# Isolation and Characterization of Two Nonaflatoxigenic Classes of Morphological Variants of *Aspergillus parasiticus*

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## ABSTRACT

Serial transfer of mycelial macerates of aflatoxigenic strains of *Aspergillus parasiticus* produced 2 morphological variants. The "fan" variant type showed flat growth, a gradient of sporulation density, absence of mycelial pigmentation and deep furrows on the back of the colony. The "fluff" variants displayed abundant aerial mycelium, sparse sporulation, absence of mycelial pigmentation and furrows on the reverse of the colony. Neither "fan" nor "fluff" produced detectable aflatoxin. In a marked mutant strain, the appearance of "fan" and "fluff" and their concomitant loss of aflatoxin production occurred whereas spore color and auxotrophic markers were retained. A parasexual diploid synthesized from the mutant strain with the "fan" phenotype produced high levels of aflatoxin whereas a parasexual diploid containing the "fluff" phenotype produced low levels of aflatoxin. The unusual mode of isolation, the differential retention of aflatoxin and other genetic markers in a mutant strain, and the different levels of aflatoxin production in parasexual diploids with "fan" and "fluff" imply an unusual genetic mechanism for the control of the aflatoxin pathway.

Aflatoxins constitute a family of biologically potent metabolites produced by certain strains of the closely related fungal species *Aspergillus flavus* and *A. parasiticus*. Aflatoxins were originally discovered as the etiological agents in a poultry pathology called "turkey X disease." Subsequently, turkey X disease was recognized as one specific form of a larger toxigenic syndrome in vertebrates now collectively called "aflatoxicoses" (1). In addition to acute and chronic toxicity, aflatoxins display carcinogenic, mutagenic and teratogenic activity in a wide range of animal species (2,3).

Aflatoxins and other well known fungal toxins such as the ergot alkaloids, the trichothecenes and patulin, are all examples of secondary metabolites. These natural products are distinguished from primary metabolites (which are necessary for growth and are ubiquitous) because they are generally produced by one species, or a small number of closely related species, have no known function in the metabolism of the producing organism, and are frequently synthesized after active growth has ceased during a period of morphological differentiation. The role of secondary metabolites in nature has perplexed microbiologists and natural products chemists for decades (4-6).

For several practical reasons, aflatoxins provide a useful model system for studying secondary metabolism. Sensitive and accurate assays are available. The late Walter A. Pons, Jr., in whose honor this symposium is held, pioneered and refined many of the relevant extraction and assay procedures for aflatoxins from oilseeds (7,8). As natural toxicants, aflatoxins cannot be excluded totally from the food supply, and the danger to human and veterinary health requires continuous surveillance. Consequently, more abundant data on occurrence, distribution and ecological parameters are available for aflatoxins than for secondary metabolites of lesser economic importance. In addition, the aflatoxin biosynthetic pathway has attracted considerable attention. Aflatoxins are produced through a polyacetate-malonate pathway; the methoxymethyl group is derived from methionine (9). Using radiolabeling techniques, 6 compounds have been identified as polyketide intermediates: norsolorinic acid, averantin, averufin, versiconal hemiacetal acetate, versicolorin A and sterigmatocystin. Four of these compounds (norsolorinic acid, averantin, averufin and versicolorin A) are accumulated in blocked mutants, indicating that mutational analysis is a fruitful approach to research on the aflatoxin biosynthetic pathway (9-12).

In this study, 2 novel classes of nonaflatoxigenic variants were characterized. These nonaflatoxigenic variants were distinguished from previously described blocked aflatoxin mutants by their mode of isolation, by the differential loss of aflatoxigenicity in auxotrophically marked strains, and by their behavior in parasexual crosses. A preliminary report of this work has been presented (13).

## EXPERIMENTAL PROCEDURES

### Strains

The wild-type strain of *Aspergillus parasiticus* was SU-1 (NRRL 5862), which is aflatoxigenic and prototrophic, with green conidiospores. Two ultraviolet (UV)-light-derived spore color and auxotrophic mutants isolated by Bennett (14) were also studied. The white-spored, pyridoxine-requiring mutant was designated *wh-1 pdx-2*; the brown-spored, adenine-requiring strain that accumulated norsolorinic acid was designated *br-1 nor-1 ade-1*. Nonaflatoxigenic mutants were induced in SU-1 and *wh-1 pdx-2* by serial transfer of mycelial macerates as described later. Parasexual diploids were synthesized between the auxotrophic strains using methods previously described (14).

### Media and Culture Conditions

The complete medium (CM) was potato dextrose agar (Difco) plus 0.5% yeast extract. CM was used for stock cultures of wild type and mutant strains and for viable counts.

The liquid defined medium (AM) devised by Adye and Mateles (15) in which 50 g sucrose was substituted for glucose, was used for aflatoxin assays and for most of the serial maceration experiments. In serial maceration experiments using the auxotrophic strain *wh-1 pdx-2*, pyridoxine was added to AM (1 mg/100 mL AM) or liquid potato dextrose broth (PD) was substituted for AM. All cultures were incubated at 30 C with high humidity in the dark.

### Mycelial Maceration

Flasks containing 100 mL of the appropriate liquid medium (AM for SU-1; AM plus pyridoxine or PD for *wh-1 pdx-2*) were inoculated with $10^6$-$10^7$ spores. Cultures were grown in a reciprocal shaker for 7 days. The entire contents of the flasks (both mycelial pellets and culture medium) were blended at high speed in a Waring blender for 2 min. Two mL of the resulting mycelial macerate were used to reinoculate fresh flasks containing 100 mL of liquid medium. One mL of the mycelial macerate was appropriately diluted and

plated on CM to obtain viable counts of colony forming units (cfu). The procedure was repeated every 7 days for a total of 3 transfers. Each line of mycelial macerates was maintained separately and labeled series A-C for SU-1 and series D-G for *wh-1 pdx-2*.

In another group of experiments, 10 flasks each containing 100 mL AM were inoculated with dense spore suspension of SU-1 and grown in reciprocal shaker culture. At intervals of 1, 2, 3, 4 and 20 weeks, pairs of flasks were removed and the mycelial pellets and medium were blended together for 2 min at high speed in a Waring blender. Appropriate dilutions of mycelial macerates were plated on CM to obtain viable counts of cfu.

### Ultraviolet Light Irradiation

Spore suspensions of SU-1 and 2 nonaflatoxigenic mutants derived from SU-1 were placed 30 cm from a 30-W germicidal UV bulb (G.E. G30T8) which delivered 215 $\mu W/cm^2$ at this distance. The spore suspension was agitated with gentle swirling at 2-min intervals. Aliquots of irradiated spores were withdrawn after 3, 6 and 9 min, were diluted appropriately and plated on CM to obtain viable counts and to observe morphological characteristics.

### Aflatoxin Assays

Fungi were cultured in duplicate in 100 mL AM, or AM supplemented with pyridoxine or adenine, in 250 mL Erlenmeyer flasks. After 1 week of incubation in shaker culture, mycelial pellets were separated from the culture filtrate by gravity filtration through cheesecloth. The mycelium was combined with 50 mL of acetone and macerated in an explosion-proof Waring blender for 1 min. The resulting slurry was filtered through a Buchner funnel; additional acetone was added to the ground mycelium until the mycelium was colorless. The filtrate was diluted to contain 70% water. This aqueous acetone mixture was shaken twice with 75-mL portions of chloroform in a separatory funnel. The aqueous phase was discarded and the acetone/chloroform phase was evaporated to dryness at room temperature in a hood. Chloroform solutions of the dried aflatoxin extracts were appropriately diluted and assayed on Adsorbosil-1 silica gel plates by thin layer chromatography (TLC) using ether/methanol/water (96:3:1, v/v/v) as the developing solvent. Aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ were quantified densitometrically by comparison of fluorescent intensities with known standards according to the method of Pons et al. (8).

## RESULTS

Wild-type *A. parasiticus* grown in AM medium with high sucrose and ammonium sulfate as the sole nitrogen source does not sporulate, but grows abundantly in the form of mycelial pellets. These pellets were macerated in a Waring blender and reinoculated into AM medium; cfu were monitored by viable counts on CM. Three distinct morphological types on CM were observed after serial transfer of mycelial macerates (Figs. 1 and 2).

On CM, wild-type *A. parasiticus* sporulates heavily and uniformly over the surface of the colony; sclerotia are sometimes observed. The reverse is light yellow with little furrowing. The "fan" variant sporulates heavily in the center of the colony, but only slightly at the edges; the reverse is ivory-colored with deep furrows (Figs. 1 and 2). Wild-type SU-1 produces high levels of aflatoxin (580-2,050 $\mu g$/100 mL AM). Neither "fluff" nor "fan" variants produced detectable aflatoxins.

The percentage of different morphological types observed after 3 parallel series of transfers of mycelial macerates is shown in Table I. After one transfer, the cfu of series B included 20% with the fluff morphology. After two transfers, both series B and C were producing fluff-type variants; in addition, series A and C included some with the fan morphology. All series produced morphological variants, but the number and ratio of each variant type varied among series.

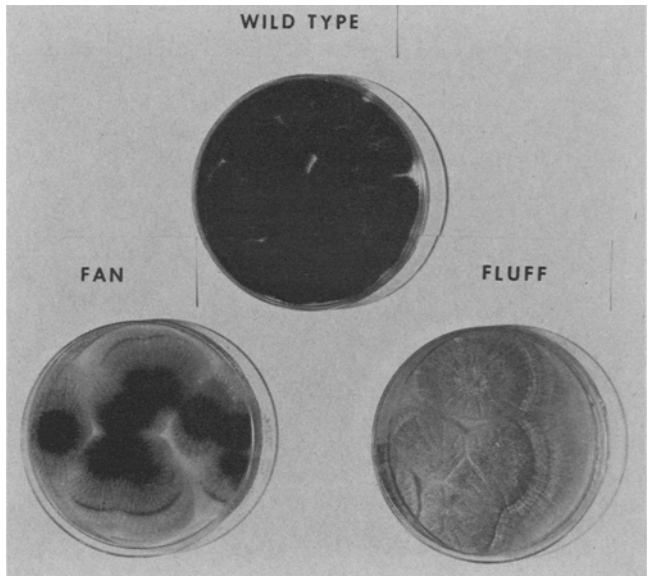


**FIG. 1. Surface view of 3 morphological types of *Aspergillus parasiticus* strain SU-1.**

In another set of experiments, 10 flasks were inoculated with spores and grown for up to 20 weeks. At the end of 1, 2, 3, 4 and 20 weeks, mycelial pellets were macerated and plated on CM. All the resulting colonies were like the original wild type. No variants were observed, indicating that, in this experiment, aging per se had no detectable effect on the morphology of colonies growing from mycelial macerates that had not been subjected to serial transfer.

To determine the stability of these nonaflatoxigenic morphological variants, their UV light sensitivity was compared with that of wild type. These results are presented in

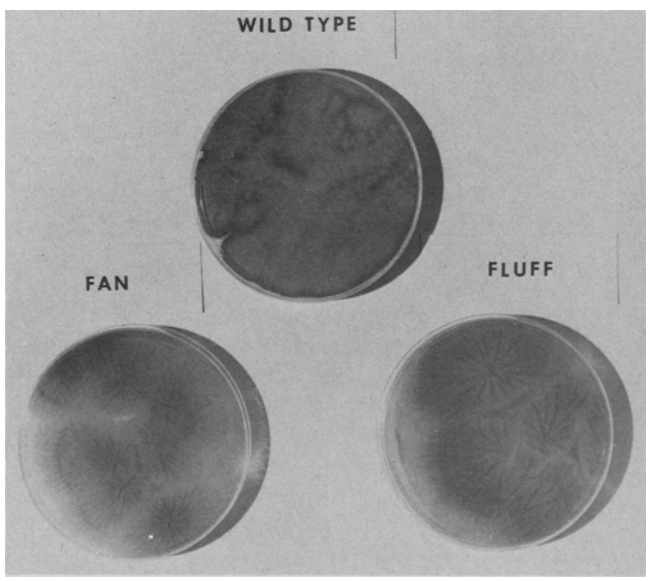


**FIG. 2. Reverse view of 3 morphological types of *Aspergillus parasiticus* strain SU-1.**

TABLE I

**Morphological Variants of Wild Type *Aspergillus parasiticus* after Serial Mycelial Maceration**

| Transfer (7 days) | Series | Morphological type (% cfu) | | |
|---|---|---|---|---|
| | | Standard[a] | Fan[b] | Fluff[c] |
| 0 | A | 100 | – | – |
| | B | 100 | – | – |
| | C | 100 | – | – |
| 1 | A | 78.7 | – | 21.3 |
| | B | 100 | – | – |
| | C | 100 | – | – |
| 2 | A | 82.0 | 9.0 | 9.0 |
| | B | 58.0 | – | 42.0 |
| | C | 88.0 | 3.5 | 8.5 |
| 3 | A | 94.4 | – | 5.6 |
| | B | 83.1 | – | 16.9 |
| | C | 37.5 | 12.5 | 50.0 |

[a]Abundant sporulation with some colonies producing sclerotia; aerial mycelium only on aging; light-yellow reverse with little furrowing.

[b]Abundant sporulation in center of colony becoming sparse at edges; no sclerotia; ivory reverse with deep furrows extending from center of colony.

[c]Sparse sporulation over entire colony; abundant fluffy aerial mycelium; ivory reverse with deep furrows extending from center of colony.

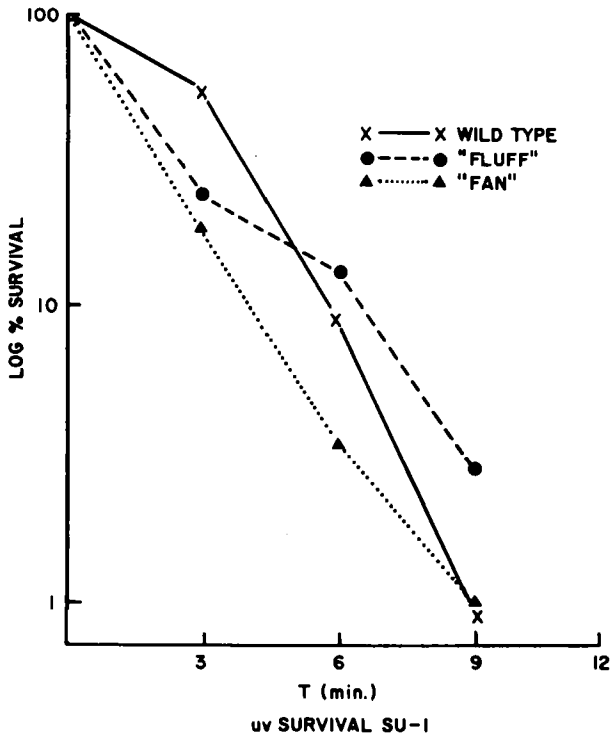


**FIG. 3. Percentage survival of UV-irradiated spore suspensions of wild type and 2 morphological variants of *A. parasiticus*.**

Figure 3. Ca. 99% kill was observed for all 3 types after a 9-min exposure to UV. Among the survivors of UV irradiation, some spore color and other mutants were seen. However, neither fan nor fluff survivors reverted to wild-type morphology in these experiments.

The aflatoxigenic mutant strain *wh-1 pdx-2* was subjected to serial transfer of mycelial macerates in PD broth (series D and E) and in AM plus pyridoxine (series F and G). These results are presented in Table II. No variants were observed from macerates transferred in PD broth, whereas both fan and fluff variants were observed after transfer in the defined medium. Microscopic examination of mycelial pellets from PD showed sporulation.

Aflatoxin production by *wh-1 pdx-2* and a single spore-derived culture of fluff and fan are presented in Table III. The white spore color and pyridoxine requirement of the morphological variants was retained, but ability to produce aflatoxin was lost in these variants.

The 3 forms of *wh-1 pdx-2* (standard, fan and fluff) were each crossed with *br-1 nor-1 ade-1* and 3 parasexual diploids were isolated. Aflatoxin production by the 3 diploids is presented in Table III. High levels of aflatoxin produced the diploids containing the standard (1,262 μg/100 mL AM) and the fan (1,930 μg/100 mL AM) form of *wh-1 pdx-2* whereas the diploid containing the fluff form produced only 116 μg/100 mL AM.

## DISCUSSION

We have shown that morphological variants can be induced in wild-type and mutant *A. parasiticus* by serial transfer of mycelial macerates. Sporulating cultures did not produce variants, nor did cultures that had been aged 1-20 weeks. This method was reproducible in the sense that the same types of variants were isolated in each experiment. However, the time of isolation, the number of each type, and the ratio of variants with respect to each other, differ among experiments.

These morphological variants do not produce aflatoxins. In the marked mutant strain *wh-1 pdx-2*, the spore color and auxotrophic markers are retained whereas aflatoxin producing ability is lost.

There are other reports concerning the effect of repeated laboratory transfers on aflatoxin production. Mayne et al. (16) demonstrated attenuation of aflatoxin production by *A. parasiticus* after serial transfer of a polysporous culture with hyphae on potato slices or agar media. When the attenuated cultures were transferred without mycelium by isolating single spores, high aflatoxin-producing ability was restored. Torres et al. (17) described the degeneration of 4 originally aflatoxigenic strains after several transfers at consecutive 72-hr intervals on crushed, moist wheat. Loss of aflatoxin synthetic capacity was accompanied by macroscopic and microscopic morphological changes. In a third study, a mutant strain of *A. parasiticus* that accumulated

TABLE II

**Morphological Variants of *wh-1 pdx-2* after Serial Mycelial Maceration in Two Media**

| Transfer (7 days) | Series | Morphological type (% cfu) | | |
|---|---|---|---|---|
| | | Standard[a] | Fan[a] | Fluff[a] |
| 0 | D[b] | 100 | – | – |
| | E | 100 | – | – |
| | F[c] | 100 | – | – |
| | G | 100 | – | – |
| 1 | D | 100 | – | – |
| | E | 100 | – | – |
| | F | 100 | – | – |
| | G | 100 | – | – |
| 2 | D | 100 | – | – |
| | E | 100 | – | – |
| | F | 77.4 | 0.2 | 22.4 |
| | G | 45.3 | 1.0 | 53.7 |

[a]See Table I for description of morphological types.

[b]Series D and series E in PD.

[c]Series F and series G in AM plus pyridoxine.

**TABLE III**

**Aflatoxin Production by Mutant and Diploid Strains of *Aspergillus parasiticus***

| Strain[a] | Aflatoxin (μg/100 mL AM) | | | | |
|---|---|---|---|---|---|
| | $B_1$ | $B_2$ | $G_1$ | $G_2$ | Total |
| *br-1 nor-1 ade-1* | 16 | 4 | 3 | ND[b] | 23 |
| *wb-1 pdx-2* | 1100 | 360 | 57 | 6 | 1525 |
| *fan wb-1 pdx-2* | Tr[c] | ND | ND | ND | Tr |
| *fluff wb-1 pdx-2* | ND | ND | ND | ND | ND |
| *wb-1 pdx-2/br-1 nor-1 ade-1* | 1010 | 225 | 22 | 5 | 1262 |
| *fan wb-1 pdx-2/br-1 nor-1 ade-1* | 1300 | 390 | 240 | 100 | 1930 |
| *fluff wb-1 pdx-2/br-1 nor-1 ade-1* | 75 | 21 | 20 | Tr | 116 |

[a]Spore color symbols: *wb* = white; *br* = brown; auxotrophic markers: *ade* = adenine, *pdx* = pyridoxine; mycelial markers: *nor* = norsolorinic acid; *fan* = absence mycelial pigmentation; flat spreading morphology with sporulation in center colony; *fluff* = absence mycelial pigmentation; sparse sporulation, abundant fluffy aerial mycelium.

[b]ND = none detected.

[c]Tr = less than 0.001 μg/100 mL AM.

the red pigment norsolorinic acid and low levels of aflatoxin produced unpigmented, nonaflatoxigenic forms after serial transfer in defined medium (18). The loss of pigment and aflatoxin production was not associated with either zinc deficiency or the presence of barium in the medium, but was associated with conditions in which the inocula contained hyphae rather than spores. These studies and our results suggest that the appearance of the fan and fluff phenotypes, with associated loss of aflatoxigenicity, is correlated with conditions in which the nuclei in the inoculum are obtained from vegetative hyphae rather than from conidiospores.

The highly specific nature of the phenotypic changes produced in this study raises the question of whether these nonaflatoxigenic isolates are "variants" or "mutants." Despite the stability of the fan and fluff forms, we prefer to call them variants because of the nature of the protocol which produced them. In fungi, specific, directed modes of induction of variants are often indicative of extrachromasomal inheritance (19). The differential loss of aflatoxin production and retention of other genetic markers, coupled with the unusual mode of isolation, could be interpreted as presumptive evidence that the genetic information for the aflatoxin pathway is extrachromasomal. In fungi, extrachromasomal and nuclear inheritance can be distinguished on the basis of a heterokaryon test. Studies in this laboratory have shown that the fan and fluff phenotypes do not segregate in a heterokaryon test (20) indicating that we are not observing simple cytoplasmic inheritance. Although the enzymes for the aflatoxin pathway appear to be associated with a dispensable and differentially inherited genetic element, a more subtle genetic mechanism than presence or absence of a cytoplasmic factor is required.

Parasexual diploids synthesized with a fan strain produced aflatoxin in concentrations similar to a control diploid (Table III), whereas a parasexual diploid containing a fluff variant produced 10-fold less aflatoxin. This implies that distinct genetic phenomena are responsible for the 2 nonaflatoxigenic phenotypes, perhaps involving regulation of the enzymes of the aflatoxin pathway.

Further genetic studies of the fan and fluff phenotype, using parasexual genetic analysis and direct examination of the nucleic acids and enzymes of these variants, will be necessary to resolve the enigmas posed. These studies will elucidate aspects of aflatoxin biosynthesis and also serve as model systems for the whole problem of strain degeneration in secondary metabolic capability among the mycelial fungi.

ACKNOWLEDGMENTS

We thank J. Floyd and P.-M. Leong for experimental assistance, and W. Carney for photographs. This research was partially supported by grants from the American Cancer Society (IN-133A), the Cancer Association of Greater New Orleans (103-80), and a Cooperative Agreement from the U.S. Department of Agriculture (58-7B30-0-246).

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