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Relationship between Aflatoxin Biosynthesis and Sporulation in Aspergillus parasiticus

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Guzmán-de-Peña, D., and Ruiz-Herrera, J. 1997. **Relationship between aflatoxin biosynthesis and sporulation in Aspergillus parasiticus. Fungal Genetics and *Biology* 21, 198–205.** Regulation of aflatoxin biosynthesis during differentiation of Aspergillus parasiticus was analyzed by using a drug that inhibits the development of the fungus and mutants affected in sporulation. Diaminobutanone, a competitive inhibitor of ornithine decarboxylase, repressed spore germination. If added after spore germination had occurred, it blocked sporulation completely and suppressed aflatoxin biosynthesis, but was only partially inhibitory of mycelial growth. Putrescine partially counteracted the inhibitory effect of the drug on both sporulation and aflatoxin biosynthesis. Analysis of mutants affected in sporulation confirmed the existence of a relationship between sporulation and aflatoxin formation. A nonsporulating mutant was unable to synthesize aflatoxins. In a sectorial mutant, the sporulating sector synthesized aflatoxins normally, whereas the asporogenous sector was unable to do so. It is suggested that regulation of aflatoxin biosynthesis is correlated with the sporulation process.

Index Descriptors: Aspergillus parasiticus; aflatoxins; sporulation; diaminobutanone; asporogenous mutants.

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Aflatoxins are secondary metabolites produced by three species of *Aspergillus: Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* (Ellis *et al.*, 1991; Kurtzman *et al.*, 1987). Aflatoxins are potent carcinogenic agents in animals and are suspected of causing cancer in humans (Linsell, 1982). Aflatoxins are produced when *Aspergilli* colonize plants in the field or during storage of agricultura1 commodities, rendering these products unsuitable for animal or human consumption (Ellis *et al.*, 1991; Shotwell, 1986). The production of aflatoxins represents a health hazard and the cause of severe economical losses.

Aflatoxins are polyketide derivatives; the initiation of their synthesis involves the condensation of acetyl and malonyl groups by polyketide synthetase, an enzymatic complex similar to fatty acid synthetase (Dutton, 1988). Recently it has been demonstrated that genes coding for aflatoxin biosynthesis in *Aspergilli* are clustered (Trail *et al.*, 1995b; Yu *et al.*, 1995). Transcription of at least some of these genes (*nor-1* and *ver-1)* is controlled by the gene *aflR*, which codes for a zinc cluster DNA binding protein (Woloshuk *et al.*, 1994). For a recent review see Trail *et al.* (1995a).

Synthesis of aflatoxins depends on strain specificity, culture conditions, and nutritional factors (Luchese and Harrigan, 1993). A large number of coinpounds have been found to inhibit aflatoxin biosynthesis, mainly tlirougli their effect on fungal growth (Zaika and Buchanan, 1987). It has beeii proposed that aflatoxin biosynthesis iiiiglit be related to development in *Aspergillus,* mainly in relation to sclerotia formation (Trail *et al.*, 1995a). Some indirect evidence appears to support such a relationship. First,

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aflatoxins are svnthesized in lower amounts in shake cultures, where no sporulation occurs (Steyn *et al.*, 1980). Second, pigments from asexual and sexual spores of *Aspergillus* are also polyketide derivatives (Brown *et al.*, 199.3; Brown and Salvo, 1994), and thus aflatoxiiis and spore pigment biosynthesis could share regulatory steps. More information exists in *Streptomyces* spp regarding a possihle association between sporulation and formation of seconclary metabolites. Kliokhlov *et al.* (1973) showed that A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) was an autoregulator necessary for botli induction of sporulation and streptomycin production by *Streptomyces griseus*. Mutants altered in A-factor production were simultaneously affected in both processes. Genetic and biochemical evidence has suggested that a coinplex regulatory switch is triggered by A-factor-binding proteins (Horinouchi ancl Beppu, 1992). Hopwood (1988) identified genes whose mutation abolished the formation of aerial mycelium antl secondary metabolites in *Streptomyces coelicolor*. Tliese genes were referred to as "pleiotropic switches." The association between developinent and secondary metabolite synthesis is not fortuitous since genetic control inechanisms for the two processes displayed an important cross-correlation.

In the present study we have analyzed the relationsliip between developinent and aflatoxin synthesis in *A. parasiticus* using two approaches: analysis of mutants affected in sporulation ancl selective inhibition of sporulation. The latter approacli involved the use of 1,4,diamino-2-butanone (DAB), a competitive inhibitor of ornithine decarboxylase (ODC), the first and more regulated enzyme involved in the synthesis of polyamines. We have previously demonstrated that polyamines are essential for differentiation in several fungi (reviewed by Ruiz-Herrera, 1994). In *Aspergillus nidulans*, an increase in the levels of ODC previous to spore gerinination has been demonstrated, suggesting tliat polyainines also play a role in the differentiation of *Aspergilli* (Stevens, 1975). The results herein reported, obtained by the use of DAB ancl mutants of A. *parasiticus* affected in sporulation, sustain the hypothesis that aflatoxin biosynthesis and sporulation are linked phenomena. Tliese results are important for our coiiiprehension of tlie regulation of toxin forination. From a practica1 poiiit of view they suggest a novel possibility to control aflatoxin accumulation under natural conditioiis. A preliminary account of these results has been presented elsewliere (Guzman-de-Peña and Ruiz-Herrera, 1994).

MATERIALS AND METHODS

Strains

The following strains were used: a monosporic isolate from A. *parasiticus* NRKL 2999 antl two rnutants, DGP10 and DGP29, isolated in tlie course of this work (see below). Strains were periodically transferred on slants or plates of PDA (Difco).

Media and Culture Conditions

Unless otherwise indicated, Erlenmeyer flasks (125 ml) containing 25 ml of inducing medium (YEC medium; Papa, 1977) were inoculated with 10^5 spores and incubated under agitation (200 rpm) at 28°C. Incubations proceedetl under normal laboratory illuinination conditions. When germ tubes reaclietl a lerigth about twice the spore diameter (about 7–8 h), tlie flasks were removed from the shaker and placed uiider static conditions for 2–7 days. In experiments where asporogenous mutants were employed, flasks were inoculated with five square pieces (ca. 3 × 3 mm) cut out from tlie inoculum plates of mutants or wild type. Flasks were incubated undisturbed for variable periods at 28°C. Where indicated, filtered-sterilized solutions of DAB and/or putrescine were added at inoculation time or once spore germination had taken place.

To measure the effect of DAB on sporulation iii solid inedia, 8-h-old gernilings were homogeneously distributed over the surface of solid inducing mediuin. Plates were incubated at 28°C, and at intervals, sterile filter paper disks soaked in 50 m*M* DAB were placed over the plates. To determine reversal of DAB effect by putrescine, 8-h-old germlings were clistributed over the surface of solid inducing medium containing 50 m*M* DAB. Plates were incubated at 28°C, and paper filter disks soaked in putrescine solutions of different concentrations were placecl over tlie plates. After 36 h, the plates were observed ancl photographed.

Determination of Spore Numbers and Dry Weight

Mycelium growii iii liquid inediuin was recovered by filtration and resuspended in 10 ml of distilletl water containing 0.1% Triton X-100, transferred to a petri dish, and gently shaken to free tlie spores. After a brief period to allow sedimentation of the mycelium, the supernatant containing tlie spoi-es was carefully recovered. The proce-

dure was repeated once again. The spores were pelleted by centrifugation, resuspended in 1 ml water, and counted with a hemocytometer. Spore-free mycelium was returned to the spent culture medium, extracted with chloroform to recover aflatoxins (see below), washed with distilled water by filtration, and dried at 70–80°C to constant weiglit. Wlien solid medium was used, spores were harvested by flushing the plates with 0.1% Triton solution followed by centrifugation. Agar plates were extracted with chloroforin for 30 min, chloroform was recovered, water was added to the plates, and tlie plates were heated in a microwave oven to liquefy the agar. The mycelium was washed with hot water, blotted dry, dried, and weighed.

Determination of Aflatoxins

Cultures treated as descnbed above were extracted witli twice their volume of chloroforin. Aflatoxins were partially purified from the chloroform layers by the procedure previously described (Cuzman-de-Peña *et al.*, 1992). In some experiments aflatoxins were separated by TLC. Silica gel plates were developed with choroform:acetone:water (85:15:1.5, by vol.), dried, and examined under a UV light lamp. Quantitative determination of aflatoxins was done by HPLC, using a Zorbax LC18 column (DuPont). The mobile phase was a mixture of water:acetonitrile:methanol (45:15:40, by vol.). Elution of aflatoxins was recorded at 364 nm. Standard solutions of aflatoxins B_1, B_2, G_1, and G_2 were run under the same conditions. Data were expressed per total culture medium. The total amount, the types of aflatoxins that accumulated, and their ratios varied from one experiment to the next. All experiments were repeated at least three times with duplicate or triplicate samples. Only reproducible results are reported.

Mutagenesis and Mutant Isolation

Spores from the wild-type strain were washed twice with sterile distilled water, pelleted by centrifugation, and then resuspended in sterile 50 m*M* citrate buffer, pH 5.5, at a final density of 3×10^6 spores per milliliter. Spore suspensions (0.9 ml) were mixed with *N*-methyl-*N*′-nitro-N-nitrosoguanidine (0.1 ml, 0.25 ing per ml) and incubated in darkness for 45 min. Sodium thiosulfate (5%) was added, and the spores were sedimented by centrifugation and washed twice with 50 m*M* phosphate buffer, pH 7.0. Under these coiiditions, about 10% survival was observed. Spores were inoculated oii PDA (Difco) plates containing 0.1% Triton and incubated at 28°C. Nonsporulating and white colonies were selected. From about 10,000 colonies scored, 30 nonsporulating or white colonies were detected. Most of tliein revealecl a delayed plienotype; after prolonged incubation there were differeiitiating green spores. Two stable strains were finally recovered: DGP10, a nonsporulating strain, similar in plienotype to *brl*⁻ mutants froin *A. nidulans* (Clutterbuck, 1969). and DGP29, a inutant forining sectorial colonies where green spore-bearing sectors alternated with wliite spore-less sectors.

RESULTS

Aflatoxin Synthesis in Agitated Liquid Cultures versus Solid Media

It has been descnbed (Coldblatt, 1968; Steyn *et al.*, 1980) that *A. flavus* synthesizes less aflatoxins in shaken than in stationary cultures. We entertained the possibility that this phenomenon was related to sporogenesis, and proceeded to compare the levels of aflatoxins made by *A. parasiticus* in shaken and solid inedia. We tested different volumes of medium in Erlenmeyer flasks of the same size to obtain variable aeration of the mediuin. In the liquid cultures, we took special precautions to avoid (by constant inanipulation) a ring of stationary-type growth from mycelium that clings to the glass above the liquid bouridary, especially when low levels of liquid medium are used. This precaution is usually overlooked and may explain some variable results. Data show that, contrary to solid medium (Table 1), only traces of aflatoxins were synthesized by *A. parasiticus* in shake cultures (200 rpm). An iniportant observation was that the lower the ratio between flask volume and medium volume, the less amounts of aflatoxins were made. In fact, practically no aflatoxin was synthesized wlien the medium volume was increased to 100 ml, conditions under which attachment of the myceliuin to the flask walls was miniinal.

Effecro f DAB on Spore Germination and Sporulation

We tested the effect of tlie drug on the gerinination of conidia of *A. parasiticus* in the liquid complex medium described under Materials and Methods. In agreement with data from Stevens *et al.* (1977) witli *A. nidulans,* no effect on germination of spores froin *A. parasiticus* was obtained by DAB concentrations lower than 1 m*M*. On thr other hand, 10 m*M* DAB inhibited spore gerinination by 35%, and 50 m*M* DAB inhibited it by more than 90%.

TABLE 1

Aflatoxiii Biosynthesis by A *parasiticus* in Shake Cultures and Solid Media

Culture conditioiis	Volume (ml)	Growth (mg D.W.[a])	Spores ($\times 10^{-8}$)	Aflatoxins (~1R) B_1	G_1	Total	Prr mg D.W.
Shaken liquid	25	147	0	4.3	0	4.3	0.029
	50	173	0	2.3	0	2 3	0.013
	100	158	0	0.1	0	0.1	0.001
Solid	50	1575	7.1	221.0	400	621.0	0.394

Note. Liquid YEG medium dispensed into 125-ml Erlenmeyer flasks or solid medium in 50-m1 petri dishes was inoculated with the same number of spores (10^5). Flasks containing liquid medium were incubated in a water bath at 28°C and 200 rpm, and petri dishes were incubated under static conditions at the same temperature. After 2 days of incubation, cultures were harvested and analyzed. Data are expressed per plate or flask. Average of duplicate samples.

[a] Dry weight

FIG. 1. Inhibition of sporulation of **A.** parasiticus by DAB. Germlings (8-h-old) were distributed over plates of solid inducing medium (YEG). Filter paper disks soaked in 50 m*M* DAB were placed at the indicated times (in hours). After **36** h of incubation, plates were photographed.

DAB effect on sporulation was tested on solid medium. To overcome the effect of the drug on germination, spores were incubated in DAB-free liquid medium for 7-8 h in shake culture. Germlings were distnbuted over plates containing complex (YEG) solid medium, and filter paper disks soaked in a 50 m*M* DAB solution were placed over the plates at 2-h intervals. Sporulation around the disks was inhibited if the drug was applied up to 8 h after germling inoculation (16 h of total incubation time; Fig. 1). If the drug was added after this time, no inhibition occurred (not shown). The effect of DAB was partially reversed by putrescine, but high concentrations were necessaxy. Best effects were observed when germlings were seeded over solid medium containing 50 m*M* DAB and filter paper disks soaked in putrescine solutions were placed over the plate. Around the disks containing over 100 m*M* putrescine, conidia developed (not shown).

Effect of DAB on *Aflatoxin* Formation

To measure the effect of DAB on aflatoxin forination, spores were inoculated in complex (YEG) liquid mediuin and incubated under agitation. After germination liad occurred (about 7–8 h), a filter-sterilized concentrated solution of DAB was added to several flasks, wliereas otliers were left as controls. Flasks were tlien inciibated stationarily to induce sporulation. After 5 days, cultures were harvested and treated as described under Materials and Methods to measure spore numbers and dxy weight. Aflatoxins were extracted and analyzed by TLC. Results from five different experiments were reproducible and showed that there was no synthesis of aflatoxins in the presence of 50 m*M* DAB as measured by HPLC (Table 2). While 50 m*M* DAB completely blocked sporulation and aflatoxin formation, mycelial growth was inhibited by only about 50%.

In order to determine whether DAB inhibited aflatoxin biosynthesis or its regulation, we added the drug at

TABLE 2

Effect of DAB oii Sporulation and Aflatoxin Formation by **A.** parasiticus

DAB added (m*M*)	Growth m D.W.[a])	Spores ($\times 10^{-6}$)	Aflatoxins (μg) B_1	G_1	Total
None	**363** ± 27	5.1 ± 1.5	10.9 ± 5.4	6.2 ± S 0	17.1 ± 5.5
50	170 ± 32	0	0	0	0

Note. Erleiiiieyer fliisks containing YEG medium were inoculated with 10^5 spores and incubated iii a shaking water bath at 200 rpm and 28°C for 8 h. Where indicated some flasks received a filter-sterilized solution of DAB to obtain the desired concentration, and all flasks were incubated under static conditions for 5 extra days. At this time cultures were harvested and analyzed. Data are expressed per flask. Mean of triplicate samples ± SEM.

[a] Dry weiglit.

TABLE 3
Inhibition of Sporulation and Aflatoxin Biosynthesis by DAB Addition at Different Intervals

Time of DAH addition (h)	Growth (mg D.W.[a])	Spores ($\times 10^{-6}$)	Aflatoxins (μg)
8	89	0	3.3
30	132	1	6.1
30	218	100	85.5
Not added	144	80	111.8

Note. Erlenmeyer flasks containing YEG inediuin were inoculated with 7.5×10^4 spores and incubated in a shaking water bath at 200 rpm and 28°C for 8 h. After this time, tlie flasks were taken to static coiiditions. At tlie indicated intervals of time, a filter-sterilized solution of DAB was carefully iritroduced into tlie inediuin, so as not to disturb tlie mycelial mat, to a final concentration of 50 m*M*. After 7 days of incubation. cultures were harvested and analyzed. Data are expressed per flask. Averages of duplicate sainples.

[a] Dry weight.

different times of incubation to liquid cultures maintained static as described in the former experiment. If added up to 30 h of incubation, DAB inhibited both sporulation and aflatoxin biosynthesis, but if added after this time, the drug did not inhibit either phenomena, but stiniulated growth and sporulation (Table 3). Similar to sporulation on solid inedium, putrescine addition partially reversed the effect of DAB on aflatoxin biosynthesis and spore formation (Table 4). Under these conditions the ratio among B_1 and G_1 aflatoxins was reversed compared to the control. This is probably due to a delay in aflatoxin biosynthesis, since G_1 is a derivative of B_1, which accumulates at later incubation periods (Yabe *et al.*, 1988).

Synthesis of Aflatoxins by Mutants Affected in Sporulation

To confirm the association between sporulation and aflatoxin biosynthesis, a search for mutants affected in sporulation was initiated. After an exhaustive screening two proinising inutants were isolated. One of them, DGP10, produced no spores (Fig. 2b). Colonies froin the DGP29 inutant appeared with green and white sectors. In contrast to green sectors that appeared as wild type (Fig. 2a), the white ones contained no spores (Fig. 2d). Synthesis of aflatoxiiis by the mutants was studied iii static liquid inedia. Results are presented in Table 5. Mutant DGP10 did not produce aflatoxins. Mycelium coming from the green sector of strain DGP29 prodiicetl significaiit ainounts of aflatoxiiis. whereas mycelium froin tlie white sector did iiot produce tlie toxins (Table 5).

DISCUSSION

Oiir results reveal a correlation in the regulation of sporulation aiid aflatoxin biosynthesis in A. *parasiticus.* Shaken cultures of the fungus, where no sporulation occurretl, syntliesized low amounts of aflatoxiiis in contrast to cultures of the fungus grown on solid inediiiin or under static conditions. The observation that decreasing the ratio between flask volume and inedium voluiiie lowered the levels of aflatoxins made is probably related to the levels of oxygen in the inediuin. A role of oxygen in aflatoxin synthesis regulation has been claiined (Steyn *et al.*, 1980). However, Landers *et al.* (cited by Diener and Davis, 1968) have reported that aflatoxins are synthesized by *A. flavus* incubated under an atinosphere made of 1% O_2 and 99% N_2. Accordingly, a more likely explanation for these results is the relationship between the oxidation state and sporulation (Hansberg and Aguirre, 1990).

As has been demonstrated for Mucorales (Ruiz-Herrera, 1994), DAB inhibited both spore germination and sporulation of *A. parasiticus.* DAB is a competitive inhibitor of omithine decarboxylase (ODC; Stevens *et al.*, 1977), the first and inost regulated enzyme of the polyamine biosynthetic pathway. Since polyamines are growth factors, it would be expected that inhibition of their synthesis would be lethal. In different fungi we have observed that this is not so. DAB can inhibit growth up to 50%, but it can inhibit completely developmental processes such as spore

TABLE 4
Partial Reversion of DAB Effect by Putrescine

Experiment	DAB (m*M*)	Putrescine (m*M*)	Growth (mg D.W.[a])	Spores ($\times 10^{-5}$)	Aflatoxins (μg) B_1	Aflatoxins (μg) G_1	Aflatoxins (μg) Total
A	Noiie	None	371	3.6	5.2	15.7	20.9
	50	None	128	0	0	0	0
	50	100	239	0.5	1.4	0	1.4
	50	200	314	1.0	3.0	0	3.0
	50	300	291	1.8	4.3	0.8	5.1
B	None	None	364	61.0	2.5	2.9	5.4
	50	None	278	0	0	0	0
	Noiie	300	790	6380.0	7.9	10.8	18.7
	50	300	551	100.0	3	0	1.3

Note. Flasks coiitaining YEG medium were inoculated with 1.2×10^5 spores, and tlie flasks were shaken for 8 h at 28°C. DAB and putrescine were added at the indicated concentrations, and cultures were incubated under static conditions for 5 days at 28°C. Data are expressed per culture. (A) Average of duplicate samples. (B) Average of triplicates.

[a] Dry weight

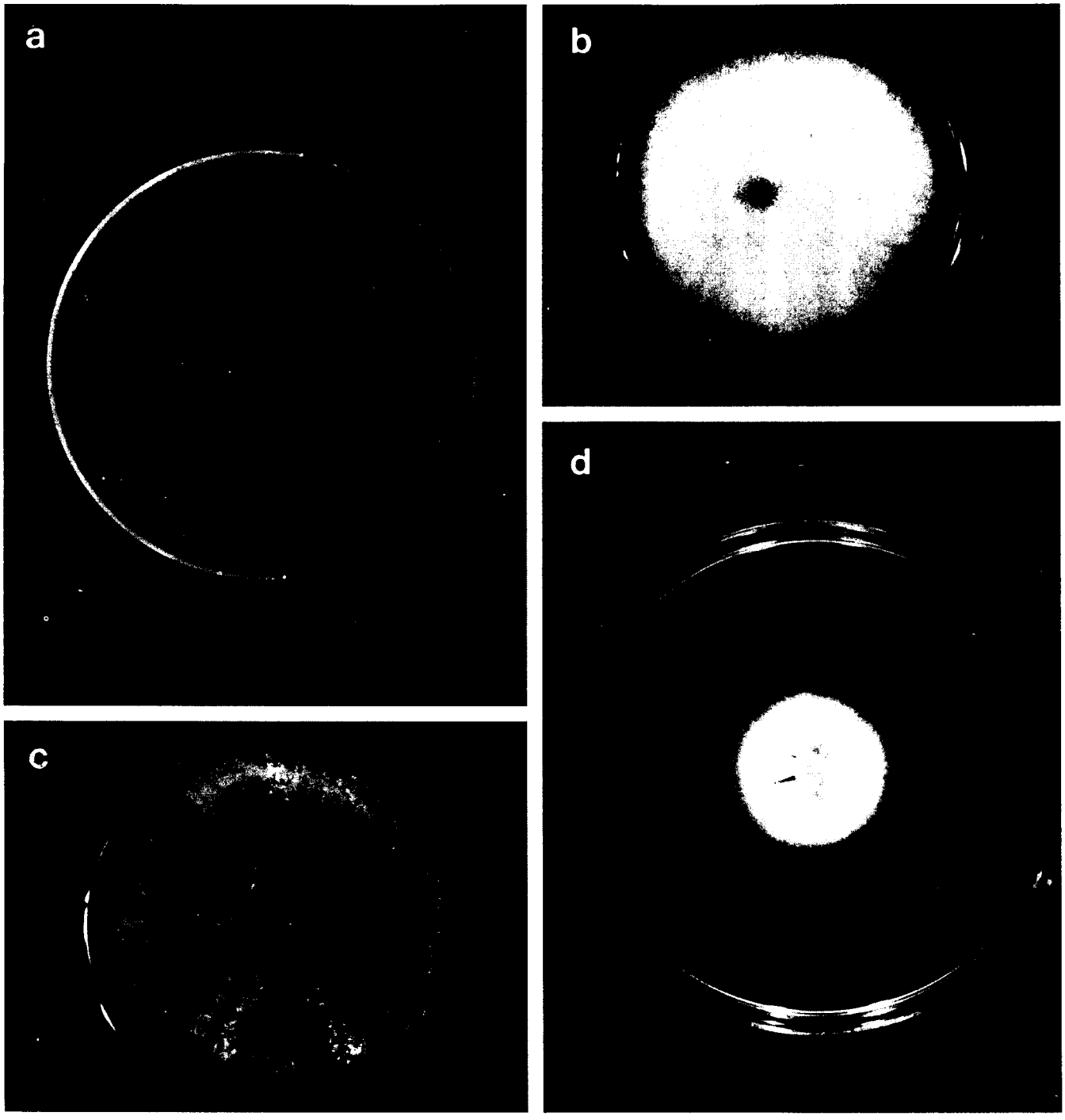

FIG. 2. Colonial morphology of *A parasiticus* strains. *(a)* Wild type; (b) DGP10; (c) DGP29; (d) DGP29 (W) grown from the white sector

TABLE 5
Synthesis of Aflatoxiiis by Mutants of *A. parasiticus*

Experiment	Strain	Growth (mg l . . ')	Spores ($\times 10^{-5}$)	Aflatoxins (μg)		
				B_1	G_1	Total
A	Wild type	594	3.9	1.8	6.8	8.6
	DGP10	411	*0*	0	0	0
	DGP29 (W)[b]	239	*0*	*0*	0	0
B	DGP10	186	0	0	0	0
	DGP29 (W)[b]	75	0	0	0	0
	DGP29 (G)[c]	124	80	3.1	25.0	28.1

Note. Squared pieces (ca. 3 × 3 mm) from inoculuin plates were inoculated into YEG liquid inediuiii. Flasks were incubated under static conditions at 28°C. Experiment A, 5 days of incubation; Experiment B, 3 days of incubation. Data are expressed per plate. Average of duplicate samples.

[a] Dry weight.
[b] White sector.
[c] Green sector.

germination, sporulation, and yeast-mycelial transition (Ruiz-Herrera and Calvo-Mendez, 1987; Martinez-Pacheco et al., 1989; Martinez et al., 1990; Reyna-Lopez and Ruiz-Herrera, 1993). We have obtained evidence that the distinctive action on growth and differentiation is due to the existence of more than one cellular pool of ODC, one of which (at least) is impermeable to DAB (Martinez-Pacheco and Ruiz-Herrera, 1993; Guevara-Olvera et al., 1993). In the present study we have eliminated the possibility that DAB inhibited aflatoxin biosynthesis itself by ineans of two types of experiments: DAB effect was counteracted by putrescine, the ODC product, and there was a limited, and the same, period of sensitivity to the drug of both sporulation and aflatoxin formation processes. If added after this time, DAB did not affect sporulation or aflatoxin formation. Our results show that DAB suppresses the onset of sporulation and aflatoxin biosynthesis in **A.** *parasiticus,* an effect similar to that observed with other fungi (Ruiz-Herrera and Calvo-Mendez, 1987; Martinez-Pacheco et al., 1989; Reyna-Lopez and Ruiz-Herrera, 1993). We have demonstrated tliat the sensitivity period correlates with the transitory increase in ODC and poly-ainiiie levels that precedes eacli differentiation process. Once they return to basal levels, the DAB-impermeable pool would be responsible for inaintaining tlie polyamine levels necessary for growth (Huiz-Herrera, 1994).

The relationship existing among aflatoxiii formation and sporulation is supported by the data obtained witli two mutants. Mutaiit DGP10, which is unable to sporulate, is also unable to form aflatoxins. As a tentative hypothesis it may be suggested that this mutant is affected in a gene commonly involved in tlie control of the sporulation pathway and aflatoxinogenesis. In A. *nidulans* it has been suggested that *brlA* is probably a transcriptional factor respoiisible for tlie expression of genes specific to the sporulation process (Tiinberlake, 1990). In turn *brlA* appears to be regulated during A. *nidulans* conidiogenesis by a set of genes (*fluG* and *flbA* tlirough E) altered in fluffy mutants (Wieser and Adams, 1995). The phenotype of sectorial mutant DGP29 is probably caused by a mutation making uristable a regulatory gene required for both sporulation and aflatoxin formation. Similar variegated mutants have been described in *A. nidulans* (Clutterbuck, 1969). This sectorial niutant provided an excellent internal control for the hypothesis that sporulation and aflatoxin formation are associated phenomena, since its sporulating sector synthesized aflatoxins, whereas the asporogenous one was unable to do so.

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