SHORT COMMUNICATION

Loss of Norsolorinic Acid and Aflatoxin Production by a Mutant of *Aspergillus parasiticus*

By J. W. BENNETT

Department of Biology, Tulane University, New Orleans, Louisiana 70118, U.S.A.

(Received 18 August 1980; revised 6 January 1981)

A mutant strain of *Aspergillus parasiticus* that accumulated the red mycelial pigment norsolorinic acid and low levels of aflatoxin was subjected to serial transfer in defined media. The isolation of unpigmented, non-aflatoxigenic forms was associated with conditions in which the inocula for serial transfers contained hyphae rather than conidia, but was not associated with either zinc deficiency or the presence of barium in the medium. The unpigmented, non-aflatoxigenic variants were stable.

INTRODUCTION

Filamentous fungi are known for their morphological and metabolic variability, particularly among strains that have been maintained in laboratory culture for prolonged periods. Such strain instability is troublesome in industrial fermentations and in experiments dealing with secondary metabolism.

Aflatoxins are biologically potent secondary metabolites produced by the closely related species *Aspergillus flavus* and *A. parasiticus*. Differences in aflatoxin-producing ability are common among strains isolated from nature and those maintained in the laboratory (Diener & Davis, 1969; Bennett, 1979). Stable mutants blocked in aflatoxin production have been isolated from aflatoxigenic wild-type strains after treatment with ultraviolet light (Bennett & Goldblatt, 1973) and nitrosoguanidine (Donkersloot *et al.*, 1972; Papa, 1979). Non-aflatoxigenic strains have also been isolated from *A. flavus* after successive transfer in a chemically defined medium containing high levels of barium (Lee & Townsley, 1968). With ultraviolet light and nitrosoguanidine, a wide variety of randomly induced mutants were isolated in addition to the non-aflatoxigenic strains. With barium treatment, only non-aflatoxigenic variants were isolated.

The instability of an aflatoxin-producing strain of *A. parasiticus* after serial transfer of polysporous cultures on defined and complex solid media has been characterized (Mayne *et al.*, 1971). The present communication describes an extension of the study of cultural instability, using a chemically defined liquid medium which supports sporulation. The strain used was a stable mutant that produces both aflatoxin and a bright-red mycelial pigment that has been identified as norsolorinic acid (Lee *et al.*, 1971). Norsolorinic acid and aflatoxin are biosynthetically related through the polyketide pathway (Hsieh *et al.*, 1976; Bennett & Lee, 1979), and in this mutant strain the two compounds are produced concomitantly (Bennett *et al.*, 1971). This correlation is useful because the red pigment is easily identified and its presence serves as a marker for aflatoxin production.

METHODS

Strains. All experiments were conducted using the mutant strain of *Aspergillus parasiticus* isolated by Bennett & Goldblatt (1973) after ultraviolet light treatment, and designated *br-1 nor-1* (ATCC 24690). This strain has brown conidia, and accumulates norsolorinic acid and moderate levels of aflatoxin.

Media. The complex medium (CM) was potato dextrose agar (Difco) plus 0.5% (w/v) yeast extract. The chemically defined liquid medium (AM) was formulated by Adye & Matales (1964). In some experiments, ZnSO_4 was omitted from AM (AM-Zn). In other experiments BaCO_3 (5 g l^{-1}) was added to AM (AM+Ba). For quick-screening aflatoxin assays, cultures were grown on the medium devised by De Vogel *et al.* (1965) as modified by Bennett & Goldblatt (1973). For quantitative aflatoxin assays, cultures were grown on shredded wheat as described by Mayne *et al.* (1971).

Serial transfers. Cultures in AM-Zn were maintained in stationary culture for 7 d at 30 °C. The medium was then decanted, leaving behind the sporulating mycelial mat. This was flooded with 0.025% (v/v) Tween 80 in physiological saline and vigorously shaken ten times. The resulting spore suspension was used both to obtain a viable count on CM and as an inoculum for aflatoxin assays and for the next successive generation of growth on AM-Zn. Serial transfers were conducted weekly for 7 or 9 weeks.

Experiments in AM+Ba were maintained in shake culture for 7 d at 30 °C. The entire contents of the culture flask (both mycelium and medium) were then transferred to a sterile Waring blender and blended at high speed for 2 min. The resulting mycelial macerate was appropriately diluted to obtain viable counts, and used to reinoculate the next successive generation of growth in AM+Ba. Serial transfers in AM+Ba were conducted weekly for 5 weeks. Parallel control cultures of mycelial macerates in standard AM medium were run as controls.

The number of pigmented and unpigmented colonies from viable counts on CM were counted after 7 d at 30 °C.

Aflatoxin assays. Aflatoxins were extracted from shredded wheat (replicate samples) with aqueous acetone by the method of Pons *et al.* (1966). Final extracts of aflatoxins were suspended in chloroform and appropriately diluted samples were assayed by thin-layer chromatography on Adsorbosil-1 silica gel plates developed in chloroform/acetone (95:5, v/v). Aflatoxins B_1 , B_2 , G_1 and G_2 were densitometrically quantified according to the method of Pons *et al.* (1968). Aflatoxin data are presented as totals.

RESULTS AND DISCUSSION

The first series (A) of successive transfers in AM-Zn produced 6% unpigmented forms after two transfers. No pigmented colonies were seen after the seventh transfer (Table 1). Loss of pigmentation was accompanied by a reduction in and then loss of aflatoxin production, a result similar to that obtained by Lee & Townsley (1968) with cultures grown in a defined medium with added barium. These authors reported on two series of transfers. In one series, the entire population of *A. flavus* lost its ability to produce aflatoxin and yellow pigment after six successive transfers. In the second series, the culture remained aflatoxigenic for five successive transfers, but after seven transfers, 28% of the colonies had lost the ability to produce aflatoxin.

Two additional series (B and C; see Table 1) in AM-Zn gave different results. After nine transfers, series B had produced no unpigmented forms, and aflatoxin production had risen from $48.5 \mu\text{g (g shredded wheat)}^{-1}$ at the beginning of the experiment to $104.5 \mu\text{g g}^{-1}$ at the end. In series C, a few unpigmented forms were observed after eight transfers, and after the ninth transfer 11% of the colonies were unpigmented. There was also a drop in aflatoxin production after the ninth transfer in series C.

The loss in aflatoxigenicity and pigmentation after serial transfer in AM-Zn was associated with differences in gross morphology. In series A, sporulation was reduced after the third transfer, and after six transfers, when aflatoxin production was almost completely lost, both mycelial growth and sporulation were greatly decreased. In series B, there was no change in the morphology of colonies on AM-Zn or in pigmentation and aflatoxin production increased over the course of nine transfers. In series C, morphology remained unchanged for the first seven transfers, during which time no unpigmented forms were observed. Only when a decrease in sporulation was observed (eighth transfer) did unpigmented forms appear and decreased aflatoxin production occur.

To see if instability of aflatoxin production was favoured in the absence of sporulation, strain *br-1 nor-1* was grown in standard AM medium and in AM+Ba in shake culture.

Table 1. *Development, pigmentation and aflatoxin production of A. parasiticus br-1 nor-1 during three series of successive transfers in a defined medium lacking zinc*

Cultures were transferred weekly in AM-Zn. At each transfer, mycelium and spore development were scored, plates of CM were inoculated to obtain counts of pigmented colonies, and shredded wheat was inoculated for the aflatoxin assay.

Transfer	Mycelium and spore development*			Percentage of pigmented colonies			Aflatoxin production [$\mu\text{g (g shredded wheat)}^{-1}$]		
	Series A	Series B	Series C	Series A	Series B	Series C	Series A	Series B	Series C
0	—	—	—	100	100	100	83.5	48.5	67.0
1	+++	+++	+++	100	100	100	79.5	56.1	68.0
2	+++	+++	+++	94	100	100	—†	50.9	66.0
3	++	+++	+++	45	100	100	12.5	68.0	69.0
4	++	+++	+++	1.3	100	100	4.4	56.0	79.5
5	++	+++	+++	0	100	100	0.041	58.5	67.5
6	+	+++	+++	0.1	100	100	<0.001	86.0	75.0
7	+	+++	+++	0	100	100	<0.001	82.0	60.5
8		+++	++		100	97		132.0	59.0
9		+++	++		100	89		104.5	34.0

* +++, Pad of mycelium about 4 mm thick, heavy sporulation; ++, pad of mycelium about 4 mm thick, sparse sporulation; +, film of mycelium about 1 mm thick, sparse sporulation.

† Sample lost.

Table 2. *Production of unpigmented colonies by A. parasiticus br-1 nor-1 during serial transfer in a defined medium with and without barium*

Cultures were transferred weekly in AM and AM+Ba. At each transfer, plates of CM were inoculated to obtain counts of pigmented colonies.

Transfer	Percentage of pigmented colonies			
	AM		AM + Ba	
	Series D	Series E	Series F	Series G
0	100	100	100	100
1	39	29	95	93
2	19	4	62	44
3	27	0	21	26
4	31	0	53	24
5	0	0	19	24

Sporulation does not occur under these culture conditions. In standard AM medium, both transfer series (D and E) exhibited loss of pigment formation after the first transfer (Table 2): in series E the entire culture was unpigmented after the third transfer, in series D after the fifth transfer. Similar results were obtained in the series (F and G) transferred in the medium containing barium: unpigmented forms were obtained in both series after one transfer; after five transfers, over 75 % of the colonies in these series were unpigmented.

To determine if the unpigmented forms remained non-aflatoxigenic after passage through a conidial phase, ten single spores were isolated from unpigmented colonies on CM (from series D) and assayed for aflatoxins on shredded wheat. No aflatoxins were detected. The assay was extended by sampling 50 single spores from unpigmented colonies and 50 from pigmented colonies obtained from both the standard AM and AM+Ba series, and growing them on the quick-screening agar of De Vogel *et al.* (1965). None of the unpigmented colonies exhibited

the fluorescence associated with aflatoxin production; all the pigmented colonies gave the characteristic bright blue fluorescence.

These results confirm the correlation between pigment and aflatoxin production. Loss of aflatoxigenicity was associated with conditions in which the inocula for serial transfers contained hyphae rather than conidia. Ironically, the original zinc-deficient medium and stationary culture had been selected because it was a regime which supported sporulation.

A relationship between sporulation and the maintenance of good aflatoxin production has also been reported by Mayne *et al.* (1971). In their study, aflatoxin-producing ability was attenuated in a wild-type strain of *A. parasiticus* after serial transfer of mycelia and spores. However, when the attenuated polysporous culture was transferred without mycelium by isolating single spores, there was a return to high aflatoxin-producing ability.

In contrast to the study of Mayne *et al.* (1971), in the experiments described here unpigmented, non-aflatoxigenic forms of strain *br-1 nor-1* did not revert to pigmented, aflatoxigenic forms, even after passage through a conidial phase. Stock cultures of these non-aflatoxigenic strains have remained stable after 6 years in the laboratory. Since norsolorinic acid is a relatively early intermediate in the aflatoxin biosynthetic pathway, the simultaneous loss of both substances observed in the current study suggests that information required for the pathway is coordinately controlled.

I am grateful to Tony Franz and Claire Cooper for technical assistance, and to Eivind Lillehoj for help with the preparation of the manuscript. Portions of the work were conducted during a National Research Council Agricultural Research Service Postdoctoral Research Associateship at the Southern Regional Research Center, New Orleans, Louisiana. The study was completed with the support of a Cooperative Agreement from the U.S. Department of Agriculture (58-7B-8-28).

REFERENCES

- ADYE, J. & MATALES, R. I. (1964). Incorporation of labelled compounds into aflatoxins. *Biochimica et biophysica acta* **86**, 418–420.
- BENNETT, J. W. (1979). Aflatoxins and anthraquinones from diploids of *Aspergillus parasiticus*. *Journal of General Microbiology* **113**, 127–136.
- BENNETT, J. W. & GOLDBLATT, L. A. (1973). The isolation of mutants of *Aspergillus flavus* and *A. parasiticus* with altered aflatoxin producing ability. *Sabouraudia* **11**, 235–241.
- BENNETT, J. W. & LEE, L. S. (1979). Mycotoxins—their biosynthesis in fungi: aflatoxins and other bisfuranoids. *Journal of Food Protection* **42**, 805–809.
- BENNETT, J. W., LEE, L. S. & VINNETT, C. (1971). The correlation of aflatoxin and norsolorinic acid production. *Journal of the American Oil Chemists Society* **48**, 368–370.
- DE VOGEL, P., VAN RHEE, R. & KOELLENSMID, W. A. A. (1965). A rapid screening test for aflatoxin synthesizing aspergilli of the *flavus-oryzae* group. *Journal of Applied Bacteriology* **28**, 213–220.
- DIENER, J. L. & DAVIS, N. D. (1969). Aflatoxin formation by *Aspergillus flavus*. In *Aflatoxin. Scientific Background, Control and Implications*, pp. 13–54. Edited by L. A. Goldblatt. New York: Academic Press.
- DONKERSLOOT, J. A., MATALES, R. I. & YANG, S. S. (1972). Isolation of averufin from a mutant of *Aspergillus parasiticus* impaired in aflatoxin biosynthesis. *Biochemical and Biophysical Research Communications* **47**, 1051–1055.
- HSIEH, D. P. H., LIN, M. T., YAO, R. C. & SINGH, F. (1976). Biogenesis of aflatoxins: conversion of norsolorinic acid and other hypothetical intermediates into aflatoxin B₁ by *Aspergillus parasiticus*. *Journal of Agricultural and Food Chemistry* **24**, 1171–1174.
- LEE, E. G. H. & TOWNSLEY, P. M. (1968). Chemical induction of mutation or variation in aflatoxin-producing cultures of *Aspergillus flavus*. *Journal of Food Science* **33**, 420–423.
- LEE, L. S., BENNETT, J. W., GOLDBLATT, L. A. & LUNDIN, R. E. (1971). Norsolorinic acid from a mutant strain of *Aspergillus parasiticus*. *Journal of the American Oil Chemists Society* **48**, 93–94.
- MAYNE, R. Y., BENNETT, J. W. & TALLANT, J. (1971). Instability of an aflatoxin-producing strain of *Aspergillus parasiticus*. *Mycologia* **63**, 644–648.
- PAPA, K. E. (1979). Genetics of *Aspergillus flavus*: complementation and mapping of aflatoxin mutants. *Genetical Research* **34**, 1–9.
- PONS, W. A., CUCULLU, A. F., LEE, L. S., ROBERTSON, J. A., FRANZ, A. O. & GOLDBLATT, L. A. (1966). Determination of aflatoxins in agricultural products: use of aqueous acetone for extraction. *Journal of the Association of Official Analytical Chemists* **49**, 554–562.
- PONS, W. A., CUCULLU, A. F., FRANZ, A. O. & GOLDBLATT, L. A. (1968). Improved objective fluorodensitometric determination of aflatoxins in cottonseed products. *Journal of the American Oil Chemists Society* **45**, 694–699.