Molecular Microbiology (2002) **46**(3), 801–812

Requirement of spermidine for developmental transitions in *Aspergillus nidulans*

Yuan Jin,[1] Jin Woo Bok,[2] Doralinda Guzman-de-Peña[3] and Nancy P. Keller[2]*

[1]*Department of Plant Pathology, Texas A & M University, College Station, TX 77843-2132, USA.*

[2]*Department of Plant Pathology, University of Wisconsin, 1630 Linden Drive, Madison, WI 53706, USA.*

[3]*Departamento de Genetica y Biologia Molecular, Centro de Investigacion y Estudios Avanzados del IPN, Km 9.6 Libramiento Norte, Carretera Irapuato-Leon, Apdo. Postal. 629, Irapuato, Gto., Mexico.*

Summary

Deletion of the spermidine synthase gene in the fungus *Aspergillus nidulans* results in a strain, *ΔspdA*, which requires spermidine for growth and accumulates putrescine as the sole polyamine. Vegetative growth but not sporulation or sterigmatocystin production is observed when *ΔspdA* is grown on media supplemented with 0.05–0.10 mM exogenous spermidine. Supplementation of *ΔspdA* with ≥0.10 mM spermidine restores sterigmatocystin production and ≥0.50 mM spermidine produces a phenotype with denser asexual spore production and decreased radial hyphal growth compared with the wild type. *ΔspdA* spores germinate in unsupplemented media but germ tube growth ceases after 8 h upon which time the spores swell to approximately three times their normal diameter. Hyphal growth is resumed upon addition of 1.0 mM spermidine. Suppression of a G protein signalling pathway could not force asexual sporulation and sterigmatocystin production in *ΔspdA* strains grown in media lacking spermidine but could force both processes in *ΔspdA* strains supplemented with 0.05 mM spermidine. These results show that increasing levels of spermidine are required for the transitions from (i) germ tube to hyphal growth and (ii) hyphal growth to tissue differentiation and secondary metabolism. Suppression of G protein signalling can over-ride the spermidine requirement for the latter but not the former transition.

Accepted 2 August, 2002. *For correspondence. E-mail npk@plantpath.wisc.edu; Tel. (+1) 608 262 9795; Fax (+1) 608 263 2626.

© 2002 Blackwell Publishing Ltd

Introduction

Polyamines are small aliphatic molecules involved in cell growth and development in a wide range of organisms (Tabor and Tabor, 1985; Ruiz-Herrera, 1994). The three most commonly occurring natural polyamines are putrescine, spermidine and spermine. In fungi, these compounds have been reported to be important in cell differentiation processes including sporulation, spore germination and dimorphic transition (Guevara-Olvera *et al*., 1993; Reyna-Lopéz and Ruiz-Herrera, 1993; Ruiz-Herrera, 1994; Lopez *et al*., 1997). For example, polyamine biosynthesis increases during zoospore germination in *Blastocladiella emersonii* (Mennucci *et al*., 1975). In *Sclerotium rolfsii*, mycelial growth and sclerotium germination were positively correlated with increased putrescine levels, whereas sclerotium formation was accompanied by a marked increase in spermine content (Shapira *et al*., 1989). High levels of cellular polyamine (putrescine and spermidine) lead to hyphal growth rather than yeast-like growth in the dimorphic fungus *Mucor racemosus* (Inderlied *et al*., 1980). Disruption of the ornithine decarboxylase gene (i.e. *odc,* required for conversion of ornithine to putrescine) in the corn smut fungus *Ustilago maydis* resulted in a failure of the *odc* mutant strain to shift from the yeast to mycelial stage (Guevara-Olvera *et al*., 1997).

Several studies suggest polyamines are involved in the regulation of not only sporulation but also mycotoxin biosynthesis in *Aspergillus* species. Aflatoxin (AF) and sterigmatocystin (ST) are carcinogenic secondary metabolites produced by *Aspergillus* fungi. Treatment of *A. nidulans* (ST producer) and *A. parasiticus* (AF producer) with 1,4-diamino-2-butanone (DAB), a competitive inhibitor of ornithine decarboxylase, repressed both sporulation and mycotoxin production. The DAB effects could be counteracted by adding exogenous putrescine, the ornithine decarboxylase product, to the growth media (Guzman-de-Peña and Ruiz-Herrera, 1997; Guzman-de-Peña *et al*., 1998). Further studies in *A. nidulans* showed polyamine depletion resulted in the failure of *brlA*, a transcription factor required for asexual sporulation (Prade and Timberlake, 1993), to be expressed (Guzman-de-Peña *et al*., 1998).

The concomitant effect of polyamine deprivation on *brlA* expression, sporulation and ST production is reminiscent of a conserved G protein-mediated growth pathway that links asexual development to ST/AF production in

Aspergillus spp. (Hicks *et al*., 1997). *Aspergillus* mutants containing an activated α subunit of a heterotrimeric G protein, FadA (Yu *et al*., 1996a), exhibit a purely vegetative growth phenotype with no sporulation or ST/AF biosynthesis (Hicks *et al*., 1997). Both sporulation and ST/AF production require that this FadA signalling pathway be inactivated and this requires FlbA, a RGS domain protein which is postulated to enhance the endogenous GTPase activity of FadA (Lee and Adams, 1994; Yu *et al*., 1996a). Both FadA activated mutants and FlbA loss-of-function mutants cannot express *brlA* or *aflR*, a transcription factor required for ST/AF biosynthesis (Yu *et al*., 1996b). Guzman-de-Peña and Ruiz-Herrera (1997) therefore proposed that polyamine effects on sporulation and ST/AF production might be mediated in part through the FadA/FlbA signalling pathway.

Various arguments have been proposed suggesting a role for polyamines in the transduction of extracellular messages. Polyamines can act as an intracellular signal enhancing Ca^{2+} influx across the plasma membrane and Ca^{2+} efflux from the mitochondria in mouse heart, liver and kidney (Koenig *et al*., 1983). Polyamines also have been found to stimulate Gα protein GTPase activity in calf brain (Bueb *et al*., 1991; Bueb *et al*., 1992), mitogen-activated kinase activity in human breast epithelial cells and in mouse 10T1/2 fibroblasts and serine/threonine CK2 activity in chicken COS cells (Kubota *et al*., 1997; Leroy *et al*., 1997; Manni *et al*., 1997). Furthermore, polyamines have been implicated in depressing cAMP synthesis in bacteria (*Escherichia coli*; Wright and Boyle, 1982) and fungi (*Magnaporthe grisea* and *Mucor* spp.; Orlowski, 1995; Choi *et al*., 1997).

Our goals in this study were, first, to investigate a role for polyamines in both *A. nidulans* spore development and ST production at a genetic level by analysing a spermidine synthase mutant, and, second, to explore a possible relationship between polyamine metabolism and the FadA/FlbA signal transduction pathway in this fungus.

Results

Analysis of the A. nidulans spdA *gene*

Cosmid pW30B01 was identified by hybridization to an *A. nidulans* EST with homology to spermidine synthases. Sequencing of a 5.2 kb *Xho*I fragment from cosmid pW30B01 revealed an internal open reading frame (ORF) of 1488 bp encoding a putative 291-amino-acid protein interrupted by four introns, 159, 86, 107 and 257 bp in length (data not shown). This ORF was named *spdA* and encoded the putative spermidine synthase of *A. nidulans*. The deduced amino acid sequence not only showed high identity with spermidine synthases of *Saccharomyces cerevisiae* (Hamasaki-Katagiri *et al*., 1997) and *Schizosaccharomyces pombe* (Barrel *et al*., 1995) (~ 63%), but also shared significant identity with spermidine synthases of bacteria and mammals (*c*. 24%).

Disruption of A. nidulans spdA *gene*

Transformation of *A. nidulans* strain PW1 with the *spdA* disruption construct pYJ3 yielded 250 transformants. Sixteen of these 250 transformants grew well and sporulated on media supplemented with 1.0 mM spermidine but grew poorly and did not sporulate on 0.1 mM spermidine media. Genomic DNA was isolated from these 16 putative *spdA* mutants, digested with *Xho*I and probed with the 5.2 kb *Xho*I pYJ2 fragment containing the entire *spdA* locus. Strain TYJ3.119 had the 4.4 and 2.4 kb fragments predicted if the *Sph*I–*Sac*I fragment of *spdA* was replaced by *argB*. *spdA* disruption was confirmed by *Eco*RV digestion and polymerase chain reaction (PCR) amplification (data not shown).

For further examination of the function of different polyamines in cells of *A. nidulans*, we constructed a *puA, spdA* double mutant strain TYJ3.174 (*puA2*, *ΔspdA*) by transforming RYJ6 with pYJ3 using the same transformation procedures as above. Mutants at the *puA* locus of *A. nidulans* are crippled in the ability to synthesize putrescine because they are deficient in ornithine decarboxylase activity (Stevens, 1975). *puA2*, which was created by mutagenesis with nitrous acid by C. Herman, is allelic to *puA1* isolated by Sneath (1955).

Analysis of polyamines in Aspergillus *mutants*

Table 1 shows the chemically unconjugated polyamine composition of wild-type TPK1.1 and TYJ3.119 (*ΔspdA*). The wild type accumulated no putrescine but increasing levels of spermidine were accumulated as the spermidine

Table 1. Chemically unconjugated polyamine accumulation in wild-type and *ΔspdA* strains of *Aspergillus nidulans*.

Strain	Spermidine added (mM)	Concentration (μM mg^{-1} protein) Putrescine[a]	Spermidine[a]	Wet weight (mg)[a]
TPK1.1	0.0	0.0	0.96	163
(wt)	0.1	0.0	1.93	170
	0.5	0.0	0.55	220
	1.0	0.0	6.80	206
	3.0	0.0	14.02	267
TYJ3.119	0.0	3.8	0.0	45
(ΔspdA)	0.1	1.8	0.0	41
	0.5	1.1	0.0	49
	1.0	5.2	0.0	147
	3.0	5.8	0.12	147

a. Numbers are the average of two replications. Wet weight reflected weight of mycelium or, in the case of TYJ3.119 at 0–0.5 mM spermidine, germlings.

concentration increased in the media. TYJ3.119 accumulated putrescine but no spermidine (with the exception of cells incubated with 3.0 mM spermidine). FGSC682 (*puA2*) is a leaky mutant accumulating some putrescine and the polyamine content of the double mutant TYJ3.174 (*puA2*, *ΔspdA*) was very similar to TYJ3.119 with accumulation of putrescine (data not shown).

Spermidine is essential for A. nidulans *growth and development*

We tested the growth of the *spdA* mutant TYJ3.119 on putrescine, spermidine and spermine containing media (Table 2 and data not shown). TYJ3.119 was unable to grow on any concentration of putrescine and grew only poorly on high concentrations of spermine (data not shown). In contrast, weak hyphal growth of this mutant was observed at spermidine concentrations of 0.05 and 0.1 mM. One, 3 and 6 mM spermidine allowed some radial growth (Table 2). Substantial conidial production required spermidine concentrations ≥0.5 mM (Table 2). Interestingly, although radial growth was less than wild type, conidia production per unit area was significantly higher in TYJ3.119 at spermidine concentrations ≥0.5 mM.

The phenotype of increased asexual spore production and decreased radial growth of TYJ3.119 at high spermidine supplementation was unexpected. We further examined this phenotype by examining the *puA2* mutants. TPK1.1 (wild-type alleles), FGSC682 (*puA2*), TYJ3.119 (*ΔspdA*) and TYJ3.174 *(puA2*, *ΔspdA*) were grown on minimal medium plates containing 0.0, 0.05 mM or 1.0 mM spermidine (Table 3 and data not shown). FGSC682 showed some growth on 0.0 and 0.05 mM spermidine media and was completely restored to wild-type vegetative growth and sporulation in media amended with 1.0 mM spermidine. Colony growth of TYJ3.119 was more vigorous than TYJ3.174 on minimal medium containing either 0.05 mM or 1.0 mM spermidine although both lagged behind TPK1.1 and FGSC682. Both TYJ3.119 and TYJ3.174 produced more conidia per unit area than either wild type or FGSC682 when grown on 1.0 mM spermidine. Considering the leaky nature of the *puA2* allele, these data suggest that the observed phenotype was largely dependent on the *ΔspdA* allele.

Table 2. Effects of different concentrations of spermidine on the growth and conidiation of an *Aspergillus nidulans* wild-type and a spermidine synthase mutant after 72 h incubation.

	Diameter (cm)		Spores 10^7 cm^{-2}	
Spermidine concentration (mM)	TPK1.1 wt	TYJ3.119 (*ΔspdA*)	TPK1.1 wt	TYJ3.119 (*ΔspdA*)
0.00	7.12[a]	0.00	5.01	0.00
0.05	7.21	0.39	5.04	0.00
0.10	7.16	0.47	4.44	0.43
0.50	7.30	3.34	4.17	7.83
1.00	6.90	5.00	3.96	6.57
3.00	7.28	5.94	5.19	7.89
6.00	6.65	5.71	4.98	8.25

a. Values are means of three replicates. Means were analysed by *t*-test (TPK1.1 versus TJY3.119) for each concentration of spermidine at $P = 0.05$. The *ΔspdA* strain was significantly different from the wild type at every concentration of spermidine.

As other studies described a relationship between asexual and sexual spore production in *A. nidulans* (Champe *et al*., 1987; Calvo *et al*., 1999) where an increase in production of one type of spore is associated with a decrease of production of the other spore, we examined ascospore production at day 10 in WIM126 (wild type) and RYJ4 (*ΔspdA*) in YGT medium supplemented with 1.0 mM spermidine, an amount of spermidine yielding significantly more conidia in *ΔspdA* than in wild type. As expected, WIM126 produced a lawn of cleistothecia (the sexual body containing ascospores) where a 1-cm core contained 10^4–10^5 ascospores (Calvo *et al*., 1999). Although RYJ4 produced the same number of cleistothecia, they were barren (data not shown).

Conidiophore initiation but not conidia formation is delayed in A. nidulans *polyamine mutants*

Because all three polyamine requiring strains FGSC682,

Table 3. Diameters and spore densities of *Aspergillus nidulans* strains after 6 days growth on minimal medium.

	Spermidine					
	0 mM		0.05 mM		1.0 mM	
Strains	Diameter (cm)	Spores cm^{-2}	Diameter (cm)	Spores cm^{-2}	Diameter (cm)	Spores cm^{-2}
TPK1.1 (wt)	6.47[a]	6.0×10^7	6.52	5.0×10^7	7.02	5.54×10^7
FGSC682 (*puA2*)	0.40	0	1.12	2.0×10^5	7.10	5.48×10^7
TYJ3.119 (Δ*spdA*)	0.00	0	0.57	0	5.13	8.26×10^7
TYJ3.174 (*puA2*, Δ*spdA*)	0.00	0	0.00	0	3.93	8.62×10^7
$LSD_{0.05}$[b]	0.1869	0.9494	0.1759	0.4747×10^5	0.5244	0.9375×10^7

a. Values are means of three replicates.
b. Means were analysed using Fisher's protected least significant difference at $P = 0.05$.

TYJ3.119 and TYJ3.174 required higher levels of spermidine supplementation for asexual sporulation than for vegetative growth, it appeared that a specific level of spermidine is required for activation of *A. nidulans* conidiation, the process of asexual spore development. To further examine the nature of spermidine requirement in conidiation, we grew FGSC682, TYJ3.119, TYJ3.174 and wild-type strain TPK1.1 on 1.0 mM spermidine plates and recorded the timing of conidiophore initiation and conidia formatioo. Colonies were point inoculated and data only taken from a centre core so the fungal material vas of the same approximate age. Conidiophore development was delayed in all three polyamine mutants (Table 4) with increasing delays in this order: *puA2* > *ΔspdA* > *puA2*; *ΔspdA.* However, once conidiophore differentiation was initiated, the time required for conidia formation (4 h) was identical in all four isolates.

Table 4. Time of conidiophore formation and conidiation of *Aspergillus nidulans* strains grown on minimal medium containing 1.0 mM spermidine.

Strains	Time of conidiophore formation[a] (h)	Time of conidiation[a] (h)
TPK1.1 (wt)	16[b]	20
FGSC682 (*puA2*)	20	24
TYJ3.119 (*ΔspdA*)	26	30
TYJ3.174 (*puA2*, *ΔspdA*)	30	34

a. Hours post incubation in 37°C.
b. Data based on counts of 100 conidiphores or conidia.

Effects of polyamine starvation on spore germination of A. nidulans

Although the *ΔspdA* strains did not grow on media without spermidine, they remained alive for at least 3 days on this media. When transferred to medium supplemented with 1.0 mM spermidine after 3 days, growth and conidiation resumed in these strains. In an effort to determine the developmental step at which spermidine starvation affected the growth process of *A. nidulans*, we grew TYJ3.119 (*ΔspdA*) and TYJ3.174 (*puA2*; *ΔspdA*) and the wild-type strain TPK1.1 in minimal shake cultures containing 0, 0.05, or 1.0 mM spermidine for 24 h at 37°C. Asexual spore germination of both polyamine requiring strains was delayed by ~ 2 h (a spore was considered germinated when the germ tube was the same diameter as the spore, Table 5). However, the per cent germination was not affected.

Microscopic examination of these strains showed that enlarged conidia formed in the two polyamine requiring strains grown in 0.0 and 0.05 mM spermidine media (Fig. 1D–I and data not shown). These cells were not present when TYJ3.119 and TYJ3.174 were grown in 1.0 mM spermidine (Fig. 1J–L and data not shown). The diameter of the enlarged conidia ranged from 8.2 to 13.0 μm whereas wild-type conidia average ~ 3 μm. In medium lacking spermidine, germ tube extension of most of the *ΔspdA* conidia typically slowed at 8–10 h and then stopped shortly thereafter with no further cell division. At the same time germ tube growth slowed (~ 8 h), the germinating spores began to swell. At 12 h, the enlarged conidia were obviously distinct from other spores and mycelia. The growth and differentiation of the enlarged conidia could be restored by adding 1.0 mM exogenous spermidine to the culture.

SpdA mRNA levels do not vary during A. nidulans *conidiation*

As described above, *spdA* and its product are required for vegetative growth as well as for activation of conidiophore formation. To determine if the expression of *spdA* is developmentally regulated, *spdA* transcript was examined in wild-type strain FGSC26 during conidiophore formation. As shown in Fig. 2, a 1.1 kb mRNA corresponding to *spdA* is present and the levels remained relatively constant at all time points examined. This suggests spermidine levels

Table 5. Effect of spermidine starvation on spore germination of *Aspergillus nidulans* strains.

	Strain								
	TPK1.1 (wt)			TYJ3.119 (*ΔspdA*)			TYJ3.174 (*puA2, ΔspdA*)		
Spermidine	0	0.05 mM	1.0 mM	0	0.05 mM	1.0 mM	0	0.05 mM	1.0 mM
Time (h)									
2	1[a]	0	0	0	0	0	0	1	1
4	3	4	7	1	2	0	1	0	1
6	66	68	69	12	10	14	14	12	12
8	93	96	91	56	59	53	58	56	56
10	92	97	93	92	94	96	91	89	91

a. Per cent of germinated spores, 100 spores were counted for each test.

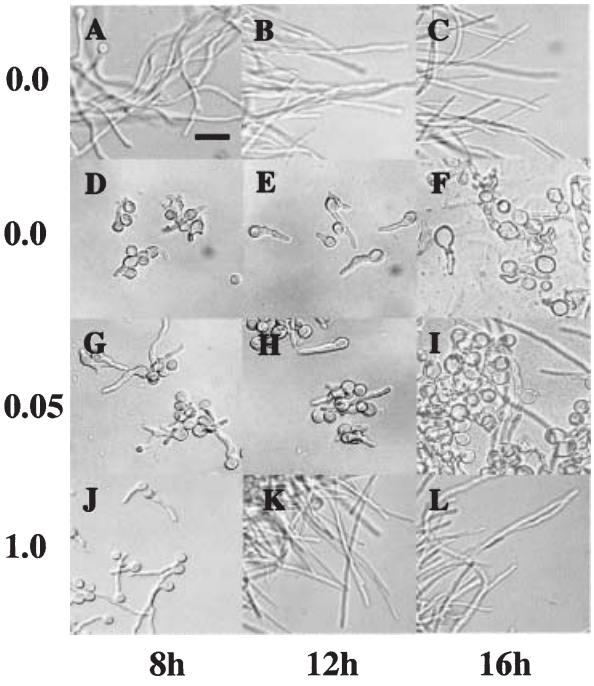

Fig. 1. Spermidine starvation causes formation of enlarged conidia. Strains TPK1.1 (A–C; wild type), TYJ3.119 (D–L; *ΔspdA*) and TYJ3.174 (*puA2*; *ΔspdA*) were grown in shake minimal medium containing 0.0 mM spermidine (A–F), 0.05 mM spermidine (G–I) or 1.0 mM spermidine (J–L). Micrographs shown were taken at 8, 12 and 16 h post inoculation time points. As TYJ3.119 and TYJ3.174 gave identical results, only TYJ3.119 micrographs are shown. The scale bar, shown in A, is 16.0 μm.

are not transcriptionally regulated, at least at this level of detection. This transcript was not detected in the *spdA* disrupted strain TYJ3.119 (data not shown).

Polyamines control sterigmatocystin production in A. nidulans

Having observed the extreme affects that *spdA* disruption has on both vegetative growth and conidiation, we next examined ST production and *stc* gene transcription in TYJ3.119 as these processes are jointly repressed by polyamine inhibitors (Guzman-de-Peña *et al*., 1998). Figure 3 shows that *aflR* and *stcU* are not expressed in the *ΔspdA* strain unless the media is supplemented with ≥0.10 mM spermidine. *stcU,* formerly called *verA* (Keller *et al*., 1994), encodes a highly expressed biosynthetic gene required for ST synthesis. Furthermore, compared with wild type, the expression of *stcU* was delayed in TYJ3.119 in amended medium.

ST production followed a concomitant course as *stcU* expression. TLC data clearly show that, while 0.10 mM and 1.0 mM spermidine allowed the *ΔspdA* strain to produce ST, no ST was produced in lower concentrations of spermidine. Also when ST was produced in TYJ3.119, it was delayed compared with wild type.

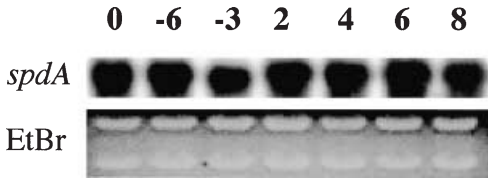

Fig. 2. Northern analysis of the *spdA* mRNA. Wild-type strain FGSC26 was grown in shake minimal medium for 20 h at 37°C and then transferred onto agar plates containing the same medium and incubated at 37°C for the times indicated. Total RNA was isolated from FGSC26 at the time of the mycelial transfer (0 h), −6 h and −3 h before the transfer and 2, 4, 6, 8 h after the transfer and then hybridized with a 0.7 kb *spdA* specific cDNA probe. The lower panel shows equal loading of total RNA as evaluated by ethidium bromide staining.

Complementation of ΔspdA *with a functional copy of the gene restores wild-type phenotype*

To confirm that the characteristics described above are solely due to the deletion of *spdA*, we transformed the *ΔspdA* strain RJY13 with a functional copy of *spdA*. All transformants yielded wild-type growth. Two transformants, TJW28, in which the wild-type *spdA* was introduced at the *trpC* locus, and TJW32, in which the wild-type *spdA* was integrated ectopically, were further analysed. There were no significant difference in colony diameter, conidia or ascospsore production between wild-type RYJ12 and the transformants TJW28 or TJW32 (Table 6 and data not shown). Polyamine analyses of TJW28 and TJW32 showed that they produced similar levels of spermidine and ST as wild type (data not shown). Thus, the *ΔspdA* phenotype appeared to be a direct result of loss of SpdA activity.

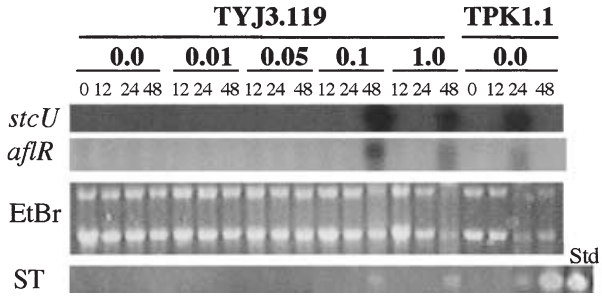

Fig. 3. Spermidine is required for sterigmatocystin biosynthesis. RNA and ST from TPK1.1 (wild type) and TYJ3.119 (*ΔspdA*) were extracted from cultures 0, 12, 24 and 48 h after shifting from 1.0 mM spermidine shake media to shake media containing 0, 0.01, 0.05, 0.10 or 1.0 mM spermidine. A 0.75 kb *Sst*II–*Sma*I fragment from plasmid pRB7 (Yu *et al*., 1996b) was used as a *stcU*-specific probe; a 1.2 kb *aflR* fragment from plasmid pAHK25 (Yu *et al*., 1996b) was used as an *aflR*-specific probe. Loading of total RNA (20 μg lane^{-1}) was evaluated by ethidium bromide staining; ST was separated by TLC and the sample labelled Std is the ST standard (Sigma).

Table 6. Wild-type *spdA* allele rescues the *ΔspdA* phenotype.

Strains	0 mM Spermidine		1.0 mM Spermidine	
	Diameter (cm)	Spores cm^{-2}	Diameter (cm)	Spores cm^{-2}
RYJ12 (wt)	7.425[a]	9.10×10^7	7.825	8.50×10^7
RYJ11 (Δ*spdA*)	0.000	0.0	4.225	17.5×10^7
TJW 28 (Δ*spdA*; *spdA::trpC*)	7.700	9.00×10^7	7.950	8.39×10^7
TJW 32 (Δ*spdA*; *spdA::trpC*)	7.800	9.06×10^7	8.000	$8.30 \times 1\ 0^7$
LSD 0.05[b]	0.1316	0.5299×10^7	0.7342	$0.8563 \times 1\ 0^7$

a. Values are means of three replicates. Diameters and spore densities of *Aspergillus nidulans* strains were calculated after 6 days growth on minimal medium.
b. Means were analysed by using Fisher's protected least significant differences at $P = 0.05$.

Requirement of spermidine for suppression of a G protein signalling pathway

The loss of asexual sporulation and ST production in the *ΔspdA* strains is not unique to this genetic lesion. Mutants in genes (e.g. *fluG* and *fadA*) involved in a G protein signalling pathway in *A. nidulans* are also defective in sporulation, *stc* gene expression and ST production (Hicks *et al*., 1997). However, sporulation and/or *stc* gene expression and ST production can be restored in these mutants by overexpressing *flbA* under the control of the threonine-inducible promoter, *alcA* (Hicks *et al*., 1997; K. Shimizu, J. K. Hicks and N. P. Keller, unpublished data). Overexpression of *flbA* results in decreased activity of FadA – and presumably other Gα proteins – through facilitation of endogenous GTPase activity. We were interested to ask if overexpressing *flbA* in a *ΔspdA* background could restore conidiation and ST production in the spermidine mutant.

To test this, near-isogenic strains TPK1.3, RYJ2 (both wild-type *spdA*) and TYJ3.35 (*ΔspdA*), containing an inducible *flbA* gene, were examined for conidiophore development and ST production in 0.0, 0.05 or 0.10 mM spermidine. As expected, TPK1.3 and RYJ2 produced conidiophores (Fig. 4A–C and data not shown) and ST (Fig. 5) at all spermidine concentrations when grown in *flbA* inducing conditions. In contrast, *flbA* overexpression did not restore development and ST production in TYJ3.35 grown in media lacking spermidine (Fig. 4D–F and data not shown). However, conidiophore development and ST production were restored in TYJ3.35 when the strain was grown in 0.05 and 0.10 mM spermidine (Fig. 4G–I and Fig. 5 and data not shown). Conidiophore vesicles and sterigmata were observed by 12 h after *flbA* induction in TYJ3.35 in 0.05 spermidine minimal medium (Fig. 4H) and complete conidiophores were observed by 24 h post induction (Fig. 4I). This was the same time frame for development in wild types TPK1.3 and RYJ2.

mRNA analysis confirmed visual and TLC results. Figure 5 shows that *brlA* and *stcU* had accumulated in TYJ3.35 after 12 h post induction in 0.05 mM spermidine but that these transcripts were not present in this strain when grown in media without spermidine. As expected, both *brlA* and *stcU* were expressed in TPK1.3 and RYJ2 in media without spermidine. Interestingly, *alcA* expression in TYJ3.35 required no addition of spermidine.

Discussion

The absolute requirement for polyamines in growth and development in eukaryotic cells has driven the interest in understanding the actual molecular functions of the polyamines in biological processes. The broad physiolog-

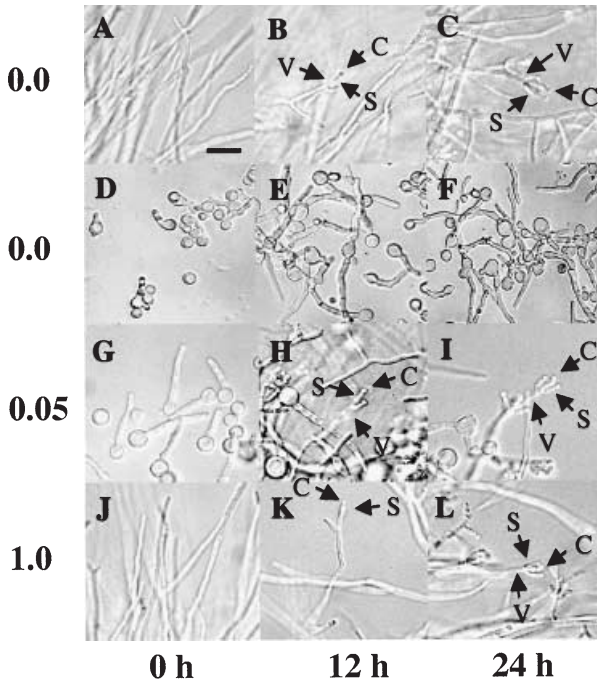

Fig. 4. Overexpression of *flbA* causes conidiophore development in the *ΔspdA* mutant. TPK1.3 (A–C: *alcA*(*p*)*::flbA*) and TYJ3.35 (D–L: *alcA*(*p*)*::flbA*; *ΔspdA*) were grown for 18 h in *alcA*(*p*) repressing shake medium (glucose) containing 0 mM (A–F), 0.05 mM (G–I) or 1.0 mM spermidine (J–L) and then shifted to *alcA*(*p*)-inducing shake medium (threonine) with the same spermidine concentrations. Cultures were observed and photographed at the time of the shift (0 h; A, D, G and J), 12 h (B, E, H and K) and 24 h (C, F, I and L) after *alcA*(*p*) induction. The scale bar in A is 16.0 μm and all other panels are the same scale. V, vesicle; S, sterigmata; C, conidia.

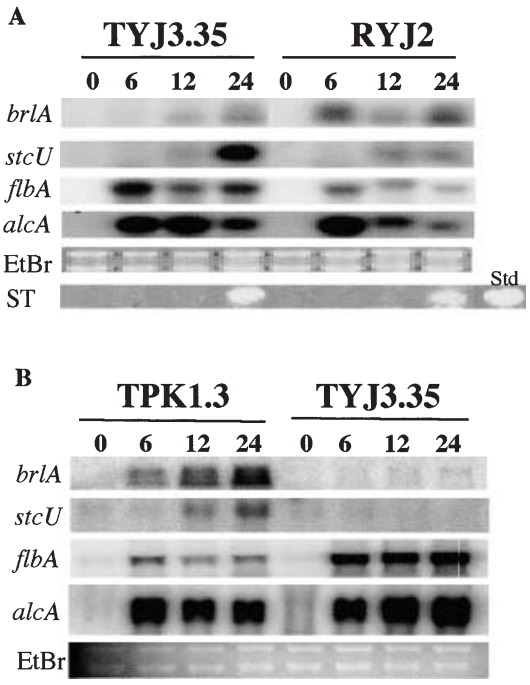

Fig. 5. Activation of *brlA and stcU* by overexpression of *flbA* in the *ΔspdA* strain requires 0.05 mM spermidine.
A. Strains grown in 0.05 mM spermidine.
B. Strains grown in 0.0 mM spermidine. Total RNA from RYJ2 (*alcA*(*p*)::*flbA*), TPK1.3 (*alcA*(*p*)::*flbA*) and TYJ3.35 (*alcA*(*p*)::*flbA*; *ΔspdA*) was isolated from cultures 0, 6, 12 and 24 h after shifting from glucose shake medium to *alcA*-inducing shake medium. A 1.9 kb *Sal*I–*Hind*II fragment from pJA139 was used as a *brlA*-specific probe; a 0.75 kb *Sst*II–*Sma*I fragment from plasmid pRB7 (Yu *et al*., 1996b) was used as an *stcU*-specific probe; a 2.5 kb *EcoR*I fragment from pBN30 was used as an *flbA*-specific probe; plasmid pJA1 (Adams and Timberlake, 1990) was used as an *alcA*-specific probe. Equal loading of total RNA (20 μg lane^{-1}) was evaluated by ethidium bromide staining. ST was separated by TLC and the sample labelled Std is the ST standard (Sigma).

ical effects of polyamines suggest that these compounds may have profound intracellular regulatory properties. Although spermidine has been found to be the major polyamine in most fungi (Tabor and Tabor, 1985; Walter *et al*., 1997), the exact role of spermidine and its importance in fungal growth and development remains unclear. In our studies, we cloned and characterized the *A. nidulans spdA* gene that encodes spermidine synthase, the enzyme required to produce spermidine. Our results demonstrate that in the filamentous fungus *A. nidulans*, spermidine is an essential polyamine for viability and suggest that certain threshold levels of spermidine are required for specific *A. nidulans* developmental processes.

Aspergillus nidulans follows a well-defined programme of development. Wild-type conidia placed on solid medium initially germinate, produce germ tubes which branch to produce vegetative hyphae followed by aerial hyphae and then conidiophores. When grown in liquid medium the conidia germinate, giving rise to germ tubes that branch to produce mycelia but not conidiophores. Our results indicate that spermidine plays a significant role in regulating these developmental transitions in this organism. At different stages of development, the level of polyamines determines the progression through to the next stage of development. We propose that several intracellular spermidine threshold levels exist. One level is that concentration (~0.05 mM exogenous spermidine) required for development to proceed past germ tube formation and another level is that concentration (greater than 0.05 mM exogenous spermidine) required for development to proceed past vegetative hyphal growth to conidiophore and ST production. Results from the overexpression of *flbA* also indicate a requirement for a minimal amount of spermidine to allow FlbA activation of asexual sporulation and ST biosynthesis to proceed. Polyamine threshold levels were also observed for *U. maydis* (Guevara-Olvera *et al*., 1997). When putrescine concentrations lower than 0.5 mM were employed, the *U. maydis odc* null mutant grew at a normal rate but was unable to engage in the yeast-to-mycelium dimorphic transition. Reversion to normal dimorphic phenotype required higher concentrations of putrescine or spermidine (Guevara-Olvera *et al*., 1997).

Not all *Aspergillus* developmental processes were sensitive to spermidine availability. The rate of conidial germination, mycelial formation and conidiophore initiation in the *ΔspdA* strains are delayed by spermidine starvation, while the time of conidial development following conidiophore formation, per cent conidial germination and percent germ tube emergence were the same as those of wild type (Tables 5 and 6). This result is consistent with previous observations that conidial germination and appressorial formation of *Uromyces viciae-fabae* (Reiz *et al*., 1995) and *Magnaporthe grisea* (Choi *et al*., 1997) are unaffected when treated with DFMO whereas somatic hypha growth, conidiophore formation and conidiation were affected. Apparently polyamines differentially affect various steps in fungal development.

Neither vegetative growth nor conidiation of the *ΔspdA* strains, either TYJ3.119 (*ΔspdA*) or TYJ3.174 (*puA2*, *ΔspdA*), could be returned to wild-type phenotype by exogenous spermidine (Tables 2 and 3). Interestingly, the *ΔspdA* mutant had greater asexual spore densities than wild type but could not be compensated for ascospore production when grown on 1 mM spermidine. The alteration in asexual to sexual spore production in the supplemented *ΔspdA* strain was reminiscent of *Aspergillus* response to sporogenic fatty acids (Champe *et al*., 1987; Calvo *et al*., 1999). These developmental aberrations could be a reflection of an altered spermidine to putrescine ratio as alterations in this ratio affect mycelial growth versus conidiation in *A. flavus* (Khurana *et al*., 1996) and developmental switches in other fungi (Shapira *et al*., 1989; Guevara-Olvera *et al*., 1997). Exogenous

spermidine is known to reduce the rate of ornithine decarboxylase synthesis and increase the rate of ornithine decarboxylase protein degradation in many organisms including fungi (Barnett *et al*., 1988; Gupta *et al*., 2001). Perhaps the exogenous spermidine led to a perturbation in putrescine production that when coupled with the *ΔspdA* allele altered polyamine ratios in a manner leading to alterations in production of spore type.

Another interesting morphological variation of the *ΔspdA* and *ΔspdA*; *puA2* strains was the formation of enlarged conidia when these mutants were grown in liquid shake medium (Figs 1 and 4). We found that these enlarged conidia originated from spores after they had germinated. The appearance of these cells was similar to swollen spores produced by an *A. nidulans rasA* mutant, *rasA*^{G17A}, where development is arrested (Som and Kolaparthi, 1994). When stained with DAPI, both the *rasA*^{G17A} and the *ΔspdA* germlings showed multiple nuclei in the conidium and inhibition of cell division (Som and Kolaparthi, 1994 and data not shown), a condition indicative of cell cycle arrest. Spermidine may be important in cell division as it has been shown to remediate spermine-induced cell cycle arrest in *Chlamydomonas* (Theiss *et al*., 2002). It is possible that spermidine is important in cell cycle progression in *A. nidulans* as cell division was resumed in the *ΔspdA* strains upon addition of 1.0 mM spermidine.

As described in the introduction, polyamines affect many proteins in signal transduction pathways (Wright and Boyle, 1982; Bueb *et al*., 1991; 1992; Orlowski, 1995; Choi *et al*., 1997; Kubota *et al*., 1997; Leroy *et al*., 1997), including G α proteins. Although we did not test activity of signalling proteins or concentration of second messengers in our study, the results presented in this communication indicate that a relationship exists between G protein signalling control and polyamine control of sporulation and ST biosynthesis. Overexpression of *flbA*, leading to the suppression of G protein activity (Lee and Adams, 1994; Hicks *et al*., 1997), could force asexual spore and ST production in the *ΔspdA* strain TYJ3.35 but this was dependent on the concentration of spermidine in the medium. ST production and asexual development could not be forced in TYJ3.35 grown in medium lacking spermidine. Yet development and ST production was forced in TYJ3.35 grown in a spermidine concentration (0.05 mM) that did not support sporulation and ST biosynthesis in the *ΔspdA* strains containing a wild-type *flbA* allele. It appears that a certain level of spermidine – less than required for ST and conidiophore development – is required for normal function of the FlbA/FadA signalling pathway. Moreover, microscopic observations of TYJ3.35 in 0.05 mM spermidine showed that the conidiophores emerged from matured mycelia and not the enlarged conidia (Fig. 4). This suggests that conidiophores cannot arise from germ tube or spore tissue and that some level of cellular maturity, in part controlled by spermidine levels, is required before suppression of the G protein pathway can instigate asexual development. Our interest is to continue to characterize the requirements of polyamines in *Aspergillus* development and further explore the interaction of polyamines and signal transduction processes in this genus. Further knowledge of this interaction could be fruitful in developing means to control fungal sporulation and mycotoxin biosynthesis.

Experimental procedures

Fungal strains and growth conditions

Table 7 lists all fungal strains used in this study. Some strains are not discussed in text but used for sexual crosses to obtain the strains of interest. Sexual crosses of *A. nidulans* strains were conducted according to Pontecorvo *et al*. (1953). All strains were maintained as silica stocks and/or glycerol stocks and were grown at 37°C on minimal medium (Cove, 1976) amended with appropriate supplements (Hicks *et al*., 1997).

Cloning of the A. nidulans spdA *gene*

Examination of expressed sequence tag (EST) sequences from the *A. nidulans* genome project (http://www.genome.ou.edu/fungal.html) identified an EST clone, e4a02a1, that showed homology to spermidine synthases. Using primers designed from e4a02a1, a DNA fragment was amplified from *A. nidulans* genomic DNA. This fragment was sequenced to confirm spermidine synthase identity and then used to probe an *A. nidulans* cosmid library. One cosmid located on chromosome VIII, pW30B01, was identified. The *spdA* containing cosmid pW30B01 was digested with *Xho*I and probed by hybridization with the labelled *spdA* PCR product. A 5.2 kb DNA fragment which hybridized to the *spdA* probe was subcloned in pYJ1 to form pYJ2. pYJ1 is a modified pBluescript KS vector in which the *Sac*I and *Sma*I sites were eliminated.

DNA sequence analysis

The 37.3 kb cosmid pW30B01 was sequenced and is available on the OU-ACGT website (Lewis *et al*., 1998). The sequence of pYJ2 was compared to the nucleotide sequence of pW30B01, and the size of the spdA gene was determined by comparing with the spermidine synthase genes of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The deduced amino acid sequence of SpdA was determined by analysis of the translational products of genomic and cDNA clones, using the CLUSTALX software system from NCBI. The GenBank number of this sequence is AY050641.

Construction of the A. nidulans *spdA vectors*

Plasmids were generated using standard techniques. pYJ2 was digested with *Sph*I and *Sac*I to remove the 0.2 kb inter-

Table 7. *Aspergillus nidulans* strains used in this study.

Fungal strains	Genotype	Source
FGSC26	*biA1; veA1*	FGSC[a]
FGSC682	*wA3, mauB4, puA2; veA1*	FGSC
PW1	*biA1; argB2; methG1; veA1*	P. Weglenski
RBN138	*wA3; pyroA4; veA1, alcA(p)::flbA::trpC*	Lee and Adams (1994)
RJH26	*biA1; argB2; ΔstcE::argB; veA1*	J. Hicks
RYJ2	*biA1; wA3; argB2; methG1; veA1, alcA(p)::flbA::trpC*	This study
RYJ4	*pabaA1, yA2; argB2; spdA::argB*	This study
RYJ6	*biA1; wA3, puA2; argB2; methG1*	This study
RYJ11	*biA1; argB2; ΔstcE::argB, ΔspdA::argB, veA1*	This study
RYJ12	*biA1; argB2; ΔstcE::argB, veA1*	This study
RYJ13	*biA1; argB2; ΔstcE::argB, ΔspdA::argB, veA1, trpC801*	This study
TJW28	*biA1; argB2; ΔstcE::argB, ΔspdA::argB, veA1, spdA::trpC*	This study
TJW32	*biA1; argB2; ΔstcE::argB, ΔspdA::argB; spdA, trpC; veA1, trpC801*	This study
TPK1.1	*biA1; methG1; veA1*	This study
TPK1.3	biA1; *wA3; argB2; methG1; veA1, alcA(p)::flbA::trpC*	This study
TYJ3.35	biA1; *wA3; argB2; methG1; ΔspdA::argB, veA1, alcA(p)::flbA::trpC*	This study
TYJ3.119	*biA1; argB2; methG1; ΔspdA::argB, veA1*	This study
TYJ3.174	*biA1; wA3, puA2; argB2; methG1; ΔspdA::argB, veA1*	This study
WIM126	*pabaA1, yA2*	L. Yager

a. Fungal Genetics Stock Center.

nal *Sph*I–*Sac*I fragment of the *spdA* ORF. The 0.2 kb fragment was replaced by the 1.8 kb *argB* fragment obtained by digesting pJY3 with *Sma*I. pJY3 was created by moving the 1.8 kb *Sma*I *argB* fragment from pJYArgB2 (Shimizu and Keller, 2001) into pK19 (Pridmore, 1987). The resulting plasmid pYJ3, the *spdA* disruption construct, was made by blunt-end ligating of the *Sph*I–*Sac*I digested pYJ2 and the 1.8 kb *argB* fragment. pYJ26-4, containing the *spdA* gene in a 1/2 *trpC* vector (to direct the gene to the *trpC* site), was created by ligating the 5.2 kb *Xho*I *spdA* containing fragment from pYJ2 into *Xho*I-digested pSH96 (Wieser and Adams, 1995).

Fungal transformation procedures *spdA* disruption strains TYJ3.119, TYJ3.35 and TYJ3.174 were generated by the following steps. First, pYJ3 was digested with *Pvu*I and the obtained linear DNA fragment was utilized to transform the *A. nidulans* strain PW1 to create TYJ3.119, strain RYJ2 to create TYJ3.35 and strain RYJ6 to create TYJ3.174. RYJ2 was generated by crossing RBN138 to PW1. RYJ6 was generated by crossing FGSC682 to PW1. Transformation, extraction of DNA from transformants and wild-type DNA, restriction enzyme digestion, gel electrophoresis, Southern blotting and hybridization were performed using standard methods (Miller *et al*., 1985; Sambrook *et al*., 1989). To facilitate isolation of the *spdA* disruptants, all the transformants were tested on two types of minimal medium plates, one supplemented with 1.0 mM spermidine and one with 0.1 mM spermidine.

Complementation of *ΔspdA* strains was achieved by transforming them with a wild-type copy of the *spdA* gene. First TYJ3.119 was crossed to RJH26 to create RYJ13. RYJ13 was co-transformed with pYJ2 (containing the wild-type *spdA* gene) and pTA11 (containing the entire trpC on a 4.4 kb *Xho*I fragment cloned into pIC19H, Marsh *et al*., 1984) to produce TJW32. RYJ13 was also transformed with pYJ26-4 (a plasmid containing the wild-type *spdA* gene ligated to the half *trpC* gene) to produce TJW28.

Polyamine analysis of Aspergillus *strains*

Polyamines were calculated for *A. nidulans* wild-type TPK1.1, the polyamine requiring strains FGSC682, TYJ3.119 and TYJ3.174, and the two complemented strains TJW28 and TJW32. All strains were grown on solid minimal medium supplemented with 0.0, 0.1, 0.5, 1.0 or 3.0 mM spermidine. Each test was repeated twice.

Spores for inoculation were obtained from 1.0 mM spermidine plates. Four hundred microlitres of 2.3×10^9 spores ml^{-1} were spread on a 0.45 μ millipore membrane placed on top of the solid media, two plates per strain. Plates were incubated 19 h at 37°C. From each plate, one half of the mycelium was used for dry weight and protein determination and the other half was used for polyamine analysis using wet mycelium.

Polyamine extraction followed that of San-Blas *et al.* (1996). Mycelium was filtered and washed twice with distilled water. Then samples were extracted with 2 ml of 6% perchloric acid for 3 h at room temperature and centrifuged at 1500 *g*. The supernatant was placed in glass tubes and air dried in an oven at 80°C. The product was resuspended in 1 ml of 6% perchloric acid and filtered through a 0.45 μ millipore membrane. Chemically unconjugated polyamines were determined by adding 1 ml of 2 M NaOH and 10 μl of benzoyl chloride to 0.5 ml of the perchloric acid sample. The mixture was shaken vigorously and incubated at room temperature for 10 min. The reaction was stopped with 2 ml of saturated NaCl. This mixture was extracted with 4 ml of diethylether. The ether phase was recovered, evaporated in a water bath until dry and 200 μl of HPLC-grade methanol was added to each tube to recover the benzoylated derivatives. Samples were filtered through a C18 column (QC 498 Alltech) and then analysed by HPLC using an ODS-C18 (4.6 mm × 150 mm) column. The solvent system was isocratic 50% methanol–water. The column temperature was 35°C and UV detector was fixed at 254 nm. Benzoylated

putrescine, spermidine and spermine were used as external standards. Polyamine concentrations were calculated from a standard curve and expressed as μmol mg^{-1} protein.

Thin-layer chromatography analysis

Sterigmatocystin production of *A. nidulans* wild-type strains and polyamine-deficient strains were examined by TLC analysis. Sterigmatocystin was extracted from either shake cultures or stationary cultures using published procedures (Keller *et al*., 1994; Hicks *et al*., 1997). For solid media, a cork borer with diameter of 1.1 cm was used to collect samples at the centres of these plates. Each sample was homogenized and mixed well with 2 ml of double distilled H_2O. Then 2 ml of $CHCl_3$ was added and agitated with a vortex. After centrifugation for 5 min at 1000 r.p.m., the separated organic phase was transferred to an Eppendorf tube. All samples were dried down and resuspended in 100 μl of $CHCl_3$. Twenty-five μl of each extract was spotted on a TLC plate and separated in toluene–ethyl acetate–formic acid (40 : 60 : 0.5). The TLC plates were sprayed with aluminium chloride to enhance ST fluorescence upon exposure to longwave (365 nm) UV light (Stalk and Rodricks, 1971).

For cultures grown in liquid media, mycelia were lyophilized and then weighed. Lyophilized mycelium (0.02 g) of each sample was ground into fine powder. Four hundred microlitres of acetone was added to the powder, mixed well with a vortex and centrifuged for 10 min at 7000 r.p.m. Supernatants (350 μl) were transferred to new tubes and 30 μl of each extract was separated on a TLC plate for ST analysis.

Time course study of spdA *mRNA during fungal development*

Total RNA of *A. nidulans* strains was extracted with Trizol (Gibco BRL). Mycelium was collected, lyophilized in liquid nitrogen, and pulverized, then total RNA was extracted following the manufacturers recommended procedure.

Wild-type strains, FGSC26 and RAMC22, as well as the *spdA* disruption strain, TYJ3.119, were inoculated at a density of 10^5 spores ml^{-1} in liquid minimal medium containing appropriate supplements, and shaken at 300 r.p.m. at 37°C for 20 h. Mycelia were harvested on miracloth, and transferred onto Fisher P8 filter paper disks placed on 1.2% agar minimal medium and incubated at 37°C. Samples were taken at the time of the mycelia transfer (0 h) and −6, −3, 2, 4, 6, 8 h before or after transfer for microscopic observation and RNA analysis.

Morphological development of polyamine mutant strains

Three microlitres of 10^5 spores ml^{-1} of *A. nidulans* wild-type strain TPK1.1 and the *spdA* disrupted strains TYJ3.119 and TYJ3.174 were inoculated in the centres of plates supplemented with one of the following concentrations of polyamines: 0.0 polyamine; 0.10, 1.0 or 3.0 mM spermidine; 0.10, 1.0 or 3.0 mM putrescine; 0.10, 1.0 or 3.0 mM spermine. The diameters of colonies were measured and conidia (the asexual spore of *A. nidulans*) were counted after 72 h incubation at 37°C. Tests were performed in triplicates and analysed by standard *t*-tests.

For microscopic observations, *A. nidulans* polyamine requiring strains FGSC682, TYJ3.119, TYJ3.174 and wild-type strain TPK1.1 were inoculated in minimal medium containing 0, 0.05 or 1.0 mM spermidine at 10^6 spores ml^{-1} and shaken for 24 h at 300 r.p.m. at 37°C. Samples were examined at 2 h intervals. Germination rate was determined by counting 100 conidia. Pictures of fungal development were taken at 8, 12 and 16 h after inoculation.

For TPK1.3 and TYJ3.35 (strains overexpressing *flbA* in the presence of threonine), microscopic observations were made at 0, 12 and 24 h after these strains were transferred to threonine media (for details, see *Overexpression of* flbA *in the* A. nidulans spdA *mutant strain*).

Effect of polyamine starvation on differentiation and ST production of A. nidulans

Three μl of 10^5 spores ml^{-1} spores of *A. nidulans* wild-type TPK1.1 and polyamine requiring strains FGSC682, TYJ3.119 and TYJ3.174 were inoculated on the centres of minimal medium agar plates containing 0.0, 0.05, 0.10 or 1.0 mM spermidine. The diameters of colonies were measured and conidia were counted after 6 days (144 h) incubation at 37°C. Tests were performed in triplicate and analysed by standard *t*-tests.

Overexpression of flbA *in the* A. nidulans spdA *mutant strain*

TYJ3.35 was constructed by transformation of RYJ2 with pYJ3, whereas TPK1.3 was generated by transformation of RYJ2 with the *argB*-containing plasmid pPK1. Spore suspensions of both strains were filtered through miracloth to remove conidiophores and mycelium. Then 2×10^6 spores ml^{-1} of each strain was inoculated in 500 ml of minimal medium (1% glucose as carbon source) containing 0.0, 0.05 or 1.0 mM spermidine and shaken at 300 r.p.m. at 37°C for 18 h. Mycelium was collected onto miracloth, washed once with minimal medium lacking glucose, divided into equal parts, transferred to flasks containing 100 ml of minimal medium with 100 mM L-threonine as the sole carbon source or 1% glucose as the sole carbon source and shaken for 48 h at 37°C. The same concentration of spermidine was included in the transfer media. For medium with L-threonine as sole carbon, samples were harvested for RNA analysis at the time of the medium shift (0 h) and 6, 12 and 24 h after the shift. For glucose medium, samples were harvested for RNA analysis at time of the medium shift (0 h) and 6, 12, 24, 48 and 72 h after the shift. Sterigmatocystin was also examined in these samples as described above.

Acknowledgements

Yuan Jin and Jin Woo Bok contributed equally to this manuscript. This research was partly funded by USAID under the Peanut CRSP Grant LAG-G-00-96-90013-00 and by NSF Grant MCB-9874646 to N.P.K. Centro de Investigacion y Estudios Avanzados del IPN funded DG. We acknowledge

technical help and useful discussions with our colleagues Drs Daren Brown and Doris Kupfer.

References

Adams, T.H., and Timberlake, W.E. (1990) Developmental repression of growth and gene expression in *Aspergillus*. *Proc Natl Acad Sci USA* **87:** 5405–5409.

Barnett, G.R., Seyfzadeh, M., and Davis, R.H. (1988) Putrescine and spermidine control degradation and synthesis of ornithine decarboxylase in *Neurospora crassa*. *J Biol Chem* **263:** 10005–10008.

Barrel, B.G., Rajandream, M.A., and Walsh, W.V. (1995) *Schizosaccharomyces pombe chromosome I sequencing project.* Vol GenBank Z54140. Sanger Centre, Hinxton Hall, Hinxton, Cambridge, UK.

Bueb, J.L., Mousli, M., and Landry, Y. (1991) Molecular basis for cellular effects of naturally occurring polyamines. *Agents Actions* **33:** 84–87.

Bueb, J.L., Da Silva, A., Mousli, M., and Landry, Y. (1992) Natural polyamines stimulate G-proteins. *Biochem J* **282:** 545–550.

Calvo, A., Hinze, L., Gardner, H.W., and Keller, N.P. (1999) Sporogenic effect of polyunsaturated fatty acids on *Aspergillus* spp. development. *Appl Environ Microbiol* **65:** 3668–3673.

Champe, S.P., Rao, P., and Chang, A. (1987) An endogenous inducer of sexual development in *Aspergillus nidulans*. *J Gen Microbiol* **133:** 1383–1388.

Choi, W.B., Kang, S.H., Lee, Y.W., and Lee, Y.H. (1997) Cyclic AMP restores appressorium formation inhibited by polyamines in *Magnaporthe grisea*. *Biochem Cell Biol* **88:** 58–61.

Cove, D.J. (1976) Chlorate toxicity in *Aspergillus nidulans*. Studies of mutants altered in nitrate assimilation. *Mol Gen Genet* **146:** 147–159.

Guevara-Olvera, L., Calvo-Mendez, C., and Ruiz-Herrera, J. (1993) The role of polyamine metabolism in dimorphism of *Yarrowia lipolytica*. *J Gen Microbiol* **139:** 485–493.

Guevara-Olvera, L., Xoconostle-Cazares, B., and Ruiz-Herrera, J. (1997) Cloning and disruption of the ornithine decarboxylase gene of *Ustilago maydis*: evidence for a role of polyamines in its dimorphic transition. *Microbiology* **143:** 2237–2245.

Gupta, R., Hamasaki-Katagiri, N., Tabor, C.W., and Tabor, H. (2001) Effect of spermidine on the in vivo degradation of ornithine decarboxylase in *Saccharomyces cereviseae*. *Proc Natl Acad Sci USA* **98:** 10620–10623.

Guzman-de-Peña, D., and Ruiz-Herrera, J. (1997) Relationship between aflatoxin biosynthesis and sporulation in *Aspergillus parasiticus*. *Fungal Genet Biol* **21:** 198–205.

Guzman-de-Peña, D., Aguirre, J., and Ruiz-Herrera, J. (1998) Correlation between the regulation of sterigmatocystin biosynthesis and asexual and sexual sporulation in *Emericella nidulans*. *Antonie Van Leeuwenhoek* **73:** 199–205.

Hamasaki-Katagiri, N., Tabor, C.W., and Tabor, H. (1997) Spermidine biosynthesis in *Saccharomyces cerevisiae*: polyamine requirement of a null mutant of the SPE3 gene (spermidine synthase). *Gene* **187:** 35–43.

Hicks, J.K., Yu, J.-H., Keller, N.P., and Adams, T.H. (1997) *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA Gα protein-dependent signalling pathway. *EMBO J* **16:** 4916–4923.

Inderlied, C.B., Cihlar, R.L., and Sypherd, P.S. (1980) Regulation of ornithine decarboxylase during morphogenesis of *Mucor racemosus*. *J Bacteriol* **141:** 699–706.

Keller, N.P., Kantz, N.J., and Adams, T.H. (1994) *Aspergillus nidulans ver*A is required for production of the mycotoxin sterigmatocystin. *Appl Environ Microbiol* **60:** 1444–1450.

Khurana, N., Saxena, R.K., Gupta, R., and Rajam, M.V. (1996) Polyamines as modulators of microcycle conidiation in *Aspergillus flavus*. *Microbiology* **142:** 517–523.

Koenig, H., Goldstone, A., and Lu, C.Y. (1983) Polyamines regulate calcium fluxes in a rapid plasma membrane response. *Nature* **305:** 530–534.

Kubota, S., Kiyosawa, H., Nomura, Y., Yamada, T., and Seyama, Y. (1997) Ornithine decarboxylase overexpression in mouse 10T1/2 fibroblasts: cellular transformation and invasion. *J Natl Cancer Inst* **89:** 67–71.

Lee, B.N., and Adams, T.H. (1994) Overexpression of *flbA*, an early regulator of *Aspergillus* asexual sporulation, leads to activation of *brlA* and premature initiation of development. *Mol Microbiol* **14:** 323–334.

Leroy, D., Heriche, J.K., Filhol, O., Chambaz, E.M., and Cochet, C. (1997) Binding of polyamines to an autonomous domain of the regulatory subunit of protein kinase CK2 induces a conformational change in the holoenzyme. A proposed role for the kinase stimulation. *J Biol Chem* **272:** 20820–20827.

Lewis, J., Kupfer, D.M., Keller, N.P., and Roe, B.A. (1998) *Aspergillus nidulans* Chromosome VIII Cosmid Clone 2h2. http://www.genome.ou.edu/fungal.html.

Lopez, M.C., Garcia, S., Ruiz-Herrera, J., and Dominguez, A. (1997) The ornithine decarboxylase gene from *Candida albicans*. Sequence analysis and expression during dimorphism. *Curr Genet* **32:** 108–114.

Manni, A., Wechter, R., Gilmour, S., Verderame, M.F., Mauger, D., and Demers, L.M. (1997) Ornithine decarboxylase over-expression stimulates mitogen-activated protein kinase and anchorage-independent growth of human breast epithelial cells. *Int J Cancer* **70:** 175–182.

Marsh, J.L., Erfle, M., and Wykes, E.J. (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. *Gene* **32:** 481–485.

Mennucci, L., Rojas, S., and Camargo, E.P. (1975) Polyamines and ornithine decarboxylase activity during growth and differentiation in *Blastocladiella emersonii*. *Biochim Biophys Acta* **404:** 249–256.

Miller, B., Miller, K., and Timberlake, W.E. (1985) Direct and indirect gene replacements in *Aspergillus nidulans*. *Mol Cell Biol* **5:** 1714–1721.

Orlowski, M. (1995) Gene expression in *Mucor* dimorphism. *Can J Bot* **73:** s238–s333.

Pontecorvo, G., Roper, J.A., Hemmons, L.M., MacDonald, K.D., and Bufton, A.W.J. (1953) The genetics of *Aspergillus nidulans*. *Adv Genet* **5:** 141–238.

Prade, R.A., and Timberlake, W.E. (1993) The *Aspergillus nidulans brlA* regulatory locus consists of overlapping transcription units that are individually required for conidiophore development. *EMBO J* **12:** 2439–2447.

Pridmore, R.D. (1987) New and versatile cloning vectors with kanamycin-resistance marker. *Gene* **56:** 309–312.

Reiz, M., Walters, D., and Moerschbacher, B. (1995) Germination and appressorial formation by uredospores of *Uromyces viciae-fabae* exposed to inhibitors of polyamine. *Eur J Plant Pathol* **101:** 573–578.

Reyna-Lopéz, G., and Ruiz-Herrera, J. (1993) Polyamines and the sporogenesis of mucorales. *Exp Mycol* **17:** 79–89.

Ruiz-Herrera, J. (1994) Polyamines, DNA methylation and fungal differentiation. *Crit Rev Microbiol* **20:** 143–150.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning; a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory, New York.

San-Blas, G., San-Blas, F., Sorais, F., Moreno, B., and Ruiz-Herrera, J. (1996) Polyamines in growth and dimorphism of *Paracoccidioides brasiliensis*. *Arch Microbiol* **166:** 411–413.

Shapira, R., Altman, A., Henis, Y., and Chet, I. (1989) Polyamines and ornithine decarboxylase activity during growth and differentiation in *Sclerotium rolfsii*. *J Gen Microbiol* **135:** 1361–1367.

Shimizu, K., and Keller, N.P. (2001) Genetic involvement of a cAMP-dependent protein kinase in a G protein signalling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. *Genetics* **157:** 591–600.

Sneath, P.H. (1955) Putrescine as an essential growth factor for a mutant of *Aspergillus nidulans*. *Nature* **175:** 818.

Som, T., and Kolaparthi, V.S. (1994) Developmental decisions in *Aspergillus nidulans* are modulated by Ras activity. *Mol Cell Biol* **14:** 5333–5348.

Stalk, M., and Rodricks, J.V. (1971) Method for analysis and chemical confirmation of sterigmatocystin. *J Assoc Official Anal Chemist* **54:** 86–90.

Stevens, L. (1975) Ornithine decarboxylase activity in germinating conidia of *Aspergillus nidulans*. *FEBS Lett* **59:** 80–82.

Tabor, C.W., and Tabor, H. (1985) Polyamines in microorganisms. *Microbiol Rev* **49:** 81–99.

Theiss, C., Bohley, P., and Voigt, J. (2002) Regulation by polyamines of ornithine decarboxylase activity and cell division in the unicellular green alga *Chlamydomonas reinhardtii*. *Plant Physiol* **128:** 1470–1479.

Walter, D.R., Cowley, T., and McPherson, A. (1997) Polyamine metabolism in the thermotolerant mesophilic fungus *Aspergillus fumigatus*. *FEMS Microbiol Lett* **153:** 433–437.

Wieser, J., and Adams, T.H. (1995) *flbD* encodes a myb-like DNA binding protein that controls initiation of *Aspergillus nidulans* conidiophore development. *Genes Dev* **9:** 491–502.

Wright, J.M., and Boyle, S.M. (1982) Negative control of ornithine decarboxylase and arginine decarboxylase by adenosine-3′,5′-cyclic monophosphate in *Escherichia coli*. *Mol Gen Genet* **186:** 482–487.

Yu, J.-H., Wieser, J., and Adams, T.H. (1996a) The *Aspergillus* FlbA RGS domain protein antagonizes G-protein signaling to block proliferation and allow development. *EMBO J* **15:** 5184–5190.

Yu, J.-H., Butchko, R.A.E., Fernandes, M., Keller, N.P., Leonard, T.J., and Adams, T.H. (1996b) Conservation of structure and function of the aflatoxin regulatory gene *aflR* from *Aspergillus nidulans and A. flavus*. *Curr Genet* **29:** 549–555.