adams *et al.*, 1988). This demonstrates that activation of *brlA* expression during early development is a major control point in initiating sporulation.

Several mutants have been described that appear to be defective in controlling the switch from mycelial growth to conidiophore development prior to *brlA* activation (Dorn, 1970; Martinelli and Clutterbuck, 1971; Yager *et al.*, 1982; Tamame *et al.*, 1983). These mutants produce large masses of undifferentiated aerial hyphae resulting in colonies that have a cotton-like or 'fluffy' appearance. We previously described the cloning and initial characterization of one fluffy gene, *fluG* (previously called *acoD*). Under optimal growth conditions, *fluG* is required for the activation of *brlA* expression and conidiophore development. However, the requirement for *fluG*-dependent expression of *brlA* can be partially suppressed by growing colonies under nutrient-limiting conditions, leading to the suggestion that *brlA* can be activated in more than one way (Adams *et al.*, 1992). The major sporulation pathway requires *fluG* and involves nutrient-independent programmed initiation of development. When *fluG* is absent, *brlA* expression and development are controlled by a mechanism that involves sensing growth rate or nutrient status. Further analysis of *fluG* mutants indicates that *fluG* is required for production of a small diffusible factor, raising the possibility that this factor signals programmed initiation of conidiation leading to both *brlA* expression and development (Lee and Adams, 1994).

In order to understand the role of *fluG* and other fluffy genes in controlling initiation of *A. nidulans* conidiophore development, we have isolated over 100 fluffy mutants and characterized their effects on *brlA* expression (Wieser *et al.*, 1994). We have focused on a group of mutants having the most severe effect on *brlA* expression (FLB for fluffy low *brlA* expression) when compared with wild type. In this paper, we describe the cloning and characterization of one of these genes, *flbA*. We show that *flbA* is not only required for *brlA* expression during *A. nidulans* conidiation but is also needed for normal colonial growth. Loss of *flbA* gene function leads to formation of fluffy colonies with long, twisted, interwoven aerial hyphae that autolyse as colonies mature. In contrast, overexpression of *flbA* in vegetative cells leads to inappropriate activation of *brlA* expression and sporulation. *flbA* is predicted to encode a protein that has 30% identity throughout the majority of the *flbA* coding sequence to *Saccharomyces cerevisiae SST2*, a gene required for regulating the signal pathway response to yeast mating pheromone (Dietzel and Kurjan, 1987; Jackson and Hartwell, 1990; Jackson *et al.*, 1991). The sequence similarity between FlbA and SST2 leads us to propose that *flbA* could play a regulatory role in controlling the FluG-initiated signal transduction pathway that triggers *A. nidulans* asexual reproduction.

Results

Isolation and physical mapping of the flbA *gene*

Spores from a wild-type *A. nidulans* strain (FGSC26) were mutagenized with 4-nitroquinoline 1-oxide (NQO) and colonies from survivors were visually screened for the fluffy phenotype. These mutants were classified into three phenotypic classes based on their levels of *brlA* expression: (i) fluffy high *brlA* expression; (ii) fluffy moderate *brlA* expression; (iii) and fluffy low *brlA* expression (described in detail in Wieser *et al.*, 1994). To understand events required for *brlA* activation during developmental initiation, we have concentrated on characterizing fluffy low *brlA* expression mutants and have identified five new loci, designated *flbA* through to *flbE*, as well as several new alleles of *fluG* (Adams *et al.*, 1992).

We isolated the wild-type *flbA* gene by complementation of a *flbA*$^-$ mutant strain as described in Yelton *et al.* (1984). An *argB2*$^-$, *flbA13*$^-$ mutant strain (RBN85) was transformed with an *A. nidulans* genomic DNA library constructed in a cosmid vector PUI1 that contains the wild-type *argB*$^+$ gene as a selectable marker (kindly provided by Dr Bruce Miller, University of Idaho). Approximately 3000 *argB*$^+$ transformants were selected on minimal media lacking arginine and a total of four transformants producing wild-type conidiophores were identified. Genomic DNA

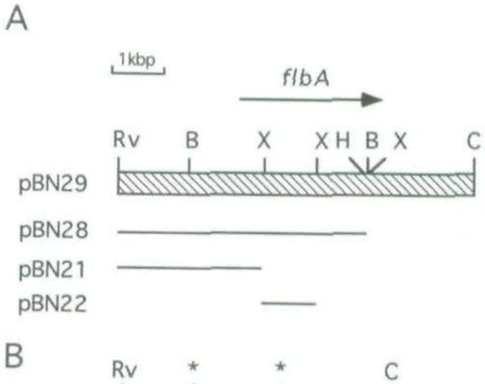

Fig. 1. Mapping and localization of the *flbA* gene.
A. A restriction map of the *flbA* complementing region is shown in the top part of the figure. DNA fragments used in co-transformation experiments to localize the *flbA* coding region are shown below. The relative frequency of *flbA13*$^-$ mutant rescue by each subclone is described in the text. The direction of *flbA* transcription was deduced by analyses of unidirectional cDNA clones and is indicated by the arrow which is positioned to represent the predicted *flbA* reading frame shown in Fig. 3.
B. pBN39 was constructed as described in the *Experimental procedures* and was used in transformation experiments to create a *flbA* deletion mutant (TBN39.5).
Restriction sites are abbreviated: RV, *Eco*RV; B, *Bgl*II; X, *Xho*I; H, *Hin*dIII; C, *Cla*I. The * represents *Bgl*II–*Bam*HI hybrid restriction sites created while replacing *flbA* with *argB*.

from two of these wild-type transformants, TBN002 and TBN003, was used to recover the transforming cosmids as described by Yelton *et al.* (1985) Restriction analysis of the cosmids recovered from TBN002 and TBN003 indicated that they represent unique clones that overlap by approximately 20 kb. These cosmids were designated pBN1 and pBN3, respectively, and both were shown to rescue the *flbA13*$^{-}$ mutation at a frequency of greater that 90%, indicating that they probably contained the entire *flbA* locus. Further analysis of these clones showed that the *flbA* gene was contained within a 7 kb *Eco*RV–*Cla*I fragment that was then subcloned into pBluescript-II KS− to make pBN29. Two overlapping fragments derived from pBN29 were subcloned and shown to rescue the *flbA13*$^{-}$ mutation but with a much lower frequency (pBN21 and pBN28; Fig. 1). This indicated that the *flbA13*$^{-}$ mutation is located within the 2.7 kb *Eco*RV–*Xho*I fragment in pBN21 and that the *flbA* gene probably extends beyond the *Hin*dIII site in pBN28. Therefore, the 1.1 kb *Xho*I fragment in pBN22 must be internal to *flbA* (Fig. 1) and was used as an *flbA*-specific probe in the experiments described below.

Deletion of flbA *results in a fluffy autolysis phenotype*

To determine the phenotype of a *flbA* null mutant, we deleted the gene from the chromosome by transforming an *argB*$^{-}$ strain (PW1) with a plasmid construct in which a 3.6 kb *Bgl*II fragment that spans *flbA* was deleted and replaced by the *argB*$^{+}$ gene (Fig. 1B). Four of twenty-five *argB*$^{+}$ transformants produced fluffy colonies that autolysed upon prolonged incubation, just as with the

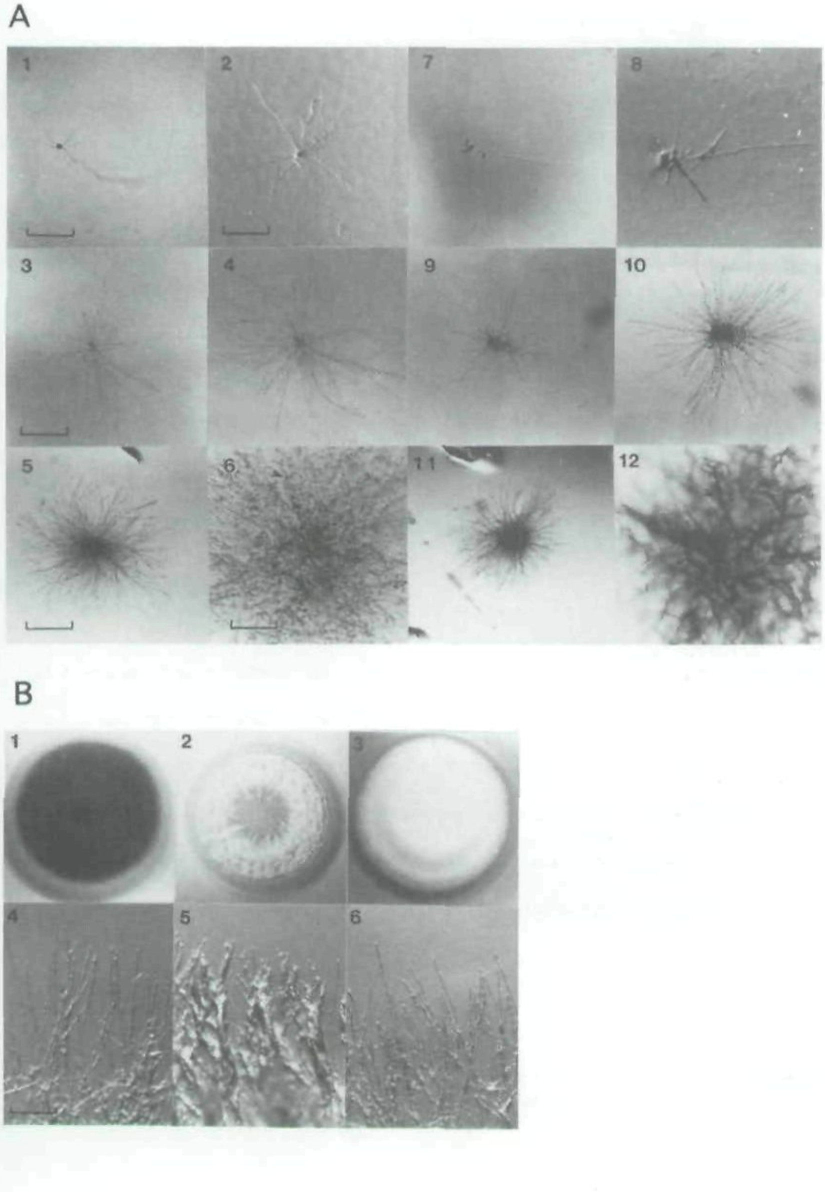

Fig. 2. *flbA* is required for colonial growth and conidiophore development.

A. Isolated spores from the wild-type strain (1–6) and a *flbA* deletion mutant strain (7–12) were allowed to germinate on complete media incubated at 37°C to follow colonial development. The same magnification of wild-type and mutant strains are shown: 12 h (1 and 7; scale bar = 125 μm), 16 h (2 and 8; scale bar = 250 μm), 20 h (3 and 9; scale bar = 500 μm), 24 h (4 and 10; scale bar = 500 μm), 28 h (5, and 11; scale bar = 1 mm; 6 and 12; scale bar = 200 μm) following inoculation. Arrows show conidiophores (panel 6) and intertwined aerial hyphae (panel 12) produced by the wild-type and the mutant strain, respectively.

B. Comparison of 3.5-day-old colonies of wild-type (FGSC26; 1 and 4), a *flbA* deletion mutant (RBN127; 2 and 5), and a *fluG* deletion mutant strain (TTA127.4; 3 and 6). The upper panels shows the whole colony and the lower panels shows the colony margins (scale bar = 250 μm).

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    1 TTGATCCTCTCCCTTCCGAAACCCTCCCTTCCTCCGATTTTCTGCCCTTTTTTTTTCGTCCCCTCTGGTTTAGTCTGATTTTCGTCATCCCGTACTGTATTTTATTTTAATTTTTTCTCA  120

                                                           M  P  T  S  I  S  T  A  P  L  S  Q  G  S  P  P  S  S  L  I  D  Y  Q
  121 TTTTACTTAGTCTGCCCTAGCCTCCCACTCCCACTCCGACCTGCACTCTTCAATGCCAACTTCCATATCTACCGCTCCCCTCAGCCAAGGATCCCCTCCCTCTTCTCTCATTGATTATCA  240

        P  Q  S  V  P  S  S  S  S  P  P  P  S  T  A  A  A  A  A  A  A  V  V  V  A  V  P  S  S  S  S  P  V  D  L  G  L  P  S  F
  241 GCCTCAGTCCGTCCCTTCTTCTTCTTCCCCTCCTCCTTCTACAGCTGCTGCTGCTGCTGCTGCGGTTGTCGTCGCTGTTCCTTCGTCTTCCTCCCCGGTCGACCTTGGCCTCCCGTCTTT  360

        T  S  T  S  S  L  I  T  S  D  V  P  A  T  T  T  T  P  S  F  T  G  S  V  I  G  S  I  S  R  R  N  R  R  S  F  A  A  L  A
  361 CACCTCCACCTCGTCCCTTATCACGTCCGACGTCCCCGCCACCACAACCACACCCAGTTTTACTGGTTCGGTTATCGGCTCCATTTCACGTCGGAATCGACGCTCTTTTGCTGCTCTCGC  480

        R  E  K  T  S  S  A  L  A  N  L  S  S  I  G  S  T  T  N  S  S  L  R  Q  S  A  S  S  G  S  L  Q  K  H  S  R  K  A  S  Q
  481 TCGAGAAAAGACTTCTAGCGCTCTCGCAAATCTCTCGTCAATCGGCTCGACCACGAATTCCTCCCTCCGGCAGTCCGCCTCATCCGGGAGTCTACAGAAGCATTCGCGCAAGGCTTCGCA  600

        L  S  V  G  E  I  S  G  F  V  P  L  S  P  P  L  S  D  G  S  G  S  S  E  Q  S  S  S  A  P  F  E  P  L  S  A  V  T  E  Q
  601 ACTGAGTGTGGGCGAAATCTCAGGCTTTGTTCCACTCTCCCCGCCATTGTCCGACGGTAGCGGCAGCAGCGAGCAATCGAGCTCTGCCCCGTTCGAACCTTTATCAGCGGTCACTGAGCA  720

        P  N  P  A  A  E  R  R  R  Q  T  I  Q  L  V  P  P  I  S  E  N  I  P  V  S  P  A  K  M  H  Q  T  S  S  R  L  L  R  M  T
  721 GCCCAACCCTGCCGCAGAACGGCGCCGCCAGACCATCCAGCTTGTACCGCCCATCTCTGAGAACATACCTGTTTCTCCCGCCAAAATGCATCAAACCTCGTCAAGGCTGTTGCGCATGAC  840

        E  D  D  R  P  F  T  K  D  F  M  D  L  F  S  T  L  M  V  S  L  K  L  D  S  H  R  V  R  F  T  K  Y  D  H  T  F  T  S  E
  841 GGAGGATGATCGTCCTTTCACCAAGGATTTCATGGATCTTTTCTCTACTCTTATGGTCAGCTTGAAACTGGATTCGCATCGCGTTCGCTTTACGAAATATGACCACACTTTCACATCGGA  960

        E  A  I  N  N  L  G  S  L  K  F  S  Q  S  N  R  M  P  D  P  K  D  P  S  R  I  V  T  T  T  T  T  T  F  S  M  A  K  E
  961 GGAAGCCATTAACAATCTGGGCTCCCTCAAATTCTCTCAATCGAACCGGATGCCAGACCCGAAAGACCCCTCCCGCATTGTAACCACTACTACAACAACTACCTTCTCAATGGCGAAAGA  1080

        M  A  R  S  V  C  Q  R  F  V  D  A  R  F  I  E  S  V  D  G  K  Y  S  H  T  F  P  L  K  G  A  L  Y  Q  L  T  P  K  G  I
 1081 AATGGCTCGATCGGTATGTCAGCGGTTTGTGGATGCTCGCTTCATTGAGTCTGTGGATGGGAAATACTCGCACACGTTTCCGCTGAAGGGCGCTTTATATCAACTTACTCCGAAAGGTAT  1200

        N  I  L  Q  R  F  C  Q  R  N  G  I  T  A  R  H  V  I  D  V  L  E  S  P  R  N  T  M  Q  L  V  N  L  E  R  D  T  E  T  D
 1201 CAACATTTTGCAGAGGTTCTGTCAGAGAAACGGCATCACTGCGCGCCATGTTATTGACGTCCTGGAGTCGCCCCGCAACACTATGCAACTCGTCAACTTGGAGCGCGATACCGAAACCGA  1320

        K  L  S  H  D  R  A  T  I  E  V  I  F  R  R  F  A  G  Q  D  G  P  N  V  K  S  S  V  S  S  S  D  S  D  S  L  S  D  Y  S
 1321 CAAGCTCTCCCACGACCGAGCGACGATCGAAGTCATCTTCCGTCGATTCGCAGGGCAGGACGGCCCTAATGTGAAGAGCAGTGTCTCGAGCTCGGATTCAGATTCCCTCAGCGACTACTC  1440

        N  G  L  V  G  V  K  M  A  R  E  R  K  V  G  D  K  I  C  A  N  T  F  T  G  K  A  A  V  D  W  L  M  D  C  S  T  T  I  E
 1441 AAATGGTCTGGTTGGAGTAAAGATGGCTCGGGAGCGCAAAGTCGGCGACAAAATTTGCGCCAATACTTTCACTGGGAAGGCTGCGGTTGACTGGCTGATGGACTGTTCGACAACCATTGA  1560

        P  R  E  T  V  L  I  A  E  L  F  V  K  Y  G  L  I  T  V  L  Q  E  D  R  S  M  P  Q  V  E  N  S  L  V  A  F  Q  P  S  K
 1561 GCCCCGGGAGACGGTTCTGATCGCGGAACTTTTTGTTAAGTATGGATTGATCACCGTACTTCAGGAGGACCGGTCAATGCCGCAGGTCGAGAACTCACTAGTTGCTTTCCAACCCTCGAA  1680

        N  A  I  Y  A  I  T  E  R  G  Q  R  V  C  G  W  L  A  R  D  K  P  R  D  T  T  T  Y  D  S  R  G  I  P  R  D  S  N  N  A
 1681 GAACGCGATCTACGCCATCACTGAGCGTGGCCAGCGGGTTTGCGGATGGCTCGCTCGCGACAAGCCTCGCGACACGACGACCTATGACAGCCGTGGCATCCCACGGGATTCGAATAACGC  1800

        R  L  N  H  I  L  Q  D  P  A  L  R  L  L  F  R  E  F  L  R  F  S  L  C  E  E  N  L  S  F  Y  I  D  V  S  E  F  T  T  Q
 1801 ACGCTTGAACCATATCTTGCAAGACCCTGCCCTACGCCTACTATTCCGAGAATTTCTCCGTTTCTCACTCTGCGAAGAAAACCTGTCATTCTACATTGACGTCTCCGAGTTCACCACCCA  1920

        Y  H  K  F  D  K  V  G  H  F  K  K  P  D  A  V  R  E  T  L  A  A  A  Y  G  L  Y  N  A  F  L  A  P  G  S  P  C  E  L  N
 1921 GTATCATAAATTCGACAAGGTGGGCCACTTCAAGAAGCCCGACGCTGTACGGGAAACGCTTGCGGCAGCATATGGCCTTTACAATGCGTTCCTAGCACCCGGGTCTCCCTGCGAGCTCAA  2040

        I  D  H  A  L  R  N  S  L  A  S  R  M  T  K  A  V  G  D  D  D  S  M  L  K  S  L  Q  E  V  V  Q  L  F  E  M  A  Q  T  S
 2041 CATTGACCACGCGCTCCGTAACAGTCTGGCCAGTCGCATGACGAAAGCTGTTGGCGACGACGACTCAATGCTCAAGAGTCTCCAAGAAGTAGTCCAGCTGTTTGAGATGGCACAAACATC  2160

        V  F  K  L  M  S  S  D  S  V  P  K  F  L  R  D  P  K  Y  S  A  I  L  Q  E  H  D  V  D  D  L  I  G  G  G  R  S  Y  S  P
 2161 GGTATTCAAGCTTATGTCAAGCGATTCTGTTCCCAAATTCCTGCGGGATCCGAAATACTCCGCAATCCTCCAGGAGCACGACGTTGATGACCTGATCGGTGGTGGAAGATCTTACTCGCC  2280

        T  P  G  N  V  P  E  R  S  M  S  R  S  Q  R  S  *
 2281 TACGCCTGGAAACGTCCCGGAACGATCGATGAGTCGCTCACAACGTTCATGACAGTAATTATCTACACGCGTGATGGCTACGCCCATCGTACCCTATCGGTCGATATTACTTTCTAGGCA  2400

 2401 CGATTTTGTTCCACGCGATACGTTTTAATATCTTCATTCCACGTCTGTGATATGAACGCCCTCCGCATTGTTTCCTATATATAATCCACCCTCGAGACACGACACCTATTTCCCCCGACG  2520

 2521 AGCTTCCTGCAAGCCATCACGAGATATGAAAGACATTATGATCCACGGATCATGTTGATAGAGACGCCTGGGCAGAAATTTTGCTGATGTTTTCTGTCGCCTTTTCTTTTATCCATTCGC  2640

 2641 ACGGATTCCACTGGGATAGAAATGCGGAAAGCCCAAAACATAATTGTTGAGCAGCATTAGAGTGGCGTTTCTTCCTGCTTCATTTACTCTTTTTCATCTCATTTGTCGCACTATTCTCCC  2760

 2761 ATGTATGCACGAAGCTAAGACCTCAATTTCCTTACTTCATTTTCCTTTTATTTCATCTATTCACTTCGGGACATCAAGTCGACATGTAGGTGCCCTCGTCTTATGTTCTCTCTGCACGAG  2880

 2881 CATCTAGCGTCTTCCAAGCAATCTGCACGCATCTATCTGGTTTCGCATTTCAGGGTTCAGCGCTGTCTGGCTATCTGTTCTATGCAGCTACGGCGATTCGATTCTCGTTTGCCTTATCTT  3000

 3001 CTCTTCTTGACCGAGGTTCCCTGCTGTTCTTGTATACATATGATATGATTCACGGAATTC  3060
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Fig. 3. Sequence of *flbA* coding region. The DNA sequence of the 3060 bp region corresponding to *flbA* and the predicted amino acid sequence of the FlbA polypeptide are shown. The first 205 bp were derived from genomic sequence and the remainder from cDNA clones. The accession number for this sequence is L24395.

flbA13$^-$ mutant. Because this *Bgl*II fragment includes approximately 500 bp of *flbA* upstream sequence, it is possible that the mutant phenotype results from disruption of a flanking gene. However, we consider this unlikely based on the complementation results described above.

Southern blot analysis showed that the four fluffy autolytic transformants contained the expected deletion while all the wild-type transformants contained an intact *flbA*$^+$ gene (data not shown). A heterozygous diploid constructed between one of the *flbA* deletion mutants (TBN39.5) and an *flbA13*$^-$ strain (RBN85) was also fluffy, indicating that the region identified probably corresponds to the *flbA* locus rather than to an unlinked suppressor. We have been unable to show linkage between *flbA13*$^-$ and the *flbA* deletion because heterokaryon autolysis has made it impossible to carry out meiotic crosses. However, genetic mapping of the *flbA13*$^-$ mutation showed it was located on chromosome I and the cloned sequence was also shown to physically map to chromosome I, supporting the hypothesis that this defines the same locus (Wieser *et al.*, 1994; Brody *et al.*, 1991).

Although the *flbA* deletion mutant formed fluffy aconidial colonies having abnormal mycelial development, both of these phenotypic defects could be partially remediated by growth on high osmolarity medium (e.g. 0.8 M NaCl), allowing isolation of asexual spores from *flbA*$^-$ mutants for use as inoculum. Figure 2 shows the temporal progression of colonies derived from single spores of a wild-type strain (FGSC26; Fig. 2A, panels 1–6) and an *flbA*$^-$ deletion mutant strain (RBN127; Fig. 2A, panels 7–12) grown on the surface of complete medium at 37°C. Both wild-type and *flbA*$^-$ mutant spores produced germ tubes about 6 h after inoculation. Shortly after germination, hyphae from both the wild-type and the *flbA*$^-$ deletion mutant strain began to branch, finally resulting in formation of a mycelial network. Mycelial development differed greatly in *flbA*$^-$ mutant colonies as compared with wild-type colonies, in that thick leading hyphae appeared to form more frequently in *flbA*$^-$ mutants than in the wild type (Fig. 2A, panels 2 and 8). In addition, the leading hyphae of *flbA*$^-$ mutant strains were often grouped tightly together in bundles while wild-type colonies produced regularly distributed hyphae, as described by Trinci (1974). Approximately 22 h following inoculation, aerial hyphal branches appeared within the centre of wild-type colonies. By 28 h after inoculation, some of these aerial branches had differentiated to produce complex conidiophores, observed as dark aerial spots (Fig. 2A, panel 6) (Timberlake, 1990). At the same time, *flbA*$^-$ mutant colonies had produced abundant aerial mycelia that, instead of differentiating into conidiophore stalks, formed bundles of tightly interwoven hyphae (Fig. 2A, panel 12).

Figure 2B shows 3.5-day-old colonies of a wild-type strain (FGSC26), an *flbA* deletion mutant (RBN127), and a *fluG* deletion mutant (TTA127.4). During maturation, wild-type colonies produced a lawn of conidiophores. In contrast, the *flbA* deletion mutant colony began to lyse in the centre and, as time progressed, the colony lysed completely. The *fluG* deletion mutant produced a cotton-like colony with a large mass of aerial hyphae. The bottom panel of Fig. 2B shows that, while hyphae at the margins of wild-type and *fluG*$^-$ mutant colonies were loosely spaced, *flbA*$^-$ mutant hyphae were tightly packed together.

flbA *is related to the* S. cerevisiae SST2 *gene*

The DNA sequence of the *flbA* gene region was determined and is presented in Fig. 3. The sequence contains one long open reading frame predicting a 717-amino-acid polypeptide. We compared the putative FlbA sequence with other sequences in various databases using the BLAST search algorithm (Altschul *et al.*, 1990) and found the most significant ($P[N] = 4.9e-20$) match was to the *S. cerevisiae SST2* gene (Dietzel and Kurjan, 1987). If gaps are included, the predicted *flbA* polypeptide has 30% identity and 49% similarity beginning at amino acid 205 and extending throughout the rest of the coding region to yeast *S. cerevisiae* SST2 (Fig. 4). No other proteins in the various data bases are significantly similar to either FlbA or SST2.

To test the possibility that the similarity observed between *SST2* and *flbA* reflected conservation of function, we constructed an *flbA*$^-$ mutant *A. nidulans* strain containing the *S. cerevisiae SST2* gene under the control of the inducible *alcA* promoter (Lockington *et al.*, 1985; Gwynne *et al.*, 1987). Overexpression of *SST2* in this way did not rescue the *flbA*$^-$ phenotype (data not shown).

flbA *mRNA is present throughout the asexual cycle of* A. nidulans

As described above, the *flbA* product is apparently required for some aspect of hyphal growth as well as for activation of the conidiation pathway. To determine if *flbA* is transcribed throughout the asexual cycle of *A. nidulans*, total RNA was isolated from mature conidia and from growing mycelial cultures at various times before and after inducing conidiation. Figure 5 shows that a 3.0 kb mRNA corresponding to *flbA* is present at low levels during vegetative growth by 12 h after inoculation and that message levels remained relatively constant at all time points examined, although minor increases were observed shortly after developmental induction. This transcript was not detected in an *flbA* deletion mutant indicating it was *flbA*-specific (data not shown).

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flbA   205 IPVSPAKMHQTSSRLLRMTEDDRPFTKDFMDLFSTLMVSLKLDSHR.... 250
                  :|: ||: :  | ::  || |:  ::| ::::| |   :
SST2     1 MVDKNRTLHELSSKNFSRTPNGLIFTNDLKTVYSIFLICLDLKEKKHSSD 50

                    .         .         .         .         .
       251 ......VRFTK.YDHTFTSEEAINNLGSLKFSQSNRMPDPKDPSRIVTTT 293
                   ||| :  ||| :|||  :| | :                ||
        51 TKSFLLTAFTKHFHFTFTYQEAIKAMGQLELKVD...........MNTTC 89

                    .         .         .         .         .
       294 TTTTFSMAKEMARSVCQRFVDARFIESVDGKYSHTFPLKGALYQLTPKGI 343
               :     :|| :   |   :::   :::     |    |:| ||||:
        90 INVSYNIKPSLARHLLTLFMSSKLLHTPQDR.TRGEPKEKVLFQPTPKGV 138

                    .         .         .         .         .
       344 NILQRFCQRNGITARHVIDVLESPRNTMQLVNLERDTETDKLSHDRATIE 393
            :||::    :|  :   |:| |  | | |  :||   || : |    |
       139 AVLQKYVR..DIGLKTMPDILLSSFNSMKLFTFERSSVTDSIIHSDYLIH 186

                    .         .         .         .         .
       394 VIFRRFAGQDGPNVKS.........SVSS..................... 413
           ::| :  |   ||| |         ::||
       187 ILFIKMMGAK.PNVWSPTNADDPLPCLSSLLEYTNNDDTFTFEKSKPEQG 235

                    .         .         .         .         .
       414 ..........................SDSDSLSDYSNGLVGVKMARERKV 437
                                      ||:|  :|     |:::   :
       236 WQAQIGNIDINDLERVSPLAHRFFTNPDSESHTQYYVSNAGIRLFENKTF 285

                    .         .         .         .         .
       438 GDK...ICANTFTGKAAVDWLMDCSTTIEPRETVLIAELFVKYGLITVLQ 484
           |     :   ||| ||  :|:|||      :| | :| ||:| |||  :
       286 GTSKKIVIKYTFTTKAIWQWIMDCTDIMHVKEAVSLAALFLKTGLIVPVL 335

                    .         .         .         .         .
       485 EDRSMPQVENSLVAFQPSKNAIYAITERGQRVCGWLARD........... 523
            : |  :       || |:  :: :  ||  :  | :
       336 LQPSRTDKKK....FQISRSSFFTLSKRGWDLVSWTGCKSNNIRAPNGST 381

                    .         .         .         .         .
       524 .............KPRDTTTYDSRGIPRD.....SNNARLNHILQDPALR 555
                        :    |  || |:  |     ||  :|: :| ||::|
       382 IDLDFTLRGHMTVRDEKKTLDDSEGFSQDMLISSSNLNKLDYVLTDPGMR 431

                    .         .         .         .         .
       556 LLFREFLRFSLCEENLSFYIDVSEFTTQYHKFDKVGHFKKPD........ 597
            |||  |   || |||  :|::  |      : |:   |  |
       432 YLFRRHLEKELCVENLDVFIEIKRFLKKMTILKKLIDSKHCDKKSNTSTS 481

                    .         .         .         .         .
       598 ..............AVRETLAAAYGLYNAFLAPGSPCELNIDHALRNSLA 633
                            | |  || :|  ::  |||::||| | ||  :
       482 KNNIVKTIDSALMKQANECLEMAYHIYSSYIMIGSPYQLNIHHNLRQNIS 531

                    .         .         .         .         .
       634 SRMTKAVGDDDSMLKSLQEVVQLFEMAQTSVFKLMSSDS........... 672
             |        | | | :    |::     |     || |
       532 DIMLHP....HSPL.SEHFPTNLYDPSPASAESAASSISSTEADTLGEPP 576

                    .         .         .         .         .
       673 .....VPKFLRDPKYSAILQE..HDVDDLIGGGRSYSPTPGNVPERSMSR 715
                  | | :  :|   |   |:: :   :      | :: |  |
       577 EVSLKPSKNLSNENCSFKKQGFKHQLKEYKPAPLTLAETDDDSPNASVEN 626

       716 SQ 717
           |:
       627 SH 628
```

Fig. 4. Comparison of FlbA sequence with *S. cerevisiae* SST2. A BESTFIT comparison between *flbA* and the *S. cerevisiae SST2* polypeptides is shown. Identities are illustrated by vertical dashes and conservative changes are illustrated by colons. Conserved amino acids were defined using a modified Dayhoff table (Gribskov and Burgess, 1986) and requiring a similarity value of 0.7 to consider positive.

flbA *is required for* brlA *expression*

Our primary screen to identify fluffy mutants having reduced *brlA* expression relied upon *brlA* promoter-directed *lacZ* expression (Wieser *et al.*, 1994). In this assay, no *brlA*-directed β-galactosidase activity could be detected in an *flbA*$^{-}$ mutant strain (RBN118) after developmental induction (data not shown). To confirm that *flbA* is required for activation of *brlA*, total RNA from wild-type and *flbA*$^{-}$ deletion mutant strains was isolated at various times before and after developmental induction. Figure 6 shows that *brlA* mRNA first became detectable 4 h after induction in wild-type strains and continued to be present at later time points. By contrast, no *brlA* mRNA could be detected in the *flbA*$^{-}$ deletion mutant strain even 24 h after developmental induction.

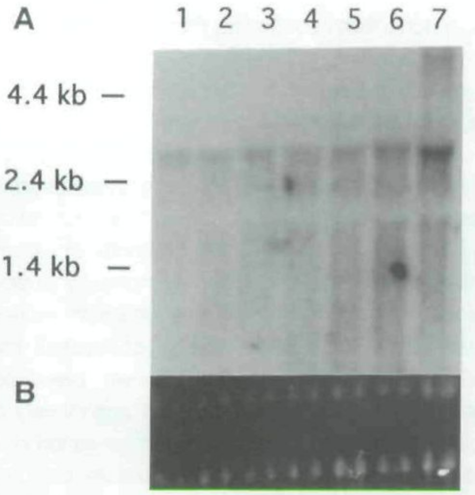

Fig. 5. *flbA* encodes a 3.0 kb message that is present throughout the life cycle of *A. nidulans*.
A. Total RNA (10 µg lane^{-1}) was isolated from a wild-type *A. nidulans* strain (FGSC26) at various times during the asexual life cycle and fractionated on a formaldehyde–agarose gel. The resultant gel blot was probed with a specific *flbA* cDNA fragment. Lane 1, RNA isolated 12 h after inoculation; lane 2, RNA isolated 20 h after inoculation; lanes 3 to 6, RNA isolated at 4, 8, 12 and 24 h after developmental induction; lane 7, RNA isolated from conidia. RNA size markers are indicated at the left.
B. Equal loading of total RNA was confirmed by ethidium bromide staining.

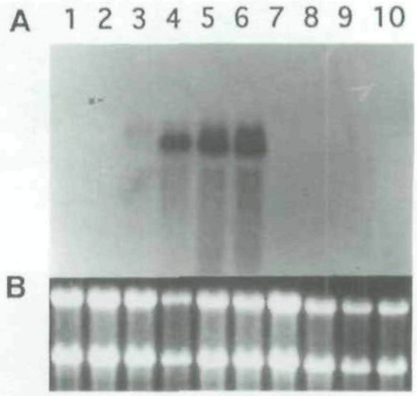

Fig. 6. *flbA* is required for activation of *brlA*.
A. Total RNA (15 µg lane^{-1}) was isolated from a wild-type strain (FGSC26, lanes 1–6) and a *flbA* deletion strain (TBN39.5, lanes 7–10) at different times during conidiophore development and fractionated on a formaldehyde–agarose gel. The resultant gel blot was probed with a *brlA* gene fragment from pBS2.5. RNA was isolated 14 h post-inoculation (lane 1), 20 h post-inoculation (lanes 2 and 7), 4 h after developmental induction (lane 3), 8 h after developmental induction (lanes 4 and 8), 12 h after developmental induction (lanes 5 and 9), and 24 h after developmental induction (lanes 6 and 10).
B. Equal loading of total RNA was confirmed by ethidium bromide staining.

Overexpression of flbA *activates development*

To examine the possibility that *flbA* is directly involved in a pathway required for activating *brlA* expression, we constructed a strain (TBN54.2) containing an extra copy of the *flbA* gene fused to the *A. nidulans alcA* promoter. *alcA* transcription is nutritionally regulated so that high levels of expression are observed when cells are grown on threonine as the sole carbon source, but this expression is repressed in the presence of glucose (Lockington *et al*., 1985; Gwynne *et al*., 1987). Figure 7A shows that *flbA* expression was induced by threonine in TBN54.2 but not in TTA292, a strain containing the *alcA(p)* fused to *brlA*, or in TBN54.3, a control strain lacking an *alcA(p)* fusion construct.

Overexpression of *flbA* in growing hyphae resulted in cellular transformations that resembled the changes that take place following forced activation of *brlA* in vegetative hyphae using the *alcA* promoter (Adams *et al*., 1988). Figure 8 shows that *alcA*-induced expression of *brlA* (B) or *flbA* (C) caused hyphal tips to differentiate into reduced conidiophores that produced spores. These changes occurred within 2 h of shifting the *alcA(p)::brlA* fusion strain to *alcA*-inducing medium (Adams *et al*., 1988) but did not occur until 6 to 9 h after shifting the *alcA(p)::flbA* fusion to *alcA*-inducing medium. By contrast, no sporulation was observed when *flbA* was overexpressed in a

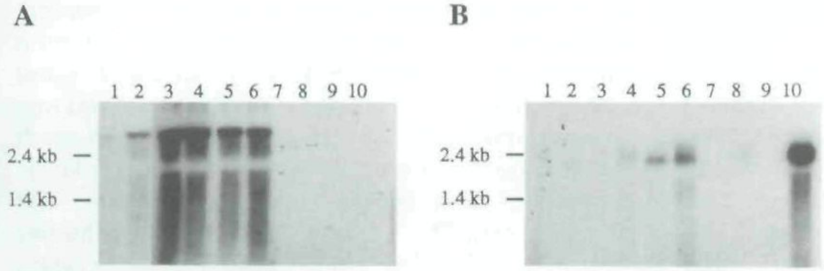

Fig 7. Overexpression of *flbA* activates *brlA* expression. Total RNA (10 µg lane^{-1}) was isolated from an *A. nidulans* strain containing an *alcA(p)::flbA* fusion (TBN54.2; lanes 1–6), a control strain lacking an *alcA(p)* fusion (TBN54.3; lanes 7 and 8), and a strain containing an *alcA(p)::brlA* fusion (TTA292; lanes 9 and 10). The strains had either been grown for 14 h in liquid minimal medium with glucose (lanes 1,7 and 9) or shifted from this medium to minimal medium with threonine and allowed to grow for 2 h (lane 2), 4 h (lane 3), 6 h (lane 4), 9 h (lane 5), or 12 h (lanes 6, 8 and 10). The blot in (A) was probed with a *flbA* specific fragment and the blot in (B) was probed with a *brlA* specific fragment. Equal loading of total RNA was confirmed by ethidium bromide staining (data not shown).

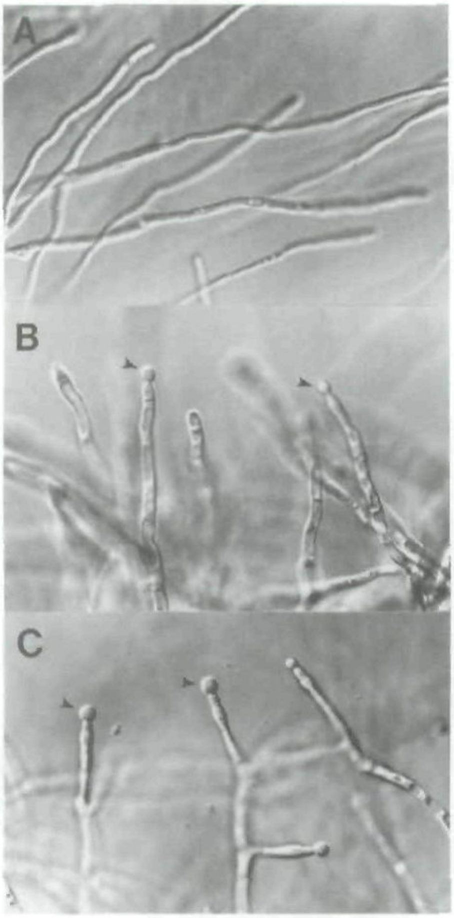

Fig. 8. Overexpression of *flbA* activates sporulation. Strains TBN54.3 (control) (A), TTA292 (*alcA(p)::brlA*), (B) and TBN54.2 (*alcA(p)::flbA*) (C) were grown for 14 h in liquid minimal medium with glucose and then shifted from this medium to liquid minimal medium with threonine and allowed to grow 9 h. Specimens were photographed using DIC optics. Arrows indicate spores forming at hyphal tips.

brlA$^-$ strain (data not shown). Figure 7B shows that *brlA* mRNA accumulated to easily detectable levels between 6 and 9 h of inducing the *alcA(p)::flbA* construct.

Discussion

Results from extensive genetic and biochemical studies of *A. nidulans* conidiation have clearly implicated only three genes, *brlA*, *abaA* and *wetA*, as essential post-initiation regulators of conidiophore development and conidium maturation (Adams *et al.*, 1988; Mirabito *et al.*, 1989; Timberlake, 1990; Marshall and Timberlake, 1991). These three genes have been proposed to form a linear, dependent regulatory pathway responsible for the co-ordinated expression of hundreds of additional conidiation-specific genes. This pathway is activated when *brlA* is first expressed shortly after developmental induction, but the genetic mechanisms controlling this crucial step in conidiation are not well defined. Here we provide evidence that a newly identified gene, *flbA*, plays a critical role in activating *brlA* expression early in development. Under normal growth conditions, *flbA*$^-$ mutant colonies produce hyphae that grow on top of one another forming interwoven hyphal masses that eventually lyse rather than activating *brlA* expression and conidiation. By contrast, in submerged vegetative hyphae, a condition that normally suppresses conidiation, overexpression of *flbA* results in activation of *brlA* expression and differentiation of hyphal tips into spore-producing conidiophores. These developmental changes require a wild-type *brlA* gene and closely resemble those taking place following forced activation of *brlA* in hyphae, although the time required before conidiation is delayed several hours as compared with *alcA(p)::brlA*-directed conidiation (Adams *et al.*, 1988). We interpret these results to mean that *flbA* has a direct role in regulating the activation of *brlA* and conidiophore development and suggest that *flbA* is part of the central developmental regulatory pathway (Timberlake, 1990).

It is important to recognize that *flbA* differs from the other genes in the central regulatory pathway in at least three ways. First, *flbA* mRNA levels remain relatively constant during vegetative growth and following developmental induction. Although there is evidence that some *brlA* transcript is present before development is induced (Han *et al.*, 1993), it is clear that regulation of transcription represents a major mechanism for *brlA* control. Given that *flbA* mRNA is not normally regulated, it is interesting that increased expression of *flbA* activates development. This might be explained if *flbA* activity is normally regulated post-transcriptionally by the products of other genes but when *flbA* mRNA is present at high levels other regulatory events are no longer required. Elucidation of the mechanisms controlling the FlbA activity required for activating development is essential to an understanding of developmental induction.

The second important difference between *flbA* and other developmental regulators in *A. nidulans* is that its function is required for hyphal growth as well as conidiophore development. As described above, *flbA*$^-$ deletion mutants are also defective in early aspects of hyphal growth and colony formation (Fig. 2). While wild-type colonies develop as well-organized structures in which growing hyphae apparently sense and respond to the position of other hyphae (Trinci, 1974; Prosser and Tough, 1991), *flbA*$^-$ mutant colonies produce hyphae that grow on top of one another forming interwoven hyphal masses. In addition, as an *flbA*$^-$ mutant colony matures, the centre of the colony initiates autolysis which continues until the entire colony is destroyed. Autolysis did not begin until at least 2 days after conidiation would normally be observed in a wild-type colony. We suspect that this autolytic phenotype results from the abnormal hyphal morphology. The close association of hyphal masses probably causes physiological stresses that could lead to cell lysis. Alternatively, autolysis represents an independent effect of *flbA* loss of function.

Finally, *flbA* is the first gene in the central regulatory pathway that is not absolutely required for conidiation. *flbA*$^-$ deletion mutants form relatively normal conidiating colonies when grown on high-osmolarity medium (data not shown). The fact that *flbA* is only required for conidiation under certain circumstances supports the idea that *brlA* can be activated in more than one way (Adams *et al.,* 1992). The major pathway requires *flbA* and *fluG* (see below) and results in developmental activation as long as cultures are exposed to air. In the absence of this pathway, other environmental conditions, such as osmotic stress or nutritional limitation (Adams *et al.*, 1992), can activate *brlA* through different mechanisms. An alternative explanation for these results is that high osmolarity could activate another gene that can partially substitute for *flbA*.

It is not yet clear how *flbA* controls activation of *brlA* expression and development but there is more than one possible mechanism. Han *et al.* (1993) showed that the two overlapping *brlA* transcription units, *brlA*α and *brlA*β, are regulated through different controls involving changes in transcription and translation. They proposed that the differences in *brlA*α and *brlA*β regulation provide a means of *separating developmental initiation from the endogenous* controls that result in developmental commitment. In this model, *flbA*-directed activation of *brlA* could take place through increased transcription of *brlA*β or *brlA*α, or by altered translation of *brlA*β. We can now begin to distinguish between these possibilities by examining the effects of overexpressing *flbA* in strains containing only *brlA*α or *brlA*β.

The similarities observed between *flbA* and the *S. cerevisiae SST2* locus provide some basis for speculation on possible *flbA* functions. *SST2* was isolated based on the fact that *sst2*$^-$ mutants are supersensitive to mating pheromone; *SST2* is believed to be important in controlling the signalling pathway that regulates the yeast mating response. The precise role of SST2 in mating remains to be understood, but *SST2* is required for desensitization, or adaptation, to pheromone following prolonged exposure (Dietzel and Kurjan, 1987). Desensitized yeast cells in some way uncouple binding of pheromone from activation of the G-protein-mediated signalling cascade that results in G1 arrest of the cell cycle (Dietzel and Kurjan, 1987; Kurjan and Dietzel, 1988; Kurjan, 1992). *sst2*$^-$ mutants fail to recover from G1 arrest, leading to the suggestion that SST2 negatively regulates some component of the signalling pathway, allowing desensitization. One possible function for *flbA* is in regulating an intracellular signalling pathway for conidiation.

In addition to *flbA*, we have identified several other *A. nidulans* fluffy mutants that are defective in conidiophore development and *brlA* activation (Adams *et al.*, 1992; Wieser *et al.*, 1994). One gene, *fluG*, is required for production of a small diffusible factor that signals programmed activation of conidiophore development (Lee and Adams, 1994). *flbA* might be required for responding to this sporulation factor, much like *SST2* is needed for normal mating-response signalling. *flbA*$^-$ mutants have normal levels of *fluG* expression and can rescue the *fluG*$^-$ conidiation defect extracellularly, suggesting that *flbA* mutants are able to make the FluG-produced signal but are defective in responding. If *flbA* is required for responding to the sporulation signal, it probably has a positive role in signal transduction. This implies that *flbA* function in responding to the conidiation signal must ultimately have the opposite regulatory effect to *SST2*-directed pheromone adaptation. Whether this reflects differences in the activities for FlbA and SST2 or differences in the mechanism of signal transduction remains to be determined.

Experimental procedures

Fungal strains and growth conditions

All *A. nidulans* strains used in this study are listed in Table 1. MBN13 is the original *flbA*$^-$ mutant strain and was isolated by NQO mutagenesis of FGSC26 (Wieser *et al.*, 1994). RBN37 *was isolated as the meiotic progeny of FGSC237 and* MBN13, and was crossed with PW1 to give RBN85, an *argB2*$^-$ *flbA13*$^-$ strain. TBN002 and TBN003 were isolated

Table 1. *A. nidulans* strains used in this study.

Strain	Genotype	Source
FGSC26	*biA1 veA1*	FGSC[a]
PW1	*biA1 argB2 methG1 veA1*	P. Weglenski
RBN85	*biA1 flbA13 argB2 methG1 veA1*	This work
RBN118	*biA1 flbA13 veA1 trpC::brlA(p)::lacZ*	This work
RBN127	*biA1 flbA::argB veA1*	This work
TBN39.5	*biA1 flbA::argB methG1 veA1*	This work
TTA127.4	*pabaA1 fluG::trpC veA1*	Lee and Adams (1994)
RBN37	*biA1 flbA13 trpC801 veA1*	This work
FGSC237	*pabaA1 yA2 trpC801 veA1*	FGSC[a]
MBN13	*biA1 flbA13 veA1*	This work
TBN002	*biA1 methG1 veA1*	This work
TBN003	*biA1 methG1 veA1*	This work
TRG27.1.C	*pabaA1 yA2 veA1 trpC::brlA(p)::lacZ*	Han *et al.* (1993)
TTA292	*biA1 argB::alcA(p)::brlA methG1 veA1*	Adams *et al.* (1988)
TBN54.2	*pabaA1 wA2 methG1 trpC::alcA(p)::flbA veA1*	This work
TBN54.3	*pabaA1 wA2 methG1 veA1*	This work
rM26	*pabaA1 wA2 methG1 trpC801 veA1*	This work

a. Fungal Genetics Stock Center.

as *argB*$^+$, *flbA*$^+$ transformants of RBN85. RBN118 is an *flbA*$^-$ mutant strain that contains a *brlA(p)*::lacZ fusion and arose from a sexual cross between RBN85 and TRG27.1.C (Han *et al.*, 1993). TBN39.5 was constructed by transforming PW1 with the *flbA* deletion plasmid pBN39. TBN54.2 was constructed by transformation of rM26 with the *alcA(p)*::*flbA* fusion plasmid pBN54. TBN54.3 represents a *trpC*$^+$ transformant from the same transformation but pBN54 sequences were not present in the genome.

Appropriately supplemented complete medium was used for all experiments in this study (Käfer, 1977; Adams *et al.*, 1992) Developmental cultures were induced as described elsewhere (Timberlake, 1980; Adams and Timberlake, 1990). For the colonial growth study, wild-type (FGSC26) spores were collected from a confluent culture and suspended in sterile deionized water. The *flbA* deletion mutant (RBN127) was induced to sporulate on medium containing 0.8 M NaCl, which partially suppresses the developmental defect, and spores were collected as described for wild type. Approximately 25 spores of each strain were then spread on the surface of supplemented complete media and a single spore was chosen for monitoring colonial development during the next 36 h. Photographs were taken every hour after germination. Induction of the *alcA* promoter in the *alcA(p)*::*flbA* fusion strain was accomplished as described previously (Adams *et al.*, 1988).

Genetic techniques and microscopy

Standard *A. nidulans* genetic (Pontecorvo *et al.*, 1953; Clutterbuck, 1974) and transformation (Yelton *et al.*, 1984; Miller *et al.*, 1985) procedures were used. Photomicrographs of young germlings and hyphal development were taken using an Olympus BH2 compound microscope and differential interference contrast optics. All other light microscopy was carried out using an Olympus SZ-11 stereo microscope and transmitted light.

Isolation and sequence analysis of flbA *cDNA clones*

About 2.4×10^5 plaques from an *A. nidulans* λgt10cDNA library (Osmani *et al.*, 1988), made using RNA isolated from a log-phase culture, were screened by standard hybridization procedures using an internal 1.1 kb *Xho*I *flbA* gene fragment from pBN22 as a probe (Fig. 1). Four positive clones were identified and analysed by the polymerase chain reaction (PCR) (Saiki *et al.*, 1988) with primers directed to λgt10 sequences (AGCAAGTTCAGCCTGGT; GAGTATTTCTTCCAGG). Amplified PCR products were transferred to a nylon membrane and probed with the same *flbA*-specific fragment. Three clones containing inserts from *flbA* were identified and the largest (F7-3-1) was chosen for further study. Phage DNA isolated from F7-3-1 was digested with *Eco*RI to remove the insert and the two *flbA Eco*RI fragments were subcloned into pBluescript KS− giving rise to pBN30 and pBN33. Three additional cDNA clones were isolated by using the same probe to screen 3×10^5 plaques from a λUNIZAP cDNA library (O'Donnel *et al.*, 1991) made from RNA from log-phase cells. The clone containing the largest insert was rescued as a phagemid and named pBN38. Restriction mapping and sequence analysis of pBN38, pBN30, and pBN33 indicated that the clones overlapped and provided information on direction of transcription. The cDNA insert in F7-3-1 lacked a polyA tract, but analysis of the cDNA insert in pBN38 identified a polyadenylation site at bp 2800 (Fig. 3). Sequences at the 5′ end (bp 1–205; Fig. 3) were derived from sequence analysis of the genomic clone pBN21 (Fig. 1). The GenBank accession number is L24395.

Nucleic acid isolation and manipulation

Total RNA was isolated as described previously (Adams *et al.*, 1988). Total RNA (10 μg or 15 μg per lane) was separated by electrophoresis on a formaldehyde–agarose gel and transferred onto a nylon membrane (Hybond-N; Amersham Corp.). Hybridization to ^{32}P-labelled random primed probes was carried out according to the procedures recommended by the membrane manufacturer. Plasmid pBS2.5 containing the 2.5 kb *Bam*HI–*Sal*I fragment from *brlA* coding region was used as a *brlA*-specific probe (Boylan *et al.*, 1987). pBN30 containing the 2.5 kb *Eco*RI *flbA* cDNA fragment was used as a *flbA*-specific probe.

The *flbA* disruption plasmid pBN39 was constructed by first deleting the 3.6 kb *Bgl*II fragment from pBN29 to give pBN36. A 1.8 kb *Bam*HI fragment from pSalArgB (Berse *et al.*, 1983) containing the wild-type *argB*$^+$ gene was then inserted into the resulting *Bgl*II site to give pBN39 (Fig. 1B). The *alcA(p)*::*flbA* fusion vector pBN54 was constructed by first amplifying a 2800 bp *flbA* fragment using pBN29 as a template and the oligonucleotides GTTTAGTCTAGATTTTCGT and TAGATGCGCTGCAGATTG as primers for the PCR. The resultant fragment was digested with *Xba*I and *Pst*I and inserted into the same sites in pBluescript-II KS− to give pBN48. A 0.5 kb *Xba*I fragment that included the *alcA* promoter was isolated from pSH51 (Han *et al.*, 1993) and inserted in the single *Xba*I site of pBN48 to give pBN53. The *alcA(p)*::*flbA* fusion construct in pBN53 was then moved as a 3.3 kb *Not*I–*Pst*I fragment to pSH93 (S. Han, unpublished), a pBluescript-II SK− derivative that contains the 1.8 kb *Sst*I–*Xho*I *trpC* fragment (Hamer and Timberlake, 1987).

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