



# Aflatoxin biosynthesis

Jiujiang Yu, Deepak Bhatnagar & Kenneth C. Ehrlich

United States Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, Louisiana, USA

**Summary** Aflatoxins are toxic and extremely carcinogenic natural secondary metabolites produced primarily by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. The biosynthesis of aflatoxins is a complex process involving multi-enzymatic reactions. Genetic studies of the molecular mechanism of aflatoxin B<sub>1</sub> biosynthesis have identified an aflatoxin pathway gene cluster of 70 kilobase pairs in length consisting of at least 24 identified structural genes including a positive regulatory gene as transcription activator. The structural genes encode cytochrome P450 monooxygenases, dehydrogenases, oxidases, methyltransferases, a polyketide synthase and two unique fatty acid synthases. The aflatoxin biosynthesis and its genetic regulation are discussed in this review. The current knowledge of the relationship between fungal development and secondary metabolism is also summarized.

**Key words** Mycotoxins, Gene cluster, Transcription factor, *Aspergillus flavus*, *Aspergillus parasiticus*

## Biosíntesis de aflatoxinas

**Resumen** Las aflatoxinas son metabolitos secundarios tóxicos y altamente carcinógenos producidos, sobre todo, por las especies *Aspergillus flavus* y *Aspergillus parasiticus*. Su biosíntesis es un proceso complejo que implica reacciones multienzimáticas. Se ha descrito mediante estudios genéticos sobre el mecanismo molecular de biosíntesis de la aflatoxina B<sub>1</sub>, un fragmento de 70 pares de kilobases de longitud, conteniendo al menos 24 genes estructurales conocidos, incluyendo un gen de regulación positiva como activador de transcripción. Dichos genes estructurales codifican monooxigenasas del citocromo P450, deshidrogenasas, oxidasas, metiltransferasas, una polyketido sintetasa y dos sintetetasas de ácidos grasos exclusivas. En esta revisión se describe la biosíntesis de las aflatoxinas y su regulación genética, además de la relación entre el desarrollo del hongo y el metabolismo secundario.

**Palabras clave** Micotoxinas, Agrupación de genes, Factor de transcripción, *Aspergillus flavus*, *Aspergillus parasiticus*

Aflatoxins were discovered about 40 years ago after the devastating loss of poultry in England (Turkey X disease) [1]. Aflatoxins are the most toxic and carcinogenic compounds among the known mycotoxins and, therefore, have been extensively studied. These compounds are a group of polyketide-derived furanocoumarins (Figure 1), with at least 16 structurally related toxins that have been characterized. These toxins are produced by a number of different *Aspergillus* species [2-5]. However, in agricultural commodities, they are primarily produced by

*Aspergillus flavus* and *Aspergillus parasiticus*. There are four major aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>. *A. flavus*, *Aspergillus pseudotamarii*, and *Aspergillus ochraceoroseus* produce only the B aflatoxins, and *Aspergillus nomius*, *Aspergillus bombycis*, *A. parasiticus* and an unnamed taxon from West Africa produce both B and G toxins. Aflatoxins were originally isolated from *A. flavus* hence the name *A-fla-toxin*. Other significant members of the aflatoxin family, M<sub>1</sub> and M<sub>2</sub>, are oxidative forms of aflatoxin B<sub>1</sub> modified in the digestive tract of some animals and isolated from milk, urine and feces [6].

## Economic significance of aflatoxin contamination

Aflatoxin contamination of foods and feeds is a serious worldwide problem [7-9] resulting either from improper storage of commodities or preharvest contamination in corn, peanuts, cottonseed and tree nuts, especially during drought years. The worldwide extent of contamination of commodities is not totally understood often because of a reluctance to report its occurrence [10].

Aflatoxin contamination in food for human consumption as well as in feed for livestock has been found in many geographically diverse regions of the world. Such contamination has resulted in serious food safety

**Dirección para correspondencia:**  
Dr. Jiujiang Yu  
United States Department of Agriculture  
Agricultural Research Service  
Southern Regional Research Center  
New Orleans, Louisiana, USA  
Tel.: +1 504 286 4405  
Fax: +1 504 286 4419  
E-mail: jiuju@srcc.ars.usda.gov

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Apdo. 699, E-48080 Bilbao (Spain)  
1130-1406/01/10.00 Euros

and economic implications for the agriculture industry. Because of the health concern [11], regulatory guidelines of 20 parts aflatoxin per billion parts of food or feed substrate (ppb) is the maximum allowable limit imposed by the U.S. Food and Drug Administration for consumption and for interstate shipment of foods and feeds. In some European countries aflatoxin levels are regulated below five ppb.

Aflatoxin contamination has been a chronic problem in some parts of USA, e.g. in Arizona cotton growing areas and Southeast USA peanut farming regions. However, sporadic severe outbreaks of aflatoxin contamination have occurred in the Midwest USA cornbelt in 1977, 1980 and 1988. The total costs associated with aflatoxin contamination in corn, both in the private and public sectors, have been estimated to be over \$200 million in bad years.

Aflatoxins have been shown to be immunosuppressive, mutagenic, teratogenic and hepatocarcinogenic in experimental animals. The mode of action, metabolism and biosynthesis of aflatoxins has been extensively studied [reviewed in 8,9,12]. The chemical binding of the liver cytochrome P450-activated aflatoxin B<sub>1</sub> forms adducts with guanidine residues in DNA [13,14] that ultimately can cause liver cancer in certain animals [15,16]. An association of hepatocellular carcinoma and dietary exposure to aflatoxins was established from patients living in high-risk areas of Kenya, Mozambique, Swaziland, Thailand, People's Republic of China, Philippines, and the Transkei of South Africa [12,15-19]. The chemistry, biochemistry and molecular biology and synthesis of aflatoxins B<sub>1</sub> and B<sub>2</sub> have been investigated in significant detail. Since aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is the most toxic of this group of toxins, extensive research has been done on its synthesis, toxicity and biological effects [8,9].

### Biochemistry of aflatoxin biosynthesis

Attempts to decipher the aflatoxin biosynthetic pathway began with the discovery of the structure of these toxins. However, the major biochemical steps and the corresponding genetic components of AFB<sub>1</sub> biosynthesis have been elucidated only in the last decade at a molecular level. Several previous reviews have described the biochemistry and genetics of aflatoxin formation [8,9, 20-26].

Various studies have determined that aflatoxins are synthesized in two stages from malonyl CoA, first with the formation of hexanoyl CoA, followed by formation of a decaketide anthraquinone [for review see 20 and 23]. A series of highly organized oxidation-reduction reactions then allows formation of aflatoxin [20,21,27]. The currently accepted scheme (Figure 1) for aflatoxin biosynthesis is: hexanoyl CoA precursor → norsolorinic acid, NOR → averantin, AVN → hydroxyaverantin, HAVN → averufin, AVF → hydroxyversicolorone, HVN → versiconal hemiacetal acetate, VHA → versiconal, VAL → versicolorin B, VERB → versicolorin A, VERA → demethyl-sterigmatocystin, DMST → sterigmatocystin, ST → O-methylsterigmatocystin, OMST → aflatoxin B<sub>1</sub>, AFB<sub>1</sub> and aflatoxin G<sub>1</sub>, AFG<sub>1</sub> (Figure 1). A branch point in the pathway has been established, following VHA production, leading to different structural forms of aflatoxins B<sub>2</sub> and G<sub>2</sub>, AFB<sub>2</sub> and AFG<sub>2</sub> [20,28-33]. A number of metabolic grids may provide alternate pathways to aflatoxins [20,30,34-38].

Several specific enzyme activities associated with precursor conversions in the aflatoxin pathway [20,21,32,

36,39-45] have been partially purified [46-48] (Figure 1, identified enzymes enclosed in boxes); whereas others such as methyltransferases [48-50] have been purified to homogeneity. Several other enzymes, which are involved in aflatoxin biosynthesis such as a reductase [51] and a cyclase [52,53], have also been purified from *A. parasiticus*. A desaturase which converts VERA to VERB has been found in cell-free fungal extracts [29,32]. Matsushima *et al.*, [54] have purified and characterized two versiconal hemiacetal acetate reductases involved in toxin synthesis, whereas Kusumoto and Hsieh [55] purified to homogeneity an esterase that converts VHA to versiconal. Bhatnagar *et al.* [46] and Chatterjee and Townsend [56] demonstrated that in the later stages of AFB<sub>1</sub> and AFB<sub>2</sub> synthesis, independent reactions and formation of different chemical precursors are catalyzed by common enzyme systems [30,43,46,57].

### Genetics of aflatoxin biosynthesis

Genetic investigations of *A. flavus* and *A. parasiticus* have been hampered by the lack of sexual reproduction in these fungi. Hyphal anastomosis and nuclear exchange is governed by a complex vegetative compatibility system [58], which further complicates genetic studies. However, mutants of *A. parasiticus* and *A. flavus* strains can be analyzed using the parasexual cycle [see 59-61 for review]. Karyotype analysis for several *A. flavus* and *A. parasiticus* strains (by pulse-field gel electrophoresis) shows that there are 6-8 chromosomes ranging in size from approximately 3 to ≥ 7 Mb [61,62].

Two fatty acid synthase genes (*fas-1* and *fas-2*) and a polyketide synthase gene (*pkSA*) are involved in the synthesis of the decaketide from malonyl CoA [20,24,45,63-68]. Once formed, the decaketide is expected to undergo ring closure to form a product, noranthrone which then must undergo oxidation to form the first stable intermediate, norsolorinic acid (NOR) [69]. No specific enzyme has yet been linked to the conversion of noranthrone to NOR, but an oxidase [20,70] should be involved. Most of the other steps in aflatoxin synthesis have been more clearly defined. The conversion of NOR to AVN [71] requires a dehydrogenase [20], encoded by the gene *nor-1* [72,73]. Additional genes, such as *norA* [74,75] or *norB* (Yu *et al.*, unpublished) are in the cluster which encode dehydrogenases that also might be capable of carrying out the conversion. The *nor-1* gene was cloned by complementation of a norsolorinic acid (NOR)-accumulating mutant [72]. The gene encoding a ketoreductase responsible for the conversion of versicolorin A (VER A) to sterigmatocystin (ST) was also cloned from the same *A. parasiticus* cosmid library [76]. It was confirmed later that the next stable intermediate is demethylsterigmatocystin (DMST) instead of ST and a cytochrome P-450 monooxygenase encoded by *verA* was required for the conversion of VERA to DMST [37,48]. A cytochrome P450 monooxygenase encoded by the gene *avnA* is required for the conversion of AVN to HAVN [77], whereas the gene *adhA* encoding an alcohol dehydrogenase [35] was found to be essential for the conversion of HAVN to AVF. The *avfA* gene encoding an oxidase is responsible for the conversion of AVF to VHA [78] in both *A. parasiticus* and *A. flavus*. Recently, a gene designated as *estA*, which could possibly encode an esterase [32,36,55,79] for the conversion of VHA to VAL, has been cloned [80]. The *vbs* gene [81,82] encodes a dehydratase that catalyzes the side chain cyclization of VHA to VERB. The conversion of VERB to VERA is catalyzed by a desaturase enco-

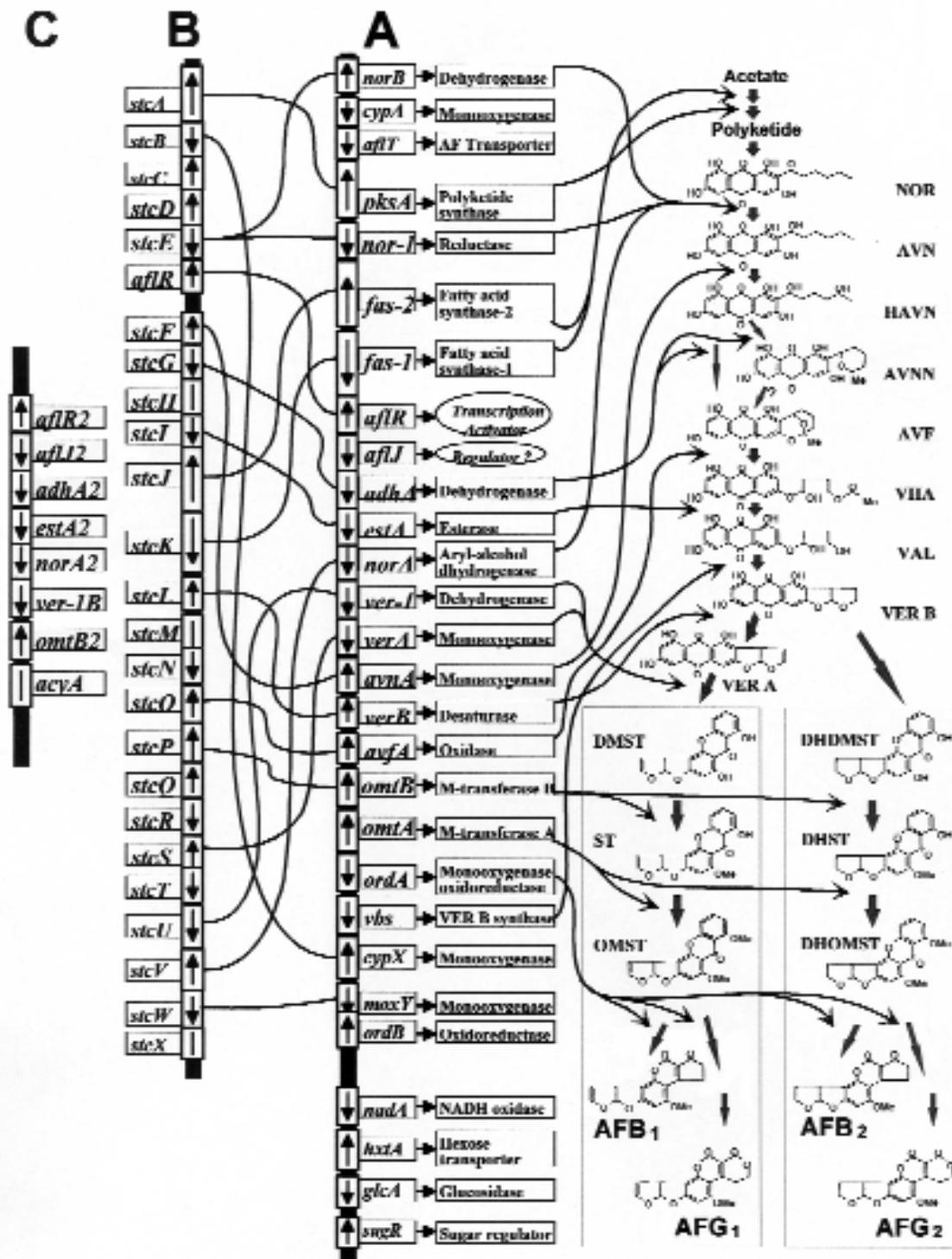


Figure 1. Generally accepted pathway for aflatoxin and sterigmatocystin biosynthesis. The corresponding genes and their enzymes are shown. The aflatoxin biosynthetic pathway gene cluster in *A. parasiticus* and *A. flavus* is shown in panel A and the sterigmatocystin biosynthetic pathway gene cluster in *A. nidulans* is shown in panel C. The partial duplicated aflatoxin pathway gene cluster in *A. parasiticus* is shown in panel B. The gene names are labeled on the side of the cluster. A putative hexose utilization gene cluster is shown 3' to the aflatoxin pathway gene cluster. The open boxes on the vertical bar represent the pathway genes and arrows inside the boxes indicate the direction of gene transcription. Arrows outside the boxes indicate the relationships from the genes to the enzymes they encode; from the enzymes to the bioconversion steps they are involved in; and from the intermediates to products in the aflatoxin bioconversion steps, respectively. The main bioconversion steps are briefly summarized as follows: the regulatory gene, *aflR*, coding for the regulatory factor (AFLR protein), controls, at the transcriptional level, the expression of the structural genes characterized so far. The *fas-1*, *fas-2* and the *pkcA* gene products, fatty acid synthases and polyketide synthase, respectively, are involved in the conversion steps between the initial acetate units to synthesis of the polyketide. The *nor-1* gene encodes a reductase for the conversion of NOR to AVN. The *avnA* gene encodes a cytochrome P450 type monooxygenase involved in the conversion from AVN to AVF. The *avfA* gene encodes an oxidase involved in the conversion of AVF to VHA. The *ver-1* and *verA* genes encode dehydrogenases for the conversion of VER A to DMST. The *omtA* gene encodes an O-methyltransferase for the conversion of ST to OMST and DHST to DHOMST. The *ordA* gene encodes an oxidoreductase involved in the conversion from OMST to AFB<sub>1</sub> and AFG<sub>1</sub> and DHOMST to AFB<sub>2</sub> and AFG<sub>2</sub>. Abbreviations: NOR, norsolorinic acid; AVN, averantin; HAVN, 5'-hydroxyaverantin; AVNN, averufanin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; VerB, versicolorin B; VerA, versicolorin A; DMST, demethylsterigmatocystin; DHDMST, dihydrodemethylsterigmatocystin; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; DHOMST, dihydro-O-methylsterigmatocystin; OMST, O-methylsterigmatocystin; AFB<sub>1</sub>, aflatoxin B<sub>1</sub>; AFB<sub>2</sub>, aflatoxin B<sub>2</sub>; AFG<sub>1</sub>, aflatoxin G<sub>1</sub>; AFG<sub>2</sub>, aflatoxin G<sub>2</sub> and M-transferase, methyltransferase.

ded by the gene, *verB* (Bhatnagar *et al.*, unpublished) which is homologous to *stcL* in *Aspergillus nidulans* [83].

In the later stage of aflatoxin biosynthesis, an O-methyltransferase required for the conversion of ST to O-methylsterigmatocystin (OMST) was detected, purified and characterized [49,50,84]. The gene encoding O-methyltransferase was cloned using antibody raised against the O-methyltransferase [85]. The genomic DNA sequence of *omtA* was also determined from both *A. parasiticus* and *A. flavus* [86]. The *omtA* was the first gene whose gene product involvement in the aflatoxin biosynthesis was confirmed and thoroughly studied by in vitro enzyme assay. Another gene named *omtB* encoding a methyltransferase required for the conversion of DMST to ST and DHDMST to DHST was identified [78]. The *omtB* gene was simultaneously cloned by Yabe's group and named *dmtA* [87]. The final step in the formation of aflatoxins is the conversion of OMST or DHOMST to aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>, steps requiring the presence of a NADPH dependent mono-oxygenase (*ordA*) [88,89]. The formation of the G toxins probably requires an additional oxidative step [20,33,89]. The gene(s) for G-group toxin formation has not yet been identified.

Other genes on the aflatoxin pathway cluster, whose functions have not yet been defined are, *cypA*, *cypX*, *moxY* and *ordB*. Some evidence exists that these genes encode monooxygenases which may be involved in catalyzing the conversion of AVF to VHA and OMST to aflatoxins B<sub>1</sub> and G<sub>1</sub>. Several of these steps probably require multiple enzyme activities [20]. Another gene *aflT* (Figure 1) encodes a protein with homology to antibiotic efflux proteins, which might be necessary for transporting the toxic aflatoxin out of the fungal cells (Chang *et al.*, unpublished).

### Clustering of the aflatoxin pathway genes

Although coordinated regulation of the aflatoxin pathway genes was initially proposed [90], the first experimental evidence for clustering of the aflatoxin pathway genes was observed when Skory *et al.* [76] found that genes coding for enzymes in the earlier part of aflatoxin synthesis, *nor-1* and *ver-1* genes, were linked on a cosmid clone. By mapping overlapping cosmid clones in *A. parasiticus* and *A. flavus*, the linkage of the genes involved in early (*nor1*), middle (*ver-1*) and later stages (*omtA*) as well as a regulatory gene (*aflR*) of aflatoxin biosynthesis pathway was demonstrated, thereby establishing that the aflatoxin biosynthetic genes are clustered [91]. This allowed rapid discovery of genes essential for aflatoxin biosynthesis. These genes for the toxin synthesis are located on approximately 70 kb DNA region in the *A. parasiticus* and *A. flavus* genomes (Figure 1). The genes *cypX*, *moxY* [92] and *ordB* (Yu *et al.*, unpublished) are at one end of the aflatoxin pathway gene cluster, whereas the yet uncharacterized genes *norB*, *cypA* and *aflT* are at the other end of the cluster (Yu *et al.*, unpublished observations). A sugar utilization gene cluster immediately follows the aflatoxin cluster at the downstream end and may be involved in some aspect of cluster gene regulation [78].

In *A. parasiticus* strain ATCC 56775, a partial duplication of the gene cluster was confirmed recently by Chang and Yu [93]. Duplication of aflatoxin genes *ver-1* and *aflR* was earlier reported by Liang *et al.*, [94]. The duplicated region consists of seven genes, *aflR2*, *aflJ2*, *adhA2*, *estA2*, *norA2*, *ver1B* and *omtB2* [76,93]. These genes were not expressed [8,93, Yu *et al.*, unpublished data]. Their lack of expression could be a result of a chro-

mosomal location unfavorable to gene expression [95, Yu *et al.*, unpublished].

Sterigmatocystin, the penultimate precursor to aflatoxin is produced by a number of non-aflatoxigenic fungi including *A. nidulans*. Brown *et al.*, [96] characterized a 60 kb DNA region in *A. nidulans* that consists of a cluster of genes responsible for 25 coregulated transcripts involved in sterigmatocystin biosynthetic pathway in this fungus. In *A. flavus* and *A. parasiticus* the order of the genes and their direction of transcription of the aflatoxin cluster genes are identical and there is a high degree of sequence conservation (>95%) at both the nucleotide and amino acid level [91]. However, the order of the genes in the *A. nidulans* sterigmatocystin gene cluster is somewhat different from that of *A. parasiticus/A. flavus* (Figure 1). The sequence homologies are much lower even though the similarity of gene function and structure is conserved [91,96]. Common to both the aflatoxin and sterigmatocystin gene clusters is the presence of a gene, designated *aflR*, that encodes a zinc binuclear cluster-type, sequence specific DNA-binding protein that has been shown to be necessary for expression of the genes in both clusters [97-99].

Genes for many other secondary metabolism pathways are also clustered [26,100]. Such pathways are for the biosynthesis of trichothecenes [101,102], melanins [103,104], fumonisins [105], paxilline, HC-toxin [106] and AK-toxin [107]. Some catabolic pathway genes have also been organized in cluster. These include the pathway for nitrate assimilation involving the nitrate reductase (*niaD*) and nitrite reductase (*niiA*) genes in *Aspergillus*, in *Penicillium chrysogenum* [108-113], and in *Leptosphaeria maculans* [114], in *Ustilago maydis* [115], sugar utilization [116], proline utilization [100,117] and the biosynthesis of antibiotics such as penicillins [118].

A primary advantage of gene clustering may be to facilitate coordinated gene expression. Clustering of genes allows regulatory elements to be shared, and is known for biosynthetic pathways such as in the penicillin and nitrate assimilation in which gene function is vital to the organism's survival. There is also evidence that gene clustering may influence gene regulation through modulation of localized chromatin structure. Gene complementation experiments in *A. parasiticus* in this and other laboratories using the aflatoxin pathway gene constructs demonstrated that the site of integration within the fungal genome affects gene expression [95, Yu *et al.*, unpublished data]. The integrated gene is expressed at proper levels only when it was introduced into the aflatoxin pathway gene cluster. When an aflatoxin pathway gene was introduced into the nitrate utilization gene cluster at the *niaD* locus, transcript levels were more than a hundred-fold lower than expected (Yu *et al.*, unpublished observations). The clustered organization of fungal pathway genes may have some intrinsic significance in gene regulation in that expression of one cluster may affect the expression of other genes adjacent to the gene cluster. The sugar utilization gene cluster adjacent to the aflatoxin biosynthetic pathway gene cluster in *A. parasiticus* [116] may be important for aflatoxin gene expression since aflatoxin production is induced by sugar utilization.

How the aflatoxin cluster first arose is still not well understood, but the difference in gene organization between the sterigmatocystin cluster in *A. nidulans* (and the aflatoxin cluster in *A. ochraceoroseus*) and the clusters in most aflatoxin B<sub>1</sub> and G<sub>1</sub>-producing *Aspergilli* suggests that horizontal transfer between such *Aspergilli* is not a common occurrence. This deviation in cluster arrangement could be a consequence of *A. nidulans* having a

sexual reproduction cycle allowing recombination to occur, whereas *A. flavus* and *A. parasiticus* do not. Genetic variation in *A. parasiticus* can only be created by parasexual recombination. It is possible that the clustered arrangement of related metabolic pathway genes in fungi has originated from limited horizontal gene transfer between prokaryotes and fungi (for example, the penicillin biosynthesis pathway) which allowed introduction of genes encoding some of the oxidative proteins. Evidence for this is based on the presence of: a) common metabolic pathway gene clusters in both prokaryotes and fungi, e.g. the penicillin and cephalosporin pathways gene clusters; b) fungal cluster genes contain features of the prokaryotic pathway genes, such as the absence of introns and high G+C content [119]. Another hypothesis was proposed by Watson [120]. In this hypothesis it was suggested that clustering confers advantages to the cluster itself thus allowing the unit to facilitate the movement of the cluster into different organism by horizontal transfer.

### Genetic regulation of aflatoxin biosynthesis

Many nutritional and environmental factors, such as temperature, pH, carbon and nitrogen source, stress factors, lipids, and trace metal salts, affect the production of aflatoxin by toxigenic *Aspergilli*. The molecular mechanisms for these effects are still not clear in spite of numerous studies [8,9,22,112,113,116,121]. Some of these factors may affect expression of the aflatoxin regulatory gene, *aflR*, or structural genes, possibly by altering the expression of globally acting transcription factors that respond to nutritional and environmental signals [122]. Some of these nutritional and environmental factors may affect aflatoxin accumulation by altering the activity of one or more of the enzymes involved in aflatoxin biosynthesis.

The pathway-specific regulatory gene, *aflR*, is found in aflatoxin- and sterigmatocystin-producing fungi [91,96-98,123-126]. Disruption of *aflR* prevented the accumulation of structural gene transcripts for aflatoxin biosynthesis. Introduction of an additional copy of the *aflR* caused the overproduction of aflatoxin biosynthetic intermediates [97].

The protein encoded by *aflR* has major domains typical of Gal4-type transcription factors. One of these domains, an N-terminal cysteine-rich domain, CTS-CASSKVRCTKEKPACARCIERGLAC, (Cys<sub>6</sub>-Zn<sub>2</sub>) [127-131], is typical of fungal and yeast GAL4-type transcription factors which are required for DNA-binding. Preceding the Cys<sub>6</sub>-Zn<sub>2</sub> domain, and close to the N-terminus, there is an arginine-rich domain, RRARK, necessary for nuclear localization. In the C-terminus, a stretch (residues 408-444) of His, Arg, and acidic amino acids (HHPASPFSLGFGSLEAN LRHRLRAVSSDIIDYLHRE) is important for transcription activation, in particular the three acidic amino acids shown in bold [132]. *Aspergillus sojae*, a non-toxigenic strain used in industrial fermentations, was found to contain a defective *aflR* gene, due to a mutation resulting in early termination of 62 amino acids from its C-terminal end [133-135].

Comparison of *A. flavus* and *A. nidulans* AflRs showed that while overall amino acid identity is only 31%, the nuclear localization signal domain and the Cys<sub>6</sub>-Zn<sub>2</sub> domain are 71% identical. Much higher identities were found between the amino acid sequences of AflRs of other aflatoxin-producing species of *Aspergillus* (>96%). Besides the zinc cluster region, the immediately downstream neighboring amino acids (linker region) are also necessary for sequence-specific DNA binding in proteins

of this type [136]. Non-conservative substitution of amino acids in the "linker region" also resulted in defective AflR [99].

The promoter region of all the aflatoxin pathway structural genes contains at least one palindromic (5'-TCGN<sub>5</sub>CGA-3') or partially palindromic sequence (5'-TCGN<sub>5</sub>CGR-3') [137,138] to which AflR binds in order to initiate transcription. In cases where more than one AflR-binding site is present in a gene promoter region, only one AflR binding site may be necessary. This is the case for expression of the *pksA* and *avnA* gene [139,140].

Based on reporter gene assays, removal of sequences in the *aflR* promoter from -758 to -280 had no apparent effect on promoter activity, but further truncation to -118 enhanced gene expression nearly 5-fold; further removal from bases -118 to -100 almost entirely eliminated reporter gene expression [141]. Therefore, a negative regulatory element may be present in the region from -280 to -118 and sequences from -100 to -118 appear to be critical for *aflR* promoter activity. Recent studies suggest that *aflR* transcription is responsive to a G-protein signaling cascade that is mediated by protein kinase A [142]. Such a signaling pathway may mediate some of the environmental effects on aflatoxin biosynthesis. Other environmentally sensitive transcription factors could also be involved in negative regulation of *aflR* expression. The presence of a putative PacC-binding site in the region close to *aflR*'s transcription start site may play some role in pH regulation on aflatoxin production. It was reported that the PacC-binding represses the transcription of acid-expressed genes under alkaline conditions [143] and aflatoxin biosynthesis in *A. flavus* occurs in acidic media, but is inhibited in alkaline media [144].

Adjacent to the *aflR* gene in the aflatoxin gene cluster, a divergently transcribed gene, *aflJ*, was also found to be involved in the regulation of transcription [93,145,146]. AflJ has no known sequence homology to proteins identified in the databases [145]. This gene encodes a protein, AflJ, that binds to the carboxy terminal region of AflR and may affect AflR activity [93,145,146]. Disruption of *aflJ* in *A. flavus* resulted in a failure to produce any aflatoxin pathway metabolites [145]. Previously, it was found [124] that *aflR* expression was enhanced in *A. parasiticus* transformants with *aflR* in which the *aflJ* region was present compared to transformants in which this region was missing. It was also found that [113] a transcription factor required for nitrate assimilation, AreA, bound to sites near the *aflJ* transcription start site in the *aflR*-*aflJ* intergenic region, suggesting that *aflJ* expression could be mediated by nitrogen source via the action of AreA. Therefore, AflJ may be an AflR coactivator. The exact mechanism by which *aflJ* modulates transcription of these pathway genes in concert with *aflR* is to be further investigated.

### Environmental and nutritional factors affecting aflatoxin biosynthesis

The PacC [143] and AreA [113] binding sites in the *aflR*-*aflJ* intergenic region are the potential evidences that gene expression is regulated by environmental signals (pH and nitrate). The nitrate effect on aflatoxin pathway gene expression may be directly caused by changes in the *aflR* or *aflJ* gene expression level. To support this observation (Ehrlich, unpublished), we found that certain strains of aflatoxin-producing *Aspergilli* respond differently to nitrate than do other strains, and that the differen-

ces could be correlated with differences in the number of possible GATA sites (ranging from five to nine) near the *aflJ* start site [147].

Other genes in the aflatoxin biosynthetic cluster have also been found to contain AreA and PacC binding sites at key positions in their promoters that may affect their expression. For example, the 1.7 kb intergenic region separating the *nor-1* and *pksA* genes has two adjacent PacC sites nearly in the middle that, from site-directed mutagenesis studies, affect expression of *pksA*, which encodes the pathway-specific polyketide synthase necessary for the first steps in formation of the polyketide backbone [148]. In *A. nidulans*, the promoter region of the gene *steU* which is necessary for conversion of versicolorin A to demethylsterigmatocystin, contains a PacC-binding site immediately upstream of its AflR-binding site and is probably involved in expression of this gene.

Whether or not nitrate suppresses aflatoxin production is not clear. The expression of nitrate reductase and nitrite reductase genes requires both the lifting of nitrogen metabolite repression and specific induction by nitrate [121,149,150]. Expression of genes involved in nitrate utilization is transcriptionally activated by the global positive-acting regulatory factor, AreA [113,151]. Another way that nitrate could affect aflatoxin production is by increasing cytoplasmic NADPH/NADP ratio, which could favor biosynthetic reductive reactions, and thus, could promote utilization of malonyl coenzyme A and NADPH for fatty acid synthesis rather than for polyketide synthesis [152]. Kachholz and Demain [153] and Orvehed *et al.*, [154] found that nitrate represses the activity of enzymes involved in the synthesis of alternariol monomethyl ether in *Alternaria alternata*. In transformants containing an additional copy of *aflR*, the transcription of aflatoxin pathway genes increases in nitrate medium [67]. This increase could result from the increased *aflR* copy number which elevates the basal levels of AFLR in the transformants. Preliminary data have shown that AFLR1 (a recombinant version of AFLR, but containing an intact zinc finger) also binds to sites in the promoter regions of several aflatoxin biosynthetic genes and may thereby activate their transcription [140].

The role of carbon utilization in the regulation of expression of genes involved in aflatoxin biosynthetic pathway is not as yet well understood. Unlike the biosynthesis of many other secondary metabolites, aflatoxin gene expression is induced by the presence of simple carbohydrates, for example glucose, sucrose, maltose, but not by peptone, sorbose, or lactose [reviewed in 22]. It is to be noted that all of the aflatoxin pathway genes so far studied lack CreA sites in their promoters and, therefore, would not be expected to be subject to carbon catabolite repression which is mediated by the transcription factor, CreA. However, an interesting possible role for CreA in *aflR* expression could be control of expression of the antisense *aflR* mRNA transcript (Ehrlich, unpublished observation), since at the start of this reported transcript are two tandem CreA-binding sites, GCGGGaGTGGGG. If carbon catabolite repression prevents the expression of this anti-sense *aflR* transcript, it would be expected not to down-regulate AflR protein accumulation by interfering with the activity of the *aflR* sense transcript. Another transcription factor that responds to simple sugars is Rgt1, a positively acting factor that has been shown to be necessary for regulation of glucose transporter molecule expression [155]. In *Saccharomyces cerevisiae* Rgt1 functions as a transcriptional repressor in the absence of glucose, but in the presence of high concentrations of glucose it functions as a transcriptional activator. A possible Rgt1 site is present in

the promoter region of *A. parasiticus aflJ*, and may be involved in regulation of its expression. Such regulation may be necessary for production of aflatoxin pathway metabolites (see above).

Another indirect role for an effect of glucose utilization on aflatoxin pathway gene expression could be related to the fact that immediately downstream of the aflatoxin gene cluster is a four gene sugar cluster (Figure 1), including genes that putatively encode a hexose transporter, a glucosidase, an NADH oxidase and a Cys<sub>2</sub>Zn<sub>2</sub>-type regulatory gene [116]. Activation of genes in this sugar cluster by an external hexose signal could create a region of active chromatin that includes the neighboring aflatoxin gene cluster [156]. To support this observation, we and others found that when individual aflatoxin biosynthetic genes insert at sites other than the aflatoxin gene cluster following fungal transformation, expression of these genes is much lower (>100-fold) than it is when the genes insert into the aflatoxin cluster [95, Yu, unpublished observation].

Another way that carbon source utilization could affect aflatoxin gene expression may be by inducing G-protein-dependent signaling in *Aspergillus* cells [157]. The G-protein signaling regulates fungal development and aflatoxin formation [142,158]. This will be discussed in more detail in the following section.

### Aflatoxin biosynthesis and fungal development

Evidence exists that secondary metabolism is associated with fungal developmental processes such as sporulation and sclerotia formation [158-160]. It was observed that the environmental conditions required for secondary metabolism and for sporulation are similar [159,160]. It was also reported that the spore formation and secondary metabolite formation occur at about the same time [24,142]. Certain compounds in *A. parasiticus* that exhibit the ability to inhibit sporulation have also been shown to inhibit aflatoxin formation [161]. Chemicals that inhibit polyamine biosynthesis in *A. parasiticus* and *A. nidulans* inhibit both sporulation and aflatoxin/sterigmatocystin biosynthesis [162]. More evidence from mutant strains of *Aspergilli* demonstrated the relationship between aflatoxin formation and sporulation. Mutants that are deficient in sporulation were unable to produce aflatoxins [59]. A *Fusarium verticillioides* mutation in FCC1 gene resulted in both reduced sporulation and reduced fumonisin B<sub>1</sub> production [163].

Since many environmental and nutritional factors affect aflatoxin formation, it is likely that one or more signal transduction pathways affect aflatoxin formation. Also, there appears to be a genetic connection between fungal development and toxin formation [59,69,164-167]. A correlation between increased pool size of cAMP and aflatoxin production had been observed previously [168,169]. Sterigmatocystin production by *A. nidulans* appears to require inhibition of FadA-dependent signaling [142]. FadA is the alpha subunit of the *A. nidulans* heterotrimeric G-protein. When FadA is bound to GTP and in its active form, sterigmatocystin production (and sporulation) was repressed. However, in the presence of FlbA, the intrinsic GTPase, activity of FadA is stimulated, thereby leading to GTP hydrolysis, inactivation of FadA-dependent signaling, and stimulation of sterigmatocystin production. A non-sporulating, "fluffy" mutant strain of *A. nidulans* was found to be deficient in sterigmatocystin formation [170,171]. Hicks *et al.*, [142] provided evidence that a G-protein signal transduction pathway mediated by

protein kinase A regulates both aflatoxin/sterigmatocystin synthesis and sporulation. The G-protein signaling pathway involves the “fluffy” gene regulators FluG and FlbA, and the conidiation gene regulators BrlA, FadA and PkaA. A gene, *brlA*, in *A. nidulans* encodes a transcriptional regulator (BrlA) believed to activate developmental genes [172] since mutation in *brlA* gene resulted in no conidiation [173]. 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 found to be involved in the regulation of both asexual development (conidiation) and sterigmatocystin biosynthesis in *A. nidulans* [142,174]. The *fluG* 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 (PKA), PkaA [142,175]. Over expression of *pkaA* (PkaA) inhibits *brlA* and *aflR* (positive regulator of aflatoxin/sterigmatocystin genes) expression [175]. The *fadA* encodes the alpha subunit of a heterotrimeric G-protein, FadA. A domain of the FlbA, the regulator of the G-protein signaling (RGS) is presumably to inhibit FadA [174]. In the overall scheme of the proposed G-protein signaling pathway, the FadA and PkaA favor vegetative growth and inhibit conidiation and aflatoxin/sterigmatocystin production; while FluG and FlbA inhibit FadA and PkaA function and promote conidiation and aflatoxin/sterigmatocystin biosynthesis [158,142,174]. This G-protein signaling pathway involving FadA in the regulation of aflatoxin production may also exist in other *Aspergilli* such as *A. parasiticus* (Keller, unpublished cited in 158).

## CONCLUSION

Biosynthesis of aflatoxin is a highly complex process governed by genes maintained in a cluster. Transcriptional regulation is controlled by a protein encoded by *aflR*. Chromosomal position effects, as well as a number of other globally acting regulatory genes may be subject to nutritional and environmental control. No evidence for horizontal transfer of the aflatoxin cluster among aflatoxin and sterigmatocystin-producing organisms is available, and no prokaryotic origin for clustered genes has yet been identified. The biological and evolutionary importance of aflatoxin production to the organism is also poorly understood. The clustering of these genes implies that the cluster plays an important role in fungal growth and survival. Otherwise these genes would be rapidly lost, due to genetic drift events and rearrangements. It is not yet understood how aflatoxins contribute to fungal survival since certain non-aflatoxigenic *A. flavus* species appear to compete favorably with aflatoxigenic species for the same niche. Other questions remain: what is the relationship between primary and secondary metabolism, how does stress affect aflatoxin gene expression, particular during fungal interaction with plants. Screening of an Expressed Sequence-Tag (EST) library using microarrays may provide a way to better understand how and why aflatoxins are produced. The *A. flavus* EST/microarray project is being actively pursued by this Research Unit in collaboration with The Institute for Genomic Research. Information obtained through this genomics project may help in devising strategy for elimination of aflatoxin contamination of crops, and thereby provide a sustainable, healthy food supply.

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