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Aflatoxins: Background, Toxicology, and Molecular Biology

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Abstract

Mycotoxins are mold poisons; aflatoxins are the best known and most widely studied mycotoxins. The contamination of foods and feeds with aflatoxin can have serious consequences for human and animal health. In general, aflatoxin exposure is most likely to occur in the developing countries where food handling and storage processes are suboptimal, where malnutrition is widespread, and where few regulations exist to protect the exposed populations. Depending on dose and other variables, aflatoxins can be mutagenic, carcinogenic, teratogenic, and immunosuppressive. Fundamental studies on the genetics, biosynthesis and molecular biology of aflatoxin producing fungi may offer insights into controlling this serious agricultural problem.

1. INTRODUCTION

Mycotoxins are difficult to define in a few words. All mycotoxins are low molecular weight natural products produced by filamentous fungi that are toxic to vertebrates in low concentrations. Many mycotoxins display overlapping toxicities to invertebrates, plants, and microorganisms *(1,2)*. Mycotoxins initially gained prominence in the early 1960s after a mysterious "Turkey X disease" killed approximately 100,000 turkey poults in England *(3–5)*. Turkey X disease was linked to a peanut (groundnut) meal contaminated with *Aspergillus flavus* and the toxic principles were named aflatoxins (*A. flavus* toxins). For a while, the study of toxic mold metabolites became a "hot topic" in agriculture. In fact the 15 yr between 1960 and 1975 were labeled as a "mycotoxin gold rush" because so many chemical prospectors joined the search for mycotoxins *(6)*. Eventually several hundred fungal metabolites with toxic properties were isolated. The best single compendium for accessing the structures and chemical profiles of these toxic compounds is the three-volume *Handbook of Secondary Fungal Metabolites (7)*.

Mycotoxins are commonly found in foods and feeds all over the world. It has been estimated that a quarter of the world's crops are contaminated to some extent with mycotoxins *(8,9)*. Kuiper-Goodman, a leading figure in the risk assessment field, ranks mycotoxins as the most important noninfectious, chronic dietary risk factor, higher than synthetic contaminants, plant toxins, food additives, or pesticide residues *(10)*.

The mycotoxin literature is enormous. Since aflatoxins are the most important mycotoxin, each major monograph devotes considerable attention to the aflatoxin problem *(11–27)*. Many reviews focus specifically on mycotoxins and risks related to human health *(2,9,28–34)*.

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2. HISTORY OF AFLATOXIN RESEARCH

The classic monograph, *Aflatoxin. Scientific Background, Control, and Implications (35)* is an excellent source for learning about the early history of mycotoxin research, and summarizes the early chemical and toxicological studies. To reiterate, the aflatoxins were isolated and characterized after the Turkey X disease was traced to a family of metabolites found in mold contaminated feed. The major aflatoxins were called B_1, B_2, G_1, and G_2 (Fig. 1) on the basis of their blue or green fluorescence under ultraviolet light, and relative chromatographic mobility during silica gel thin-layer chromatography. In addition to the four major aflatoxins produced by mold metabolism, about a dozen other aflatoxins (e.g., P_1, Q_1, B_{2a}, and G_{2a}) were described, especially as mammalian biotransformation products of the major metabolites *(7,18,36)*. For example, cows metabolize aflatoxin B_1 from cattle feed into a hydroxylated derivative called aflatoxin M_1 that is then secreted in milk *(37)*.

Early toxicological studies focused on the acute toxic effects of aflatoxins on animals, and demonstrated that ducklings, hamsters, rabbits, trout, rats, and a number of other vertebrates were all susceptible. Soon it was discovered that aflatoxins administered in lower doses over longer periods of time could induce tumors, particularly in the liver. Rats and trout were highly susceptible to the carcinogenic effect of aflatoxin B_1. Ten percent of trout fed a diet containing 20 ppb of aflatoxin B_1 for as little as 3 d had developed hepatomas a year later; and rats fed a single oral does of 5–7 mg of aflatoxin B_1 developed liver tumors. On the other hand, mice, hamsters, and, by extrapolation, many other untested mammalian species were relatively resistant *(35,38)*. In addition, the mechanisms of acute toxicity and aflatoxin-mediated carcinogenicity seemed to be quite different. Rats were more susceptible to aflatoxin-induced hepatocarcinoma than were hamsters yet the acute LD_{50}s for the species were similar *(11)*. Aflatoxin B_1 was recognized as the most powerful naturally occurring carcinogen ever discovered *(39)*.

It should be pointed out that not all the authors distinguished between the term "aflatoxin" (the generic family of toxins) and "aflatoxin B_1" (usually the major aflatoxin produced by toxigenic strains of *Aspergillus*). Most toxicological studies have been conducted using aflatoxin B_1.

3. FUNGI AND PHYSIOLOGY

Aflatoxins enter the food chains when toxigenic molds grow on foods and feeds. For several decades, *A. flavus* and *Aspergillus parasiticus* were thought to be the only species capable of producing aflatoxins. Then, in 1987, it was reported by Kurtzman et al. *(40)* that *Aspergillus nomius*, a species closely related to *A. flavus*, was also aflatoxigenic. More recently, a number of other aflatoxin-producing species have been described: *A. bombycis (41)*, *A. ochraceoroseus (42,43)*, *A. pseudotamari (44)*, and *A. tamarii (45)*, as well as *Emericella astellata (46)* and *Emericella venezuelensis* (Klich, unpublished data). Compared to *A. flavus* and *A. parasiticus*, these species are less abundant in nature and are rarely encountered in agriculture.

Within *A. flavus* and *A. parasiticus*, different strains display a great deal of qualitative and quantitative difference in their toxigenic abilities. For example, it has been estimated

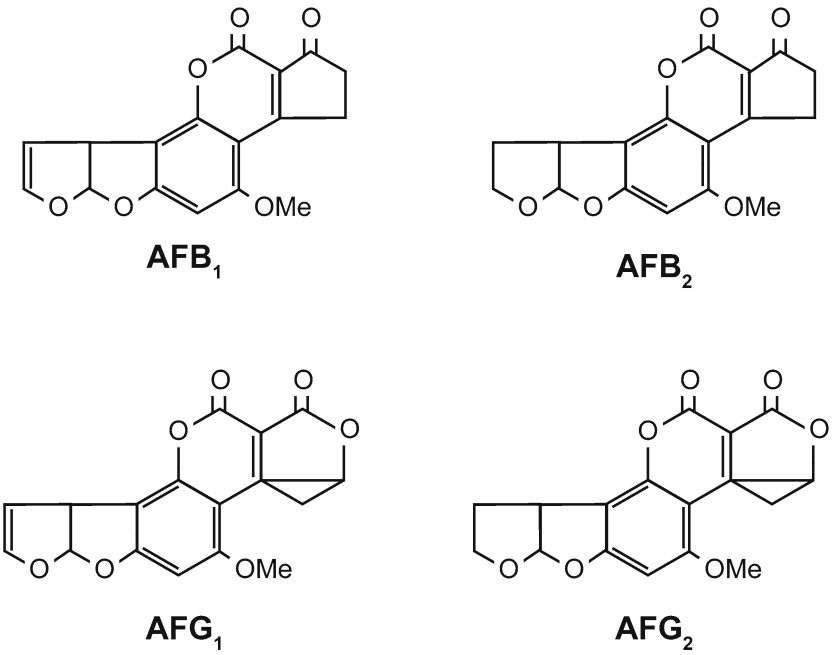

Fig. 1. Major aflatoxins.

that only about the half of *A. flavus* isolates produce aflatoxins *(47)*. The total amount of toxin biosynthesized will vary with the strain of the fungus and with the growth conditions. Moisture, temperature, and insect damage are the most important environmental variables associated with aflatoxin contamination of agricultural commodities *(48)*. Corn, peanuts, cotton, tree nuts, rice, figs, tobacco, and spices are among the most frequently contaminated crops *(49,50)*.

Crops often become contaminated with aflatoxin in the field before harvest, and especially during drought years, the plants are weakened and become more susceptible to insect damage and other insults *(50–52)*. Once harvested, stored grains are also at a high risk of being contaminated by aflatoxins. In storage, the most important variables favoring mold growth are the moisture content of the substrate and the relative humidity of the surroundings *(49,53)*.

4. TOXICOLOGY

Cytochrome P450 enzymes convert aflatoxins to the reactive 8,9-epoxide form (originally called aflatoxin-2,3 epoxide), which in turn can bind to both DNA and proteins *(54)*. In DNA, the reactive aflatoxin epoxide binds to the N^7 position of guanines, and the resultant adducts can cause GC to TA transversions. A glutathione-*S*-transferase system catalyzes the conjugation of activated aflatoxins with reduced glutathione, leading to their excretion *(55)*. Variation in the level of the glutathione-transferase system, as well as variations in the cytochrome P450 system, is hypothesized to explain the differences observed in interspecific aflatoxin susceptibility *(38,54)*.

Like all toxicological syndromes, the diseases caused by aflatoxins are categorized as either acute or chronic. Acute toxicity has a rapid onset and clearly defined symptoms. Chronic toxicity is harder to diagnose and is characterized by low-dose exposure over a long time-period resulting in cancer and other generally irreversible effects *(56)*. It is not always possible to distinguish between acute and chronic effects.

Aflatoxin is associated with both acute and chronic toxicities in human and animal populations *(54,57–59)*. The disease syndromes caused by aflatoxin consumption are termed "aflatoxicoses." Acute aflatoxicosis results in death; chronic aflatoxicosis results in cancer, immune suppression, and other "slow" pathological conditions *(60)*. In both cases, the liver is the primary target organ, and the sensitivity to aflatoxin differs from species to species. Within species, the extent of the response is influenced by age, diet, sex, exposure to pathogens, and the presence of other toxins. There is an abundance of literature on the effects of aflatoxin exposure on laboratory models and agriculturally important species *(54,57,61)*.

The best-known aflatoxin episodes are manifestations of acute effects such as Turkey X syndrome, but the main human and veterinary health burden of aflatoxin exposure is related to chronic exposure (e.g., cancer induction, impairment of liver function, immune suppression). Establishing that a human disease is associated with aflatoxin poisoning is difficult. In general, epidemiologists look for a correlation between a suspected disease condition and the presence of aflatoxin in the diet. Then laboratory scientists attempt to reproduce the characteristic disease symptoms in animal models *(60)*. Environmental and biological monitoring is important in assessing human aflatoxin exposure. In environmental monitoring, aflatoxins are assayed from foods and feeds; in biological monitoring, aflatoxin residues, adducts, and biotransformation products are assayed from blood, milk, tissues, feces, and urine samples *(60,62)*. The aflatoxin B_1- N^7-guanine adduct is a reliable urinary biomarker for detecting recent exposure, and laboratory studies have shown that carcinogenic potency is highly correlated with the extent of total DNA adducts formed in vivo *(54,63)*. Finally, it should be noted that aflatoxin effects are not limited to the liver. The nonhepatic effects of aflatoxin B_1 have been summarized by Coulombe in 1994 *(64)*.

5. AFLATOXINS AND HUMAN DISEASE

Because of the differences in aflatoxin susceptibility in test animals, it has been difficult to extrapolate data from animal models to human disease. The general consensus is that humans are relatively resistant and that acute aflatoxicosis in *Homo sapiens* is rare *(65)*. A 1974 outbreak of hepatitis in India, in which 100 people died, may have been caused by the consumption of maize that was heavily contaminated with aflatoxin *(66)*. Subsequently, it was estimated that the acute lethal dose for adults is approximately 10–20 mg of aflatoxins *(67)*. Nevertheless, at least one woman survived ingestion of over 40 mg purified aflatoxin in an unsuccessful suicide attempt *(68)*.

Kwashiorkor, a severe malnutrition disease, has been called a form of pediatric aflatoxicosis *(69)*, but animal data refute this conjecture *(70)*. Similarly, early hypotheses that aflatoxin might be involved in Reye's syndrome *(71)* have not been substantiated.

Although the quantification of lifetime individual exposure to aflatoxin is extremely difficult, several epidemiological studies have linked liver cancer incidence to estimated aflatoxin consumption in the diet, particularly in individuals already exposed to hepatitis B

infections *(59,72,73)*. Liver cancer incidence varies widely from country to country, but it is a common cancer in China, the Philippines, Thailand, and many African countries. The presence of hepatitis B virus infection complicates the epidemiological studies. In one case–control study involving more than 18,000 urine samples collected over 3.5 yr in Shanghai, it was estimated that the combination of aflatoxin and hepatitis B raised the cancer risk 30-fold over that for aflatoxin alone *(74)*. Because it is easier to vaccinate against hepatitis B virus than to remove aflatoxin from the diet, vaccination has been recommended as the most cost-effective strategy for lowering liver cancer in susceptible populations *(75,76)*.

A significant number of liver cancer patients in Africa and China have a mutation in the p53 tumor suppressor gene at codon 249 associated with a G to T transversion *(77,78)*. Because it is known that the reactive aflatoxin epoxide binds to the N^7 position of guanines and that aflatoxin B_1-DNA adducts can result in GC to TA transversions, these data add further support to the evidence that aflatoxin B_1 is a human carcinogen. Eaton and Gallagher *(63)* have written that this codon-specific change in the *p53* gene is the first example of a fixed "carcinogen-specific" biomarker.

In summary, there is no other natural product for which the evidence for human carcinogenicity is so compelling. Aflatoxin B_1 is classified as a Group I carcinogen by the International Agency for Research on Cancer *(79)*. This notoriety may explain why aflatoxins have been implicated as chemical warfare agents. In 1995, it was determined that Iraq had produced and deployed war instruments containing botulism toxin, anthrax spores and aflatoxins. International forensic teams showed that toxigenic strains of *A. flavus* and *A. parasiticus* were grown in Iraqi government sponsored facilities and aflatoxins were harvested to produce over 2300 L of concentrated toxin. The majority of this aflatoxin was used to fill warheads; the remainder was stockpiled *(80,81)*. Nonetheless, because of the large amounts of toxin necessary to cause disease and the relatively slow mode of action, aflatoxins were a strange choice for a bioterrorist *(82)*.

6. TREATMENT AND CONTROL

Most aflatoxicosis results from eating contaminated foods. Unfortunately, except for supportive therapy (e.g., diet and hydration) there are almost no treatments for aflatoxin exposure. Fink-Gremmels *(9)* has described a few methods for veterinary management of mycotoxicoses, and there is some evidence that some strains of *Lactobacillus* effectively bind dietary mycotoxins *(83–85)*. Similarly, clay-based enterosorbents have been used to bind aflatoxins in the gastrointestinal tract *(86,87)*. Selenium supplementation somewhat modified the negative effects of aflatoxin B_1 in Japanese quail *(88)*, and butylated hydroxytoluene gave some protection in turkeys *(89)*. Oltipraz, a drug originally used to treat schistosomiasis, has been tested in human populations in China with some apparent success *(76)*.

Methods for controlling aflatoxin exposure are largely prophylactic. Such methods include good agricultural practice, appropriate drying of crops after harvest and avoidance of moisture during storage *(90)*. Many agricultural scientists are trying to develop methods to minimize the preharvest contamination of crops. These approaches include developing host resistance through plant breeding and the use of biocontrol agents *(25)*.

Most efforts to address the mycotoxin problem involve analytic detection, government regulation, and diversion of mycotoxin-contaminated commodities from the food

supply. Basic research on the biosynthesis and molecular biology of aflatoxins (*see* Section 8) has been a research priority because a full understanding of the fundamental biological processes may yield new control strategies for the abolition of aflatoxin contamination of food crops.

7. ECONOMICS, FOOD SAFETY, AND REGULATION

The economic consequences of aflatoxin contamination are extreme. In developed countries, crops with high amounts of aflatoxins are either destroyed or diverted into animal feeds; aflatoxins lower the value of grains as an animal feed and as an export commodity *(21)*. When susceptible animals are fed contaminated feeds it results in reduced growth rates, illness, and death; moreover, their meat and milk may contain toxic biotransformation products. Livestock owners often take farmers and feed companies to court; legal battles can involve considerable amounts of money *(91)*.

Numerous assay methods for detecting aflatoxins have been developed utilizing virtually all of the common tools of analytical chemistry including thin-layer chromatography, high-performance liquid chromatography, gas chromatography, mass spectrometry, immunoassays, capillary electrophoresis, and biosensors. Older methods usually require solvents for clean-up steps and chromatography for quantification; more recently, immunogenic assays that can be applied to samples with little or no clean up have been developed *(92)*. Aflatoxins are nonimmunogenic but they can be conjugated to a protein carrier; a number of inexpensive antibody-based kits are now commercially available. Methods for assaying aflatoxins and other mycotoxins have been reviewed *(26,93–95)*.

Because it is normally impossible to prevent entirely the formation of aflatoxins, complete elimination is an unattainable objective. Naturally occurring toxins such as aflatoxins are regulated quite differently than food additives *(96)*. In developed countries, human populations are protected because regular surveillance keeps contaminated foods out of the food supply. Unfortunately, in countries where populations are facing starvation, or where regulations are either nonexistent or unenforced, routine ingestion of aflatoxin is common *(97)*. A joint FAO/WHO/UNEP conference report pointed out that hungry people "cannot exercise the option of starving to death today in order to live a better life tomorrow" and statistics show that the incidence of liver cancer is 2–10 times higher in developing countries than in developed countries *(75)*.

Special committees and commissions have been established by many countries and international agencies to recommend guidelines, test standardized assay protocols, and maintain up-to-date information on regulatory statutes of aflatoxins and other mycotoxins. These guidelines are developed from epidemiological data and extrapolations from animal models, taking into account the inherent uncertainties associated with both types of analysis. Estimates of "safe doses" are usually stated as a "tolerable daily intake" *(10,31,98)*. For example, in the United States, the Food and Dug Administration guideline is 20 ppb total aflatoxin in food destined for human consumption and 100 ppb is the limit for breeding cattle and mature poultry *(99)*.

Different national guidelines for safe doses have been established, and hence, there is a need for worldwide harmonization of regulations *(100)*. A compendium summarizing worldwide regulations for mycotoxins has been published by the Food and Agriculture Organization of the United Nations *(96)*; an abbreviated version was given as an appendix by Weidenborner *(101)*.

The websites for the various commissions and organizations that study mycotoxins are excellent sources for the latest information: see, e.g., the Council for Agricultural Science and Technology (CAST) (www.cast-science.org); the American Oil Chemists Society Technical Committee on Mycotoxins (www.aocs.org); the Food and Agricultural Organization (FAO) of the United Nations (www.fao.org); the International Union for Pure and Applied Chemistry section on Mycotoxins and Phycotoxins (www.iupac.org); and the US Food and Drug Administration Committee on Additives and Contaminants (www.fda.gov).

8. BIOCHEMISTRY, MOLECULAR BIOLOGY, AND GENOMICS

The severity of the potential health effects, and the magnitude of the economic losses, have been the impetus for considerable research. Many scientists, including ourselves, believe that control can best be achieved by understanding the genetic basis of aflatoxin biosynthesis and the regulatory elements that control the biosynthetic pathway. To this end, the US Department of Agriculture and other US and international funding agencies have supported decades of basic research on the molecular biology of aflatoxin biosynthesis. More recently, the US Department of Agriculture has funded an *A. flavus* genome-sequencing project that is expected to be completed by early 2005.

Molecular research has targeted the genetics, biosynthesis, and regulation of aflatoxin formation in *A. flavus* and *A. parasiticus*. Aflatoxins are biosynthesized by a type II polyketide synthase; it has been known for a long time that the first stable step in the biosynthetic pathway is the norsolorinic acid, an anthraquinone *(102)*. A complex series of post-polyketide synthase steps follow, yielding a series of increasingly toxigenic anthraquinone and difurocoumarin metabolites *(103–113)*. Sterigmatocystin (ST) is a late metabolite in the aflatoxin pathway and is also produced as a final biosynthetic product by a number of species such as *Aspergillus versicolor* and *Aspergillus nidulans*. ST is less potent than aflatoxin but is nevertheless an important mycotoxin in its own right *(114)*. Perhaps more importantly, analysis of ST biosynthesis in the genetically traceable species *A. nidulans* was pivotal in accelerating research on the cognate pathway for aflatoxin. It is now known that ST and aflatoxins share almost identical biochemical pathways. The majority of the genes for both ST biosynthesis in *A. nidulans*, and aflatoxin pathway biosynthesis in *A. flavus* and *A. parasiticus* are homologous and clustered *(106,107,111,112,115–119)*. In *A. flavus* and *A. parasiticus*, a total of 25 genes involved in aflatoxin biosynthesis, along with four sugar utilization genes, are located together within a 70-kb region of DNA *(111,112,117,120)*. Recently, a new standardized system for naming the aflatoxin pathway genes has been introduced *(111)*. A diagram depicting the clustered genes of aflatoxin and ST biosynthesis and the verified post-polyketide biosynthetic steps in this pathway is shown in Fig. 2. The expression of the structural genes in both aflatoxin and ST biosynthesis is regulated by a regulatory gene, *aflR*, which encodes a GAL4-type C6 zinc binuclear DNA-binding protein. When *aflR* is disrupted, no structural gene transcript can be detected; introduction of an additional copy leads to overproduction of aflatoxin biosynthetic pathway intermediates *(121)*. The overall amino-acid identity is 31% between the *aflR* genes from *A. flavus* and *A. nidulans*, but the nuclear localization signal domain and the cys_6-Zn_2 domain are 71% identical. The immediate downstream linker region is also highly conserved; substitution of amino-acids in

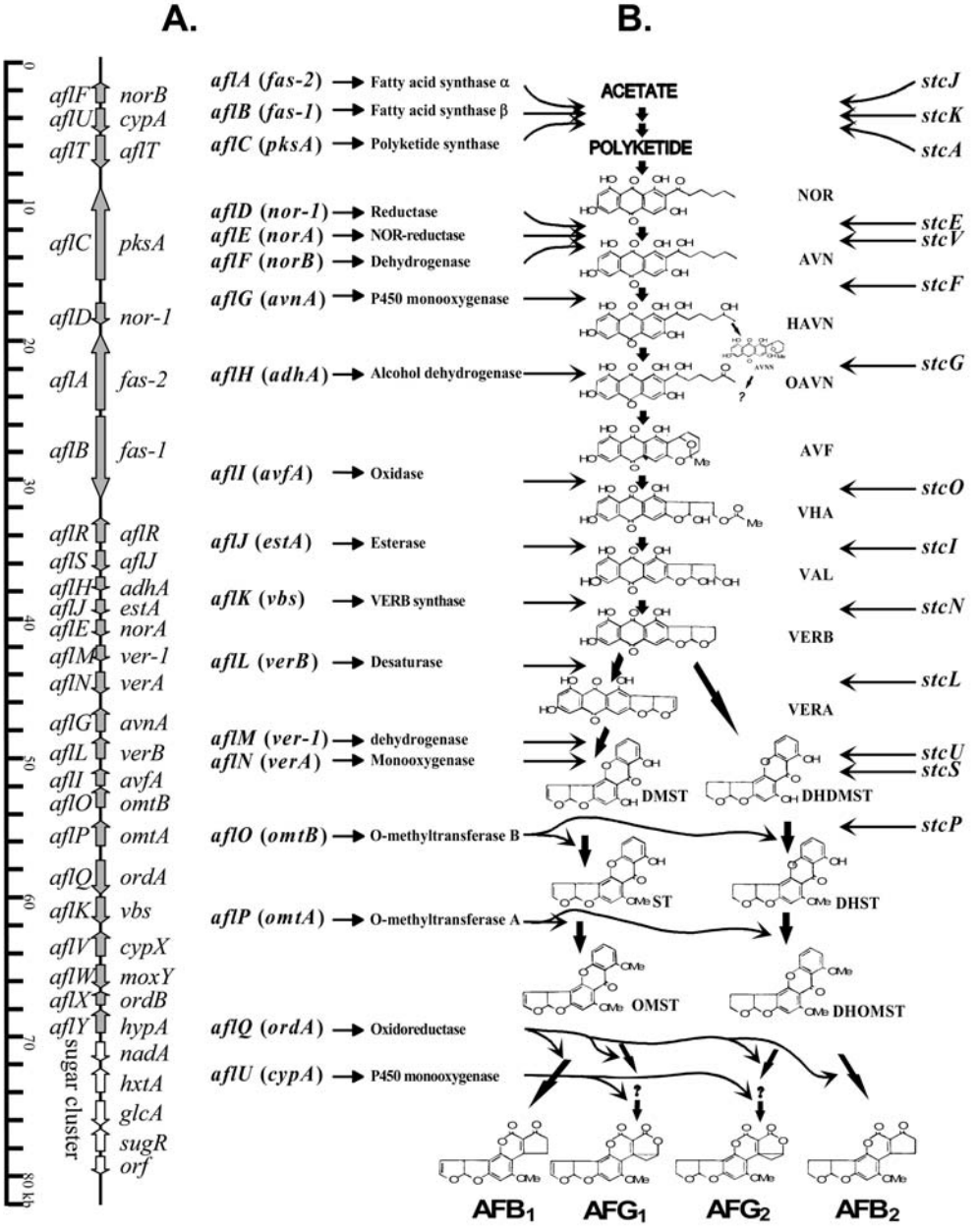

Fig. 2. Clustered genes (**A**) and the aflatoxin biosynthetic pathway (**B**). The generally accepted pathway for aflatoxin and sterigmatocystin (ST) biosynthesis is presented in Panel B. The corresponding genes and their enzymes involved in each bioconversion step are shown in Panel A. The vertical line represents the 82-kb aflatoxin biosynthetic pathway gene cluster and sugar utilization gene cluster in *A. parasiticus* and *A. flavus*. The new gene names are given on the left of the vertical line and the old names are given on the right. Arrows along the vertical line indicate the direction of gene transcription. The ruler at far left indicates the relative sizes of these genes in kilobase pairs. The ST biosynthetic pathway genes in *A. nidulans* are indicated at the right of Panel B. Arrows in Panel B indicate the connections from the genes to the enzymes they encode, from the enzymes to the bioconversion steps they are involved in, and

the linker region results in defective *aflR* expression *(122)*. Details of the promoter structure of *aflR* are reviewed by Yu et al. *(109)*.

A divergently transcribed gene, *aflJ*, is also involved in the regulation of the aflatoxin gene cluster; no aflatoxin pathway intermediates are produced when it is disrupted. The gene product of *aflJ* has no sequence homology to known proteins identified in databases *(123)*; it interacts with *aflR* but not the structural genes of the pathway *(124)*. It has been speculated that *aflJ* is an *aflR* coactivator *(109,111)*.

Aspergillus oryzae and *A. sojae* are nontoxigenic species that are widely used in Asian food fermentations such as soy sauce, miso, and sake. These food fungi are closely related to *A. flavus* and *A. parasiticus*. Although they never have been shown to produce aflatoxin, they do contain homologs of several aflatoxin biosynthetic pathway genes *(125,126)*. *A. sojae* contains a defective copy of *aflR (127,128)*. Other genetic defects have crippled the aflatoxin pathway in *A. oryzae (128,129)*.

The application of genomic DNA sequencing and functional genomics, powerful technologies that allow scientists to study a whole set of genes in an organism, is one of the most exciting developments in aflatoxin research *(130)*. The Food and Feed Safety Unit of the USDA–ARS, Southern Regional Research Center, New Orleans, LA, has sponsored an *A. flavus* expressed sequence tag (EST)/Microarray project. A normalized cDNA library was made. From over 26,000 clones sequenced, 7218 unique ESTs (genes) were identified after comparison and assembly *(131)*. Homology analysis by a BLAST search in the GenBank database indicated that 66% of these unique genes had identified homologs in the GenBank database and 34% unique ESTs had no identified homologs. Bioinformatics annotation identified many genes that are putatively involved in the aflatoxin process including signal transduction, global regulation, pathogenicity, virulence, stress response, and fungal development in addition to the genes of the biosynthetic pathway *(131)*.

These data will be useful in annotating the forthcoming genome sequence of *A. flavus* and in designing future microarray experiments to investigate the relationship between developmental and secondary metabolite genes (*see* Section 9). *A. flavus* microarrays have been constructed at The Institute for Genomic Research (TIGR), Rockville, MD. High density microarrays have been printed of 6684 short amplicons representing 5002 unique gene elements including 31 aflatoxin pathway genes. These microarrays are being used in time-course studies to detect sets of fungal genes transcribed under specific conditions at different developmental stages.

9. AFLATOXINS AND FUNGAL DEVELOPMENT

The association between fungal morphological development and secondary metabolism, including aflatoxin production, has been observed for many years *(132–135)*.

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from the intermediates to the products in the aflatoxin bioconversion steps. *Abbreviations*: NOR, norsolorinic acid; AVN, averantin; HAVN, 5′-hydroxyaverantin; OAVN, oxoaverantin; AVNN, averufanin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; VERB, versicolorin B; VERA, versicolorin A; DMST, demethylsterigmatocystin; DHDMST, dihydrodemethylsterigmatocystin; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, *O*-methylsterigmatocystin; DHOMST, dihydro-*O*-methylsterigmatocystin; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; and AFG_2, aflatoxin G_2.

The environmental conditions required for secondary metabolism and for sporulation are similar *(132,133)*, and both processes occur at about the same time *(105,136)*. Certain compounds in *A. parasiticus* that exhibit the ability to inhibit sporulation also inhibit aflatoxin formation *(137)*. Further, chemicals that inhibit polyamine biosynthesis in *A. parasiticus* and *A. nidulans* inhibit both sporulation and aflatoxin/ST biosynthesis *(138)*. Some sporulation deficient mutants of *A. parasiticus* are unable to produce aflatoxins *(139–141)*, and there is some evidence of an association between sclerotia and aflatoxin production in these species *(142)*. Similarly, a nonsporulating, "fluffy" mutant strain of *A. nidulans* is deficient in ST formation *(143,144)*. In fact, *A. nidulans*, long a well-known system for studying fungal development *(145)* is emerging as a model for studying the global regulation of both development and secondary metabolism.

Two distinct classes of *A. nidulans* mutants unable to make secondary metabolites were isolated in 1999 *(146)*. One group of mutants showed morphological defects, while the other had the wild-type parental morphology. Physiological and genetic complementation analyses of these mutants suggested that there were factors distinct from both the *aflR* gene and the developmental genes that controlled ST production. Almost simultaneously, these researchers reported that both asexual sporulation and ST production required the inactivation of proliferative growth through inhibition of the FadA (G-protein) signaling pathway *(136)* and identified a gene called *pkaA* (protein kinase A) as a component of this pathway *(147)*.

FadA is the α subunit of the *A. nidulans* heterotrimeric G-protein. When FadA was bound to GTP and in its active form, ST production and sporulation were repressed. However, in the presence of FlbA, the intrinsic GTPase activity of FadA was stimulated, thereby leading to GTP hydrolysis, inactivation of FadA-dependent signaling, and stimulation of ST production. In brief, the G-protein signal transduction pathway mediated by protein kinase A (PKA) regulated both aflatoxin/ST synthesis and sporulation *(136)*.

In the process of characterizing *A. nidulans* fluffy mutants, six loci were identified to be the results of recessive mutations in the fluffy genes *fluG, flbA, flbB, flbC, flbD*, and *flbE*. Two of these genes, *fluG* and *flbA* encoding protein factors FluG and FlbA, were involved in the regulation of both asexual development (conidiation) and ST biosynthesis in *A. nidulans (136,148)*. The *fluG* gene is involved in the synthesis of an extracellular diffusible factor that acts upstream of *flbA*. The *pkaA* gene encodes the catalytic subunit of a cyclic AMP (cAMP)-dependent protein kinase A, PkaA *(136,147)*. Over-expression of *pkaA* (PkaA) inhibits *brlA* and *aflR* expression *(147)*. The gene, *brlA*, in *A. nidulans* encodes a transcriptional regulator (BrlA) long believed to activate developmental genes *(149)*. A domain of the FlbA protein, the regulator of the G-protein signaling RGS, presumably is able to inhibit FadA *(148)*. In the overall scheme of the proposed G-protein signaling pathway, FadA and PkaA favor vegetative growth and inhibit conidiation and aflatoxin/ST production; while FluG and FlbA inhibit FadA and PkaA function and promote conidiation and aflatoxin/ST biosynthesis *(135,136,148)*. This G-protein signaling pathway involving FadA in the regulation of secondary metabolism may also exist in other aspergilli such as *A. parasiticus*.

The manner in which complex interactions among the components of this pathway (FlbA, FadA, and Pka proteins) and RasA (a member of the family of small GTP-binding proteins) influence *aflR* at both transcriptional and post-transcriptional levels is under investigation *(150)*.

Another link between sporulation and/or other aspects of development and aflatoxin biosynthesis has been studied using an unusual class of aflatoxin-negative mutants called *sec-* (for secondary metabolism minus). These strains were isolated after serial transfer of nonsporulating mycelial macerates. They exhibit reduced sporulation and no detectable aflatoxin production *(151)*. They are unable to bioconvert aflatoxin precursors to aflatoxins although Southern blot and polymerase chain reaction analysis demonstrated that the structural genes for pathway enzymes are present *(152)*. In the *sec-* strains, *aflR* expression is 5–10-fold lower than in the toxigenic strains from which they are derived, adding more evidence to the theory that *aflR* is necessary but not sufficient for aflatoxin production *(153)*. A different and possibly related morphological mutant in *A. parasiticus* called *fluP* causes a fluffy hyphal morphology, reduction of asexual spores, and a lowering of aflatoxin production *(154)*.

Finally, the *laeA* gene (for loss of *aflR* expression) is yet another intriguing discovery *(146)*. Originally isolated from *A. nidulans,* it encodes a putative nuclear methyltransferase and transcriptionally regulates several secondary metabolic pathways. Disruption of *laeA* (accession no. AY394722) in *A. nidulans* eliminates ST and penicillin biosynthesis due to the loss of gene expression (*aflR*, *stcU*) required for ST biosynthesis and a gene (*ipnA*) involved in penicillin biosynthesis. Disruption of the *laeA* in *A. fumigatus* (accession no. AY422723) and in *A. terreus* (*lovE*) eliminated gliotoxin and lovastatin biosynthesis, respectively *(155)*. The ST pathway regulator, AflR, the PKA, *(147)* and RasA, known to be involved in signal transduction and which negatively regulate sterimgatocystin biosynthesis and asexual sporulation in *A. nidulans (135,136)*, negatively regulate *laeA* expression *(155)*. It is possible that the LaeA protein is one of the global regulatory components in the signal transduction pathway that controls secondary metabolism pathways. The corresponding *laeA* gene in *A. flavus* and in *A. parasiticus* has been cloned. Although very low homology exists between *A. flavus*/*A. parasiticus* and *A. nidulans* at the nucleotide level, significant homology was observed at the amino-acid level (Yu, unpublished data). The *laeA* gene may have similar effects in regulating secondary metabolism pathway in *A. flavus*, e.g., aflatoxin biosynthesis.

The proliferation of new approaches to the study of secondary metabolism and morphological development, combined with the power of functional genomics, give us reason to hope that we are on the brink of a new era of molecular understanding of aflatoxin gene regulation.

10. SUMMARY

Aflatoxins are toxic and carcinogenic natural products biosynthesized by a polyketide pathway by certain members of the genus *Aspergillus*. When people and domestic animals eat aflatoxin contaminated foods, they could suffer both acute and chronic diseases. Of aflatoxigenic mold species, *A. flavus* and *A. parasiticus* are considered to be the most economically important. Aflatoxins can enter the food chain when these toxigenic species grow on commodities in the field, during storage, or at later points. Aflatoxin contamination is exacerbated whenever agricultural, storage, shipping, and food handling practices are conducive to mold growth. The acute and chronic effects of aflatoxin are largely avoided by preventative strategies good agricultural practice, government monitoring and regulation, and diversion of contaminated crops from the food supply. Unfortunately, strict limitation of aflatoxin contaminated food and feed

is not always an option and there are almost no treatments for aflatoxin poisoning. It is hoped that the research on the molecular biology of aflatoxigenic fungi, including a forthcoming genome sequence and microarray analysis of the *A. flavus* genome, will lead to better methods for blocking the production of this family of terrible food borne toxins.

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