Relationship between Aflatoxin Biosynthesis and Sporulation in Aspergillus parasiticus

Doralinda Guzmán-de-Peña and José Ruiz-Herrera

Departamentos de Genética y Biología Molecular e Ingeniería Genética. Centro de Investigación y de Estudios Avanzados del IPN. Unidad Irapuato. Irapuato, Guanajuato, Mexico

Accepted for publication October 18, 1996

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 sporule 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 biosynthesis. 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.

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 Aspergillus colonize plants in the field or during storage of agricultural 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 Aspergillus 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 through their effect on fungal growth (Zaika and Buchanan, 1987). It has been proposed that aflatoxin biosynthesis is simply 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,
Aflatoxins are synthesized 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., 1993; Brown and Salvo, 1994), and thus aflatoxiis and sporulation were found to be closely associated in _Streptomyces_ spp regarding a possible association between sporulation and formation of secondary metabolites. Kliokhlov et al. (1973) showed that A-factor (2-isocapryloyl-3R-hydroxyinethyl-γ-butyrolactone) was an autoregulator necessary for both 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 complex regulatory system is triggered by A-factor-binding proteins (Horinouchi and Beppu, 1992), Hopwood (1988) identified genes whose mutation abolished the formation of aerial mycelium and secondary metabolites in _Streptomyces coelicolor_. These genes were referred to as "pleiotropic switches." The association between development and secondary metabolite synthesis is not fortuitous since genetic control mechanisms for the two processes displayed an important cross-correlation.

In the present study we have analyzed the relationship between development and aflatoxin synthesis in _A. parasiticus_ using two approaches: analysis of mutants affected in sporulation and selective inhibition of sporulation. The latter approach involved the use of 1,4-diamino-2-butane (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 spor germination has been demonstrated, suggesting that polyamines also play a role in the differentiation of _Aspergillus_ (Stevens, 1975). The results herein reported, obtained by the use of DAB and mutants of _A. parasiticus_ affected in sporulation, sustain the hypothesis that aflatoxin biosynthesis and sporulation are linked phenomena. These results are important for our comprehension of the regulation of toxin forination. From a practical point of view they suggest a novel possibility to control aflatoxin accumulation under natural conditions. A preliminary account of these results has been presented elsewhere (Guzman-de-Peña and Ruiz-Herrera, 1994).

**MATERIALS AND METHODS**

**Strains**

The following strains were used: a monosporic isolate from _A. parasiticus_ NRKL 2999 and two mutants, DGP10 and DGP29, isolated in the 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 10^4 spores and incubated under agitation (200 rpm) at 28°C. Incubations proceeded under normal laboratory illumination conditions. When germ tubes reached a length about twice the spore diameter (about 7–8 h), the flasks were removed from the shaker and placed under 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 the 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 in solid media, 8-h-old germlings were homogeneously distributed over the surface of solid inducing medium. Plates were incubated at 28°C, and at intervals, sterile filter paper disks soaked in 50 mM DAB were placed over the plates. To determine reversal of DAB effect by putrescine, 8-h-old germlings were distributed over the surface of solid inducing medium containing 50 mM DAB. Plates were incubated at 28°C, and paper filter disks soaked in putrescine solutions of different concentrations were placed over the plates. After 36 h, the plates were observed and photographed.

**Determination of Spore Numbers and Dry Weight**

Mycelium grown in liquid medium was recovered by filtration and resuspended in 10 ml of distilled water containing 0.1% Triton X-100, transferred to a petri dish, and gently shaken to free the spores. After a brief period to allow sedimentation of the mycelium, the supernatant containing the spores 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 weight. When solid medium was used, spores were harvested by flushing the plates with 0.1% Triton solution followed by centrifugation. Agar plates were extracted with chloroform for 30 min, chloroform was recovered, water was added to the plates, and the 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 described above were extracted with twice their volume of chloroform. 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 chloroform: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<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub> 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 mM citrate buffer, pH 5.5, at a final density of 3 × 10<sup>6</sup> spores per milliliter. Spore suspensions (0.9 ml) were mixed with N-methyl-N′-nitro-N-nitrosoguanidine (0.1 ml, 0.25 mg per ml) and incubated in darkness for 45 min. Sodium thiourea sulfate (5%) was added, and the spores were sedimented by centrifugation and washed twice with 50 mM phosphate buffer, pH 7.0. Under these conditions, about 10% survival was observed. Spores were inoculated on 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 these revealed a delayed phenotype; after prolonged incubation there were differentiating green spores. Two stable strains were finally recovered: DGP10, a nonsporulating strain, similar in phenotype to brl<sup>−</sup> mutants from A. nidulans (Clutterbuck, 1969). and DGP29, a mutant forming sectorial colonies where green spore-bearing sectors alternated with white spore-less sectors.

**RESULTS**

**Aflatoxin Synthesis in Agitated Liquid Cultures versus Solid Media**

 It has been described (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 media. We tested different volumes of medium in Erlenmeyer flasks of the same size to obtain variable aeration of the medium. In the liquid cultures, we took special precautions to avoid (by constant manipulation) a ring of stationary-type growth from mycelium that clings to the glass above the liquid boundary, 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 important 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 when the medium volume was increased to 100 ml, conditions under which attachment of the mycelium to the flask walls was minimal.

**Effect of DAB on Spore Germination and Sporulation**

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

---

*Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.*
TABLE 1
Aflatoxin Biosynthesis by *A. parasiticus* in Shake Cultures and Solid Media

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Volume (ml)</th>
<th>Growth (mg D.W.)</th>
<th>Spores (×10^8)</th>
<th>B1</th>
<th>G1</th>
<th>Total</th>
<th>Pr</th>
<th>mg D.W.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shaken</td>
<td>25</td>
<td>147</td>
<td>0</td>
<td>4.3</td>
<td>0</td>
<td>4.3</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>liquid</td>
<td>50</td>
<td>173</td>
<td>0</td>
<td>2.3</td>
<td>0</td>
<td>2.3</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>158</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
<td>0</td>
<td>0.1</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Solid</td>
<td>50</td>
<td>1575</td>
<td>7.1</td>
<td>221.0</td>
<td>400</td>
<td>621.0</td>
<td>0.394</td>
<td></td>
</tr>
</tbody>
</table>

Note. Liquid YEG medium dispensed into 125-ml Erlenmeyer flasks or solid medium in 50-ml petri dishes was inoculated with the same number of spores (10^9). 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.

* Dry weight

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 distributed over plates containing complex (YEG) solid medium, and filter paper disks soaked in a 50 mM 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 necessary. Best effects were observed when germlings were seeded over solid medium containing 50 mM DAB and filter paper disks soaked in putrescine solutions were placed over the plate. Around the disks containing over 100 mM putrescine, conidia developed (not shown).

**Effect of DAB on Aflatoxin Formation**

To measure the effect of DAB on aflatoxin formation, spores were inoculated in complex (YEG) liquid medium and incubated under agitation. After germination had occurred (about 7-8 h), a filter-sterilized concentrated solution of DAB was added to several flasks, whereas others were left as controls. Flasks were then incubated stationarily to induce sporulation. After 5 days, cultures were harvested and treated as described under Materials and Methods to measure spore numbers and dry 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 mM DAB as measured by HPLC (Table 2). While 50 mM 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

![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 mM DAB were placed at the indicated times (in hours). After 36 h of incubation, plates were photographed.](image)

TABLE 2
Effect of DAB on Sporulation and Aflatoxin Formation by *A. parasiticus*

<table>
<thead>
<tr>
<th>DAB added (mM)</th>
<th>Growth (mg D.W.)</th>
<th>Spores (×10^8)</th>
<th>B1</th>
<th>G1</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>363 ± 27</td>
<td>5.1 ± 1.5</td>
<td>10.9 ± 5.4</td>
<td>6.2 ± S 0</td>
<td>17.1 ± 5.5</td>
</tr>
<tr>
<td>50</td>
<td>170 ± 32</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Note. Erlenmeyer flasks containing YEG medium were inoculated with 10^5 spores and incubated in 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.

* 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 stimulated growth and sporulation (Table 3). Similar to sporulation on solid medium, 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 promising mutants were isolated. One of them, DGP10, produced no spores (Fig. 2b). Colonies from the DGP29 mutant 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 aflatoxins by the mutants was studied in static liquid medium. Results are presented in Table 5. Mutant DGP10 did not produce aflatoxins. Mycelium coming from the green sector of strain DGP29 produced significant amounts of aflatoxin, whereas mycelium from the white sector did not produce the toxins (Table 5).

**DISCUSSION**

Our results reveal a correlation in the regulation of sporulation and aflatoxin biosynthesis in *A. parasiticus*. Shaken cultures of the fungus, where no sporulation occurred, synthesized low amounts of aflatoxins in contrast to cultures of the fungus grown on solid medium or under static conditions. The observation that decreasing the ratio between flask volume and medium volume lowered the levels of aflatoxins made is probably related to the levels of oxygen in the medium. A role of oxygen in aflatoxin synthesis regulation has been claimed (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 atmosphere 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 ornithine decarboxylase (ODC; Stevens et al., 1977), the first and most 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>
<thead>
<tr>
<th>Table 3: Inhibition of Sporulation and Aflatoxin Biosynthesis by DAB Addition at Different Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of DAB addition (h)</td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>30</td>
</tr>
<tr>
<td>30</td>
</tr>
<tr>
<td>Not added</td>
</tr>
</tbody>
</table>

Note. Erlenmeyer flasks containing YEG medium were inoculated with 7.5 x 10^4 spores and incubated in a shaking water bath at 200 rpm and 25°C for 8 h. After this time, the flasks were taken to static conditions. At the indicated intervals of time, a filter-sterilized solution of DAB was carefully introduced into the medium, so as not to disturb the mycelial mat, to a final concentration of 50 mM. After 7 days of incubation, cultures were harvested and analyzed. Data are expressed per flask. Averages of duplicate samples.

*Dry weight.

<table>
<thead>
<tr>
<th>Table 4: Partial Reversion of DAB Effect by Putrescine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>Noie</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>Noie</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>50</td>
</tr>
</tbody>
</table>

Note. Flasks containing YEG medium were inoculated with 1.2 x 10^6 spores, and the flasks were shaken for 8 h at 25°C. DAB and putrescine were added at the indicated concentrations, and cultures were incubated under static conditions for 5 days at 25°C. Data are expressed per culture. (A) Average of replicate samples. (B) Average of triplicates.

*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.
may be suggested that this mutant is affected in a gene commonly involved in the control of the sporulation pathway and aflatoxinogenesis. In A. nidulans it has been suggested that brlA is probably a transcriptional factor responsible for the expression of genes specific to the sporulation process (Tinberlake, 1990). In turn brlA appears to be regulated during A. nidulans conidigenesis by a set of genes (fluG and flbA through E) altered in fluffy mutants (Wieser and Adams, 1995). The phenotype of sectorial mutant DGP29 is probably caused by a mutation making uniristable a regulatory gene required for both sporulation and aflatoxin formation. Similar variegated mutants have been described in A. nidulans (Clutterbuck, 1969). This sectorial mutant 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.

ACKNOWLEDGMENTS

This work was partially supported by the Consejo Nacional de Ciencia y Tecnología, México. Thanks are given to Drs. Gerald N. Wogan (MIT, USA) and Jesús Aguirre (UNAM, México) for helpful suggestions and comments and to Dr. Salomón Bartnicki-García (University of California, Riverside) for critical reading of the manuscript.

REFERENCES


