

Characterization of some aflatoxin-deficient mutants of *Aspergillus parasiticus*

M.A. Moreno, M.C. Ramos and G. Suarez - Departamento de Patología Animal I (Sanidad Animal), Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain

Aflatoxins are polyketide secondary metabolites produced by isolates of *Aspergillus flavus* and *A. parasiticus*. Since 1960, when a wide toxic outbreak was associated with moldy feed contaminated with aflatoxins, many efforts have been concentrated on this topic because of both the carcinogenic properties of these mycotoxins, and the worldwide occurrence of aflatoxin-producing fungi in cereal, cereal products and other agricultural commodities.

Aflatoxin biosynthesis, mainly aflatoxin B₁, is a subject of continued investigation. Various experimental approaches and several mutants blocked at different steps have been very useful in the elucidation of the pathway, eight compounds being proposed as intermediates (norsolorinic acid, averantin, averufanin, averufin, versiconal hemiacetal acetate, versicolorin A, sterigmatocystin and O-methyl-sterigmatocystin).

In this paper we present the characterization of seven *A. parasiticus* mutants with little or no aflatoxin producing ability (Moreno et al. 1987 Can. J. Microbiol. 33:927-929). Using a standard natural medium (wet wheat medium, 28°C/7 days), three mutants (named M16(20-1), M39(10-0) and M73(05-10)) did not produce aflatoxins. The remaining four had attenuated aflatoxin production compared with the parental strain *A. parasiticus* NRRL 2999 in the same experimental conditions. Production expressed as a percentage of that measured in NRRL 2999 was as follows: 5% (mutant M06(20-1)), 1% (mutants M21(20-1) and M39(10-0.1)) and 0.0001% (mutant M85(20-10)).

Putative accumulation of previously-characterized aflatoxin precursors and mycelial color were investigated by growing mutants in a liquid chemically defined medium (Adye and Mateles 1964 Biochim. Biophys Acta 86:418-420) for 7 days at 28°C in a static system. At the end of the incubation period, mycelium was separated from medium by cheesecloth filtration and extracted with acetone using a standard protocol (Bennett 1979 J. Gen. Microbiol. 113:127-136). Final chloroformic extracts were analyzed by thin layer chromatography on silica-gel plates using toluene/ethyl acetate/acetic acid (80/10/10) or benzene/acetic acid (95/5) as developing solvents. Aflatoxin precursors norsolorinic acid, averantin, averufin and versicolorin A were prepared by the same procedure from the mycelium of accumulating *A. parasiticus* mutants kindly supplied by J.W. Bennett (ATCC 24690, ATCC 5674, ATCC 15517 and ATCC 36537), and were partially purified using the dry-column method (Dun et al. 1980 Biotechnol. Lett. 2:17-22); standards of aflatoxins B₁, B₂, G₁, and G₂, sterigmatocystin and O-methyl-sterigmatocystin were purchased from Sigma Chem. Co. (St. Louis, USA).

As has been previously mentioned (Bennett 1979 J. Gen. Microbiol. 113:127-136) mycelial color is related to accumulation of anthraquinone aflatoxin precursors and mutants M06(20-1), M16(20-1) and M39(10-0.1), that showed orange mycelial pigmentation, did accumulate averufin. Mutant M85(20-10), the mycelium of which did not show a differentiated color, accumulated the aflatoxin precursor versicolorin A. Mutants M21(20-1), M26(10-0.1) and M73(05-10) also did not show distinctive mycelial pigmentation and the accumulation of the precursors mentioned above was not detected.

Transformation of sterigmatocystin and O-methyl-sterigmatocystin was also investigated to complete the study of the aflatoxin-related status of the mutants. Using a modified procedure (Pro et al. 1991 Mycopathologia 116:71-75), transformation of both precursors into aflatoxin B₁ was carried out by 48 h-old mycelium of the averufin-accumulating mutants M06(20-1) and M16(20-1), the versicolorin A-accumulating mutant M85(20-10) and the mutant M21(20-1), but it was difficult to prove in mycelium of the averufin accumulating mutant M39(10-0.1). Mutants M26(10-0.1) and M73(05-10) gave negative results in these experiments and, as consequence, they have tentatively been classified as putative regulatory aflatoxin mutants.

Aflatoxin-deficient mutants became particularly interesting because they can be used as recipients in transformation experiments searching for new aflatoxin-related DNA sequences. The complementation of aflatoxin deficient mutants has recently led to the isolation of two genomic sequences related to aflatoxin production (Chang et al. 1992 Curr. Genet. 21:231-233; Skory et al. 1992 Appl. Environ. Microbiol. 58:3527-3537). As a consequence, auxotrophic (arginine) and color (white) markers have been introduced into the mutant M73(05-10) for its use as a recipient in a genetic transformation system. Using autonomously replicating plasmids derived from *A. nidulans* (Gems et al. 1991 Gene 98:61-67) a new transformation system has been developed in our laboratory (Moreno et al. 1993 BMS Aspergillus Symposium, Canterbury). Incorporation of markers into the remaining mutants is in progress.