

Isolation and Characterization of *Aspergillus parasiticus* Mutants with Impaired Aflatoxin Production by a Novel Tip Culture Method

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A convenient procedure consisting of UV photography (K. Yabe, Y. Ando, M. Ito, and N. Terakado, Appl. Environ. Microbiol. 53:230-234, 1987) and a tip culture method has been devised for the isolation and characterization of *Aspergillus parasiticus* mutants relating to aflatoxin production. With the latter procedure, the production of aflatoxins excreted into the culture medium and precursors in the mycelium were easily measured quantitatively or semiquantitatively. A total of 38 mutants in which the aflatoxigenicity was decreased or lost were obtained by UV radiation; 3 were found to be blocked mutants, which accumulated the aflatoxin precursors versicolorin A or averantin.

Aflatoxins are toxic secondary metabolites produced by certain strains of the common molds *Aspergillus flavus* and *A. parasiticus* (3, 19). To prevent the contamination of food and feed with aflatoxins, it is important to clarify the precise mechanisms of aflatoxin biosynthesis.

Several techniques including the use of blocked mutants, metabolic inhibitors, radioactive labeling experiments, and cell-free systems have been applied in these studies (3, 19). The mutants of both *A. flavus* and *A. parasiticus* with impaired production of aflatoxins are known to be very useful for the analysis of the pathway (4, 10, 11, 13-16, 22, 24). However, various detailed aspects of the biosynthetic pathway of aflatoxins are still unclear, and the factors controlling the induction of aflatoxin production have not been elucidated.

In this paper, we present a new selection method for mutants with impaired aflatoxin production, in which the UV photography method (26) and tip culture method were used stepwise. The UV photography method is a qualitative method for the analysis of aflatoxin production, whereas the tip culture method enables the measurement of aflatoxins and precursors of a large number of molds quantitatively or semiquantitatively. With this procedure, 38 mutants were obtained, and it became possible to characterize aflatoxin production as well as the accumulation of some precursors by each mutant.

MATERIALS AND METHODS

Microorganisms. The aflatoxin-producing wild strain *A. parasiticus* SYS-4 (NRRL 2999) was used in this study. *Aspergillus oryzae* SYS-2 (IFO4251), used as a control strain, does not produce aflatoxins. The mutants obtained were designated by the prefix NIAH-. Freshly prepared conidiospore suspensions were prepared as previously described (26).

Media. For the screening by UV photography, the GY agar medium was used as described before (26). In some cases, 0.05% sodium deoxycholate was added to GY agar medium (GYD) to obtain compact colonies (18). For the tip culture method, YES liquid medium (2% yeast extract, 20% sucrose) (9) was used.

Mutagenesis. Mutants were prepared by UV radiation. The conidiospores of *A. parasiticus* SYS-4 were irradiated with short-wavelength (254-nm) UV light (less than 1% viability).

Tip culture method. The tip culture method is illustrated in Fig. 1. First, a chip of the Pipetman tip (1 ml; Gilson) was stuffed with quartz wool (fine; Gas Chro Kogyo Co. Ltd., Japan), and the tip was weighed. The tip was placed in a glass tube (9 mm in diameter, 110 mm in length), and the top of the tip was covered with an aluminium cap. After the tube was autoclaved and dried, the bottom of the tip was shielded with Parafilm. Autoclaved YES medium (250 μ l) was poured onto the resultant tip. Since growth and aflatoxin production were found to be independent of the number of inoculated spores in this study, 5 μ l of the spore suspension was used for inoculation without counting the spore number of the suspension. The culture was grown for 4 days at 28°C in a box with a lid containing water. After incubation, Parafilm was removed from the tip, and the whole set was centrifuged at 900 \times g for 20 s. At this step, the culture medium containing aflatoxins excreted from mycelia was separated from the mycelia remaining in the tip. When needed, the tip containing the mycelia was weighed, and the wet weight of the mycelia was calculated. When the dry weight of the mycelia was measured, the tip was autoclaved, dried, and weighed.

For the assay of aflatoxins, 10 μ l of the filtrate was directly spotted on a silica gel plate (silica gel 60, 5721; Merck & Co., Inc., Rahway, N.J.), dried, and developed with chloroform-ethyl acetate-90% formic acid (6:3:1, vol/vol/vol) or benzene-ethyl acetate (7:3, vol/vol). Aflatoxins were also quantified by using thin-layer chromatography (TLC) and densitometry (26) or by high-performance liquid chromatography by the method of Manabe et al. (20) with slight modifications. One milliliter of water-saturated chloroform was added to the tube containing the filtrate and mixed with a Vortex mixer. A sample of the lower chloroform layer was injected into a high-performance liquid chromatography apparatus (Shimadzu HPLC LC-6A system) equipped with a silica gel column (Shim-pack CLC-SIL). The solvent system consisted of toluene-ethylacetate-formic acid-methanol (145:45:4:6, vol/vol/vol/vol). The retention times of aflatoxins B₁,

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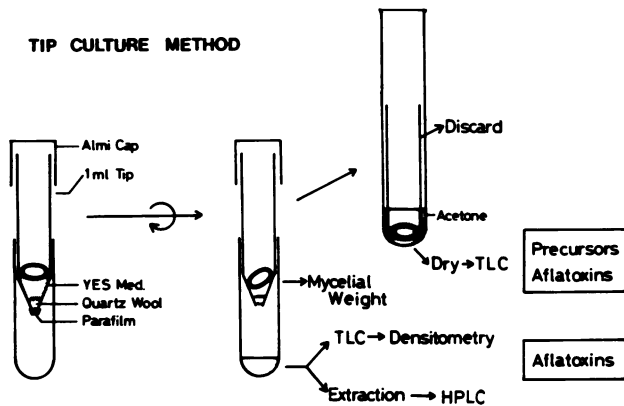


FIG. 1. Outline of tip culture method. After 4 days of culture, Parafilm was removed from the tip, and the mycelia of the mold and culture medium were separated by centrifugation. The production of aflatoxins and precursors by each mutant was detected by high-performance liquid chromatography or TLC.

B_2 , G_1 , and G_2 were compared with those of standard samples.

For the assay of the precursor pigments in the mycelia, the tip was cut immediately under the mycelial mat and transferred to a new tube. Acetone (1 ml) was added into the tube, and the precursors were extracted from the mycelia overnight. The tip containing the mycelia was removed, and then the extract was concentrated to dryness. The residue was solubilized with 0.2 ml of benzene-acetonitrile (98:2, vol/vol). A 50- μ l sample of this solution was spotted on a silica gel plate and developed with benzene-ethyl acetate (7:3, vol/vol).

Selection of mutants. A two-step method was utilized. In the first step, an appropriately diluted suspension of irradiated spores was spread on the GYD agar medium. After 3 days of culture, each petri dish was photographed by UV photography as described before (26). To determine aflatoxin production instantly, a Hasselblad 500 EL/M S-planer F5.6/120 camera with Polaroid (type 667) film or a Nikon high-resolution black-and-white CCTV camera HR-900W was used. Thereafter, the colonies showing a reduced or lost absorption compared with the wild-type strain were picked up and suspended in a small volume of the water solution containing 0.01% Tween 80.

The production of aflatoxins by these mutants was examined by the tip culture method as a second selection step. A 10- μ l sample of the culture medium was analyzed by TLC, and the mutants which did not produce or produced a smaller amount of aflatoxins than the wild-type strain on the TLC plate were selected as suitable mutants. The spores of these mutants were further purified three times by single-colony isolation on GY agar medium by observing them with a Nikon CCTV camera.

Isolation and characterization of the accumulated pigments. Three blocked mutants, NIAH-9, -16, and -204, were grown in surface culture at 28°C for 7 days. A sample (5 μ l) of the spore suspension (approximately 5×10^3 spores) was inoculated into 2 ml of YES medium in glass tubes (1.7 cm in diameter). Mycelial mats were harvested, washed, and extracted with acetone until they became colorless. The combined extracts were evaporated to dryness. The residue was dissolved and purified by Sephadex LH-20 column (2.5 by 80 cm) chromatography with methanol. The pigment fraction was further purified with a silica gel column with benzene-

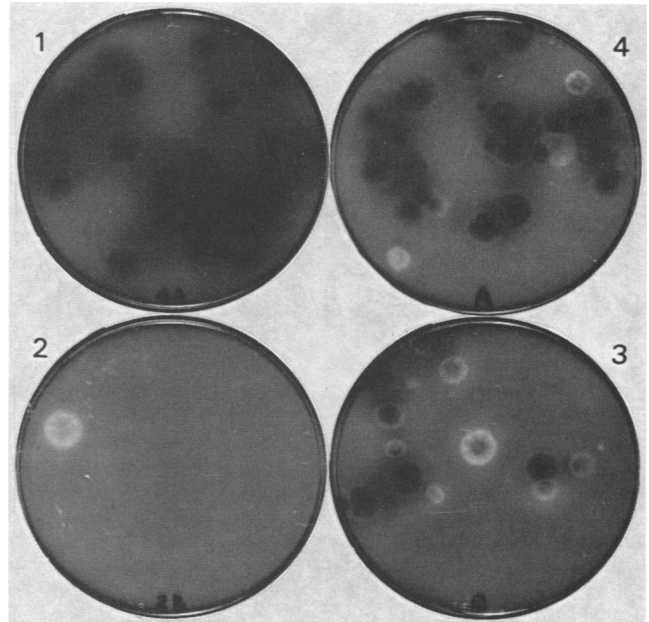


FIG. 2. First selection by using UV photography. Vegetative spores of *A. parasiticus* SYS-4 (NRRL 2999) were irradiated with short-wavelength UV light and spread on GYD agar medium. After 3 days of incubation, the petri dishes were photographed by UV photography. Petri dishes contained the following colonies: 1, wild-type strain; 2, *A. oryzae* SYS-2 (IFO 4251), which did not produce aflatoxins; 3 and 4, irradiated spores.

acetone (9:1, vol/vol), and the isolated pigment was recrystallized from chloroform.

Physical and chemical analysis was performed by comparing the chromatographic behavior with standard samples and by measuring the infrared (IR) spectrum, proton nuclear magnetic resonance, or mass spectrum. IR spectra were taken with a Hitachi 295 IR spectrophotometer with a KBr disk. Nuclear magnetic resonance spectra were recorded on a JEOL JNM-GX 270 spectrometer. Mass spectra were obtained with a Hitachi RM-50 spectrometer (ionization voltage, 70 eV) with a direct inlet system.

Fluorescence photographs were taken by using Funa-UV-light type SL-800F, a Schott 470 KV filter, and Kodak TMY 5053 film.

RESULTS

First selection by UV photography. After treatment for mutagenesis, the irradiated conidiospores were spread on the GYD agar medium and cultured. When the resultant plates were photographed by UV photography, the extent of the darkness and the size of the colonies from the irradiated spores were found to be very heterogeneous (Fig. 2). In contrast, all the colonies of the wild-type strain *A. parasiticus* SYS-4 were black and of uniform size. Since aflatoxins are the main substances absorbing UV light under these conditions (26), it was expected that in these gray and white mutants the ability to produce aflatoxins was reduced or lost. More than 10% of the resultant colonies were found to be mutants in which the UV absorption was decreased or lost in the first selection step.

In the UV photos, however, it was not possible to discriminate between the desired mutants with attenuated aflatoxigenicity and the mutants with a delayed growth rate,

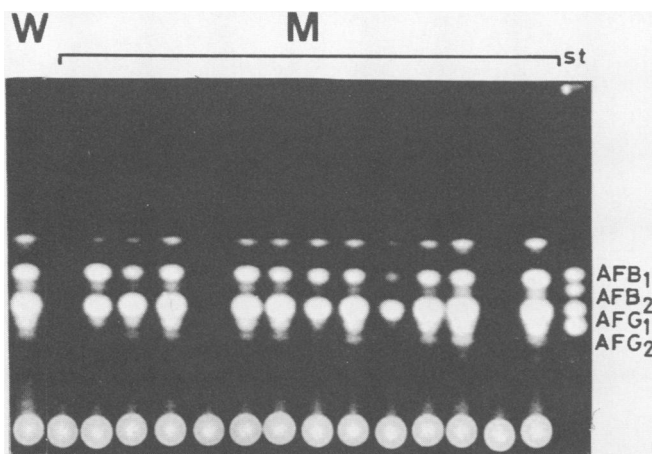


FIG. 3. Secondary selection by using the tip culture method. The conidiospores of the mutants selected first were inoculated into YES medium in the tips. After 4 days of culture at 28°C, 10 μ l of the culture medium was analyzed by TLC with a developing solution consisting of chloroform-ethylacetate-90% formic acid (6:3:1, vol/vol/vol). Then visual estimation of aflatoxin production was performed with the wild-type strain. W, Wild type strain SYS-4; M, mutants screened first. Fluorescence photographs are shown.

because secondary metabolites such as aflatoxins are usually produced after the completion of the growth phase of the organism (7). In fact, some of the white mutants changed to black after a longer incubation period based on UV photos (data not shown). To select the desired mutants specifically, the tip culture method was applied for the secondary selection.

Secondary selection by tip culture. Previous experiments had revealed that the time course of aflatoxin production and growth rate were independent of the number of inoculated spores and that aflatoxin production (total amount of aflatoxins formed per unit of dry weight of mycelia) reached maximum values after 4 days of incubation (data not shown). Therefore, the number of spores inoculated was not uniform, and the amount of aflatoxins excreted from the mycelia was determined by TLC with 4-day cultures. The results of secondary selection are shown in Fig. 3.

In the second step, approximately 10% of the mutants first selected were identified as mutants in which the aflatoxigenicity was decreased or lost. These mutants were purified by single colony isolation and used for further experiments.

Characterization of the mutants. By using the tip culture method, 38 mutants including three kinds of blocked mutants were obtained: 17 mutants did not produce aflatoxins, including blocked mutant NIAH-9; 21 other mutants produced less aflatoxin than did the wild strain; 10 mutants, including two blocked mutants NIAH-16 and NIAH-204, produced 0 to 10 ng of total aflatoxins per mg (wet weight) of mycelia; 6 mutants produced 10 to 100 ng/mg; 5 mutants produced 100 to 500 ng/mg. The wild strain produced 540 ng of aflatoxins per mg of mycelia in this condition.

The fluorescence photographs of TLC analysis of the culture medium and the extract of the mycelia of some examples are shown in Fig. 4A and B, respectively. The wild-type strain SYS-4 produced mainly aflatoxins B₁ and G₁. In contrast, the mutants NIAH-1 and NIAH-26 did not produce either aflatoxins or precursors. The remaining three mutants were blocked mutants, which accumulated red or orange pigments in the mycelia. Although other blue and green fluorescent substances were observed, they were not

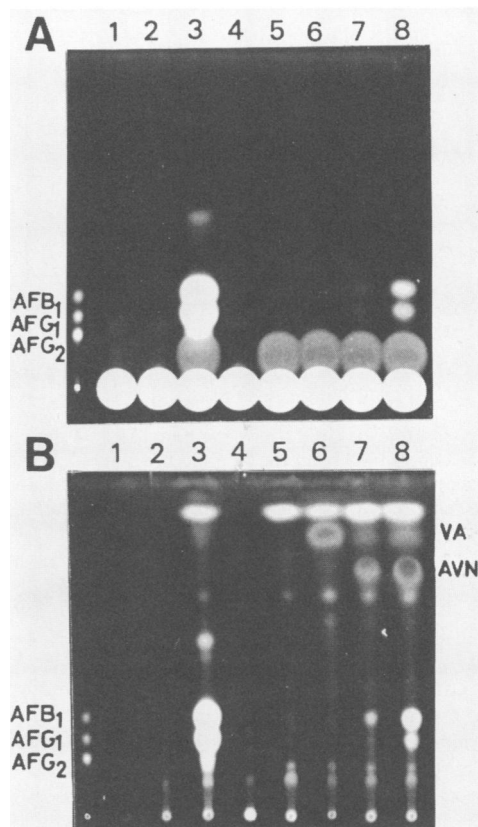


FIG. 4. Analysis of culture medium and acetone extract of mycelia. Culture medium (10 μ l) (A) and 50 μ l of benzene-acetonitrile (98:2, vol/vol) (B) in which the residue of acetone extract had become soluble were spotted on a silica gel TLC plate and developed with benzene-ethyl acetate (7:3, vol/vol). Fluorescence photographs are shown here. Lanes: 1, medium only; 2, control strain *A. oryzae* SYS-2 (IFO4251), which did not produce aflatoxins (23.4 mg of mycelial wet weight per 250 μ l of culture medium); 3, aflatoxin-producing wild-type strain SYS-4 (20.5 mg); 4 and 5, mutants which did not produce aflatoxins and precursors, NIAH-1 (18.9 mg) and NIAH-26 (21.6 mg), respectively; 6, versicolorin A-accumulating mutant NIAH-9 (18.2 mg); 7 and 8, averantin-accumulating mutants NIAH-16 (21.1 mg) and NIAH-204 (19.1 mg), respectively. The positions of the aflatoxins B₁ (AFB₁), G₁, and G₂, versicolorin A (VA), and averantin (AVN) are indicated.

considered to be related to aflatoxin biosynthesis, because the production of these substances did not show a carbon source dependency (1, 8) (data not shown).

Three pigments that accumulated in the mycelia (16.4 g of wet mycelia) of NIAH-204 were isolated and purified. The major pigment (8 mg) was identified as averantin based on the measurement of the proton nuclear magnetic resonance spectrum, IR spectrum, and mass spectrum compared with the data reported by Birkinshaw et al. (6). The second pigment (7 mg), whose nuclear magnetic resonance spectrum showed a signal at 3.47 ppm, was entirely consistent with the data reported for methoxyaverantin isolated from *A. versicolor* by Aucamp and Holzappel (2). Although the amount of averantin was almost equal to that of methoxyaverantin in this preparation, the value did not match that corresponding to the TLC pattern in Fig. 4, in which the amount of averantin was much larger than that of methoxyaverantin. This discrepancy may reflect differences in the culture time. The third pigment, which amounted to less than 1 mg, was

TABLE 1. Precursors accumulated in blocked mutants^a

Mutant	AF production	Precursor accumulated	Speculated blocked step
NIAH-9	Lost	VA	VA → ST
NIAH-16	Decreased	AVN	AVN → AVF
NIAH-204	Decreased	AVN>MAVN	AVN → AVF

^a VA, Versicolorin, A; St, sterigmatocystin; AVN, averantin; AVF, averufanin; MAVN, methoxyaverantin.

identified as norsolorinic acid based on the data of the IR spectrum compared with that of the authentic standard.

The pigment accumulated by NIAH-16 (5 g of wet mycelia) was also identified as averantin (4 mg) based on the measurement of the IR spectrum. The third mutant, NIAH-9 (5.0 g of wet mycelia), was found to accumulate versicolorin A (2 mg) based on the IR data, which were consistent with those of the standard sample isolated from *A. versicolor* (12).

DISCUSSION

In this report, we used a simple screening method for the isolation and characterization of mutants in which aflatoxin production was decreased or lost. By using this method, various kinds of mutants for aflatoxin production were obtained within a short period of time. Moreover, it was possible to detect easily the aflatoxins and precursors quantitatively or semiquantitatively for a large number of mutants by using the tip culture method. A method for the screening of mutants has been reported (17, 23) in which the toxin was produced in liquid medium, followed by some purification procedures and final detection by TLC. However, the tip culture method described herein is much simpler than those methods and is very safe, because the scale of the culture is small (250 μ l) and the amount of organic solvent required in this method is very small. Used tips containing mycelia and parafilms should be burned. The tip culture method can also be applied to other molds; for example, in *A. versicolor* various kinds of pigments accumulated in mycelia could be observed.

Although the mutants with a markedly delayed growth rate, those with a low production of conidiospores, and the mutants which showed only small changes in the aflatoxigenicity were deliberately excluded, it was possible to obtain the desired mutants corresponding to at least 1% of the spores examined. The high mutation rates observed in this experiment suggest that several reactions or pathways are involved in aflatoxin biosynthesis and that many factors affect the production of aflatoxins.

Among the 38 mutants obtained in this experiment, three were considered to be precursor-accumulating mutants. These mutants could be identified not only as gray colonies in the UV photographs but also as yellow or orange colonies under visible light, because the pigments accumulated in the mycelia. Mutant NIAH-9 was identified as a versicolorin A-accumulating mutant. Judging from the biosynthetic pathway of aflatoxins (3), this mutant seems to be blocked in the step from versicolorin A to sterigmatocystin (Table 1). Lee et al. also reported the characterization of a mutant of *A. parasiticus* that accumulated versicolorin A (15).

A. parasiticus NIAH-16 and -204 were both averantin-accumulating mutants and leaky mutants for aflatoxin production. The production of averantin was higher in NIAH-204 than in NIAH-16. Since averufanin has been reported to be a precursor of aflatoxin B₁ in the pathway between

averantin and averufin (21), both mutants are considered to have undergone a mutation at this level. Bennett et al. also reported the isolation of a mutant of *A. parasiticus* that accumulated averantin, which is different from the ones reported here, because that was a double mutant of versicolorin A-accumulating mutant and did not produce aflatoxins. In the preparation of NIAH-204 for the measurement of the IR or nuclear magnetic resonance spectrum, a large amount of methoxyaverantin was detected. However, it remains to be determined whether methoxyaverantin is related to aflatoxin biosynthesis or is a by-product of averantin.

Further characterization of the mutants obtained in this study is now in progress, and the biosynthetic relationships among the aflatoxins are discussed in the accompanying paper (25).

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LITERATURE CITED

1. Abdollahi, A., and R. L. Buchanan. 1981. Regulation of aflatoxin biosynthesis: induction of aflatoxin production by various carbohydrates. *J. Food Sci.* **46**:633-635.
2. Aucamp, P. J., and C. W. Holzapfel. 1970. Polyhydroxyanthraquinones from *Aspergillus versicolor*, *Aspergillus nidulans* and *Bipolaris sp.* Their significance in relation to biogenetic theories on aflatoxin B₁. *J. South Afr. Chem. Inst.* **23**:40-56.
3. Bennett, J. W., and S. B. Christensen. 1983. New perspectives on aflatoxin biosynthesis. *Adv. Appl. Microbiol.* **29**:53-92.
4. Bennett, J. W., and L. A. Goldblatt. 1973. The isolation of mutants of *Aspergillus flavus* and *A. parasiticus* with altered aflatoxin producing ability. *Sabouraudia* **11**:235-241.
5. Bennett, J. W., L. S. Lee, S. M. Shoss, and G. H. Boudreaux. 1980. Identification of averantin as an aflatoxin B₁ precursor: placement in the biosynthetic pathway. *Appl. Environ. Microbiol.* **39**:835-839.
6. Birkinshaw, J. H., J. C. Roberts, and P. Roffey. 1966. Studies in mycological chemistry. XIX. "Product B" (averantin) [1,3,6,8-tetrahydroxy-2-(1-hydroxyhexyl)anthraquinone], a pigment from *Aspergillus versicolor* (vuillemin) Tiraboschi. *J. Chem. Soc. C* **1966**:855-857.
7. Bu'Lock, J. D. 1975. Secondary metabolism in fungi and its relationship to growth and development, p. 33-58. *In* J. E. Smith and D. R. Berry (ed.), *The filamentous fungi*, vol. 1. John Wiley & Sons, Inc., New York.
8. Davis, N. D., and U. L. Diener. 1968. Growth and aflatoxin production by *Aspergillus parasiticus* from various carbon sources. *Appl. Microbiol.* **16**:158-159.
9. Davis, N. D., V. L. Diener, and D. W. Eldridge. 1966. Production of aflatoxin B₁ and G₁ by *Aspergillus flavus* in a semisynthetic medium. *Appl. Microbiol.* **14**:378-380.
10. Detroy, R. W., S. Freer, and A. Ciegler. 1973. Aflatoxin and anthraquinone biosynthesis by nitrosoguanidine-derived mutants of *Aspergillus parasiticus*. *Can. J. Microbiol.* **19**:1373-1378.
11. Donkersloot, J. A., R. I. Mateles, and S. S. Yang. 1972. Isolation of averufin from a mutant of *Aspergillus parasiticus* impaired in aflatoxin biosynthesis. *Biochem. Biophys. Res. Commun.* **47**:1051-1055.
12. Hamasaki, T., Y. Hatsuda, N. Terashima, and M. Renbutsu. 1965. The structure of two new metabolites of *Aspergillus versicolor*. *Agr. Biol. Chem.* **29**:166-167.
13. Leach, L. L., and K. E. Papa. 1974. Aflatoxins in mutants of *Aspergillus flavus*. *Mycopathol. Mycol. Appl.* **52**:223-229.
14. Lee, E. G.-H., and P. M. Townsley. 1968. Chemical induction of mutation or variation in aflatoxin-producing cultures of *Aspergillus flavus*. *J. Food Sci.* **33**:420-423.
15. Lee, L. S., J. W. Bennett, A. F. Cucullu, and J. B. Stanley. 1975.

- Synthesis of versicolorin A by a mutant strain of *Aspergillus parasiticus* deficient in aflatoxin production. *J. Agric. Food Chem.* **23**:1132-1134.
16. Lee, L. S., J. W. Bennett, L. A. Goldblatt, and R. E. Lundin. 1971. Norsolorinic acid from a mutant strain of *Aspergillus parasiticus*. *J. Am. Oil Chem. Soc.* **48**:93-94.
 17. Lillard, H. S., R. T. Haxlin, and D. A. Lillard. 1970. Aflatoxigenic isolates of *Aspergillus flavus* from pecans. *Appl. Microbiol.* **19**:128-130.
 18. Mackintosh, M. E., and R. H. Pritchard. 1963. The production and replicating of micro-colonies of *Aspergillus nidulans*. *Genet. Res.* **4**:322.
 19. Maggon, K. K., S. K. Gupta, and T. A. Venkitasubramanian. 1977. Biosynthesis of aflatoxins. *Bacteriol. Rev.* **41**:822-855.
 20. Manabe, M., T. Goto, and S. Matsuura. 1978. High-performance liquid chromatography of aflatoxins with fluorescence detection. *Agric. Biol. Chem.* **42**:2003-2007.
 21. McCormick, S. P., D. Bhatnagar, and L. S. Lee. 1987. Averufanin is an aflatoxin B1 precursor between averantin and averufin in the biosynthetic pathway. *Appl. Environ. Microbiol.* **53**:14-16.
 22. Papa, K. E. 1982. Norsolorinic acid mutant of *Aspergillus flavus*. *J. Gen. Microbiol.* **128**:1345-1348.
 23. Pons, W. A., A. F. Cucullu, A. O. Franz, and L. A. Goldblatt. 1968. Improved objective fluorodensitometric determination of aflatoxins in cottonseed products. *J. Am. Oil Chem. Soc.* **45**:694-699.
 24. Singh, R., and D. P. H. Hsieh. 1977. Aflatoxin biosynthetic pathway: elucidation by using blocked mutants of *Aspergillus parasiticus*. *Arch. Biochem. Biophys.* **178**:285-292.
 25. Yabe, K., Y. Ando, and T. Hamasaki. 1988. Biosynthetic relationship among aflatoxins B₁, B₂, G₁, and G₂. *Appl. Environ. Microbiol.* **54**:2101-2106.
 26. Yabe, K., Y. Ando, M. Ito, and N. Terakado. 1987. Simple method for screening aflatoxin-producing molds by UV photography. *Appl. Environ. Microbiol.* **53**:230-234.