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Aflatoxin Biosynthetic Pathway and Pathway Genes

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1. Introduction

Among the over 185 known species within the genus *Aspergillus*, *Aspergillus flavus* is the most economically important because it produces the toxic and carcinogenic aflatoxins. Its non-aflatoxigenic relative *A. oryzae* is used extensively for food fermentations (Jelinek et al., 1989). It is one of the most abundant soil-borne molds on earth. *A. flavus* fungus is a saprobe mold that is capable of surviving on many organic nutrient sources like plant debris, tree leaves, decaying wood, animal fodder, cotton, compost piles, dead insects and animal carcasses, stored grains, and even immunocompromised humans and animals (Klich, 1998). It has the ability to survive temperatures ranging from 12°C to 48°C, but the optimal growth temperature ranges from 28°C to 37°C. Its ability to grow at relatively high temperatures contributes to its pathogenicity toward humans and other warm blooded animals. For most of its lifecycle, the fungus exists in the form of mycelium or asexual spores known as conidia. Under adverse conditions such as lack of adequate nutrients or water, the fungal mycelium will transform to resistant structures called sclerotia which can survive extremely harsh environmental conditions. The fungus overwinters either as spores, sclerotia, or as mycelium in debris. When conditions become favorable the sclerotia germinate directly to produce new colonies or conidiophores with conidia (Bennett et al., 1986; Cotty, 1988; Chang et al., 2002).

Aflatoxins were first identified as the cause of a severe animal poisoning incident in England in 1960 called the Turkey X disease (Allcroft et al., 1961; Lancaster et al., 1961). *A. flavus* produces aflatoxin B₁ and B₂ whereas *A. parasiticus*, produces aflatoxins B₁, B₂, G₁, and G₂. These four major aflatoxins are named based on their blue (B) or green (G) fluorescence under ultraviolet light, and their relative mobility by thin-layer chromatography on silica gel. Aflatoxin M₁ is a hydroxylated derivative metabolized from aflatoxin B₁ by cows and secreted in milk (Van Egmond, 1989). In addition to aflatoxins B₁ and B₂, *A. flavus* also produces many other mycotoxins such as cyclopiazonic acid, kojic acid, beta-nitropropionic acid, aspertoxin, aflatrem and aspergillic acid (Goto et al., 1996).

The disease caused by ingestion of aflatoxins in contaminated food or feed is called aflatoxicosis. Acute aflatoxicosis occurs when aflatoxins are consumed at moderate to high levels. Depending on the level and duration of exposure, aflatoxins possess both hepatotoxic and carcinogenic properties. Symptoms in humans include vomiting, abdominal pain, alteration in digestion, limb and pulmonary edema, convulsions, rapid progressive jaundice,

swollen liver, high fever, coma, and death. The predominant damage is to the liver (Scholl & Groopman, 1995); (Fung & Clark, 2004; Lewis et al., 2005), but acute damage to the kidneys and heart have been found (Richard & Payne, 2003). In liver aflatoxins irreversibly bind to protein and DNA to form adducts such as aflatoxin B₁-lysine in albumin and a guanyl-N7 adduct in DNA (Skipper & Tannenbaum, 1990). Disruption of the proteins and DNA bases in hepatocytes causes the toxicity (Tandon et al., 1978; Azziz-Baumgartner et al., 2005). Major outbreaks of acute aflatoxicosis from contaminated food in humans were reported in developing countries (Centers for Disease Control and Prevention, 2004; Lewis et al., 2005). For example, in western India in 1974, 108 persons died among 397 people affected with aflatoxin poisoning in more than 150 villages (Krishnamachari et al., 1975). A more recent incident of aflatoxin poisoning occurred in Kenya in July 2004 leading to the death of 125 people among 317 reported with illness due to consumption of aflatoxin contaminated maize (corn) (Centers for Disease Control and Prevention, 2004; Lewis et al., 2005). Acute toxicosis is not the only concern. World health authorities warn that low doses and long term dietary exposure to aflatoxins is also a major risk as chronic exposure can lead to hepatocellular carcinoma (Bressac et al., 1991; Hsu et al., 1991; Wogan, 1992; Fung & Clark, 2004).

Among the four major types of aflatoxins, aflatoxin B₁ is the most toxic and the most potent carcinogen in humans and animals including nonhuman primates, birds, fish, and rodents. Chronic exposure can result in suppressed immune response, malnutrition, proliferation of the bile duct, centrilobular necrosis and fatty infiltration of the liver, hepatic lesions, and even hepatomas. In animal models, aflatoxin B₁ is modified into a more toxic and carcinogenic by-product during detoxification by a cytochrome P450 monooxygenase in liver (Ngindu et al., 1982; Hsieh, 1989; Eaton & Gallagher, 1994; Lewis et al., 2005). The epoxide form of aflatoxin binds to guanine residues in DNA, forms guanyl-N7 adducts, and induces mutations. One mutation, a G to T transversion (Baertschi et al., 1989; Bressac et al., 1991) at the third base of codon 249, a mutation hot spot of the p53 tumor suppressor gene, is generally believed to be the mechanism for initiating hepatocarcinoma formation (Busby & Wogan, 1981; Hsu et al., 1991; Ozturk, 1991; Coursaget et al., 1993). The p53 gene encodes a transcription factor involved in cell cycle regulation. It is commonly mutated in human liver cancers (Groopman et al., 1994). Aflatoxin B₁ is also a potential immunosuppressive agent (Raisuddin et al., 1993). Chronic low level exposure of growing vertebrates to aflatoxins may enhance their susceptibility to infection and tumorigenesis (Raisuddin et al., 1993). AFB₁ also affects other organs and tissues, such as the lungs and the entire respiratory system (Kelly et al., 1997). Human hepatocarcinomas are also associated with hepatitis B virus (HBV) and C virus (HCV) infections (Peers et al., 1987; Hsieh, 1989; Wild et al., 1992). Together with aflatoxins these viruses significantly increased the risk of hepatoma in hepatitis patients (Chen et al., 1996a; Chen et al., 1996b; McGlynn et al., 2003; Arsur & Cavin, 2005). In developing countries, many children are exposed to aflatoxin before birth (Turner et al., 2007), while nursing (Polychronaki et al., 2007) and after weaning (Gong et al., 2004). An association of hepatocellular carcinoma and dietary exposure with aflatoxins has been established from patients living in high-risk areas of China, Kenya, Mozambique, Philippines, Swaziland, Thailand, Transkei of South Africa (Lancaster et al., 1961; Zuckerman et al., 1967; Wong et al., 1977; Hsieh et al., 1985; Zhu et al., 1987; Huang & Hsieh, 1988; Wilson, 1989; Wogan, 1992; Eaton & Gallagher, 1994; Lewis et al., 2005).

Aspergillus flavus can grow in immunocompromised warm blooded mammals and can cause invasive and non-invasive aspergillosis in humans and animals (Denning et al., 1991; Denning, 1998; Mori et al., 1998; Denning et al., 2003). *A. flavus* is the second leading cause of

aspergillosis slightly behind *A. fumigatus*. The incidence of aspergillosis caused by *Aspergilli* is rising due to the increase of immunocompromised patients in the population due to HIV infection (Denning, 1998; Nierman et al., 2005; Ronning et al., 2005).

A. flavus is a weak and opportunistic plant pathogen, affecting many agricultural crops such as maize (corn), cotton, groundnuts (peanuts), as well as tree nuts such as Brazil nuts, pecans, pistachio nuts, and walnuts. Preharvest contamination of these crops with aflatoxins is common. *A. flavus* also causes the spoilage of post harvest grains during storage. Because *A. flavus* lacks host specificity (St Leger et al., 2000) and can attack seeds of both monocots and dicots, and seeds produced both above ground (corn) as well as below the ground (peanuts). Under weather conditions favorable for its growth, *A. flavus* can cause ear rot on maize, resulting in significant economic losses to farmers (Robens, 2001; Richard & Payne, 2003; Robens & Cardwell, 2005).

2. Economic significance

Due to the toxic and carcinogenic properties of aflatoxins, only extremely low levels of aflatoxins in foods and feeds is allowed (Council for Agricultural Science and Technology, 2003; Fung & Clark, 2004). The International Agency for Research on Cancer (IARC) has designated aflatoxin as a human liver carcinogen (Van Egmond, 1989; van Egmond & Jonker, 2005; van Egmond et al., 2007). To minimize potential exposure to aflatoxins, maximum levels of aflatoxins in many commodities have been set at levels below 20 ppb by most countries (Van Egmond, 1989; van Egmond & Jonker, 2005; van Egmond et al., 2007). Regulatory guidelines of the U.S. Food and Drug Administration (FDA) specifically prevent the sale of commodities if contamination by aflatoxins exceeds 20 ppb total aflatoxins for interstate commerce of food and feedstuff and 0.5 ppb aflatoxin M₁ in milk. The European Commission has set the limits on groundnuts subject to further processing at 15 ppb for total aflatoxins and 8 ppb for aflatoxin B₁, and for nuts and dried fruits subject to further processing at 10 ppb for total aflatoxins and 5 ppb for aflatoxin B₁. The aflatoxin standards for cereals, dried fruits, and nuts intended for direct human consumption are even more stringent, and the limit for total aflatoxins is 4 ppb and 2 ppb for aflatoxin B₁ (van Egmond & Jonker, 2005).

Aflatoxin contamination of agricultural commodities poses a potential risk to livestock and human health (Lancaster et al., 1961; Bennett & Lee, 1979; Bennett, 1987; Jelinek et al., 1989; Cleveland & Bhatnagar, 1992; Eaton & Groopman, 1994; Hall & Wild, 1994; Bhatnagar et al., 2002; Bennett & Klich, 2003; Richard & Payne, 2003). It is not only a serious food safety concern, but it has significant economic implications for the agricultural industry worldwide because of restrictions limiting the trade of contaminated crops.

Since its discovery, extensive efforts have been made and expense incurred worldwide to monitor aflatoxin occurrence and to develop control strategies (Bennett, 1970; Bennett & Goldblatt, 1973; Bennett et al., 1976b; Papa, 1976; Papa, 1979; Papa, 1984). The hallmark discovery of a color mutant that accumulates the brick-red pigment, norsolorinic acid (NOR), in *A. parasiticus* marked a milestone in the understanding the chemistry of aflatoxin biosynthesis (Bennett et al., 1971; Bennett et al., 1976a; Bennett, 1979; Bennett et al., 1983). Since NOR is the earliest and the first stable aflatoxin precursor in the aflatoxin biosynthetic pathway (Hsieh et al., 1976; Dutton, 1988; Bennett et al., 1997), this discovery led to the identification of other key aflatoxin intermediates and established the primary metabolites in the aflatoxin pathway. It provided the opportunity to isolate the first aflatoxin pathway

gene that encodes a reductase for the conversion from NOR to eventually aflatoxins (Hsieh & Mateles, 1970; Hsieh et al., 1973; Hsieh et al., 1976; Chang et al., 1992) Dutton, 1982 #357; Dutton, 1985 #313}. After the cloning of several important aflatoxin pathway genes, a 75 kb aflatoxin pathway gene cluster was established in *A. parasiticus* and *A. flavus* (Yu et al., 1995a). Discovery of the cluster promoted renewed interest in understanding aflatoxin biosynthesis by scientists all over the world. Significant progress has been made in elucidating the biosynthetic pathway, the pathway intermediates, genes, corresponding enzymes, and regulatory mechanisms (Bennett & Lee, 1979; Bennett et al., 1981; Cleveland et al., 1987; Bennett & Papa, 1988; Bhatnagar et al., 1992; Chang et al., 1993; Keller et al., 1993; Chang et al., 1995a; Chang et al., 1999a; Ehrlich et al., 1999a; Bennett & Klich, 2003; Chang, 2004; Yu et al., 2004c; Crawford et al., 2008a; Ehrlich, 2009; Ehrlich & Yu, 2009). At least 27 enzymatic steps have been characterized or proposed to be involved in bioconversion of aflatoxin intermediates to aflatoxins (Ehrlich, 2009). In this chapter, we focus on the aflatoxin biosynthetic pathway and the function of aflatoxin cluster genes. For detailed historical information on the aflatoxin pathway genes and gene cluster discovery, please refer to previous reviews (Yabe & Nakajima, 2004; Yu et al., 2004a; Yu et al., 2004c; Yu et al., 2011).

3. Aflatoxin biosynthetic pathway and genes involved

Attempts to decipher the aflatoxin biosynthetic pathway began shortly after the determination of the structure of these toxins (Goldblatt, 1969; Singh & Hsieh, 1977; FAO, 1995). The discovery of a colored mutant in *A. parasiticus* that accumulates norsolorinic acid (NOR) (Bennett et al., 1971; Lee et al., 1971; Bennett et al., 1997) paved the road for the establishment of aflatoxin biosynthetic pathway. With the rapid gene cloning and enzyme characterization, the enzymatic steps for biosynthesis of the 15 structurally defined aflatoxin pathway intermediates have been identified (Cleveland & Bhatnagar, 1987; Bhatnagar et al., 1989; Cleveland & Bhatnagar, 1990; Bhatnagar & Cleveland, 1991; Cleveland & Bhatnagar, 1991; Bhatnagar et al., 1992; Trail et al., 1995a; Yu et al., 1995a; Minto & Townsend, 1997; Townsend, 1997; Yu et al., 1997; Yu et al., 1998; Keller et al., 2000; Yu et al., 2004b; Crawford et al., 2008b). There are estimated to be 27 enzymatic steps in the aflatoxin biosynthesis (Ehrlich, 2009). As many as 30 genes are potentially involved in aflatoxin biosynthesis (Figure 1). The genes and corresponding enzymes have been extensively studied (Bennett et al., 1971; Lee et al., 1971; Yabe & Nakajima, 2004; Yu et al., 2004a; Yu et al., 2004c). In *A. flavus* and *A. parasiticus* the aflatoxin pathway genes are clustered within a 75-kb region of the fungal genome on chromosome III roughly 80 kb away from telomere (Wilson, 1989; Trail et al., 1995a; Trail et al., 1995b; Yu et al., 1995a; Townsend, 1997; Yu et al., 2004a; Yu et al., 2004c; Chang et al., 2005).

3.1 Acetate to norsolorinic acid (NOR)

Norsolorinic acid (NOR) was confirmed to be the first stable aflatoxin precursor (Bennett et al., 1971; Bennett, 1981; Bennett et al., 1983). A hexanoyl starter unit is the initial substrate for aflatoxin formation (Hsieh & Mateles, 1970). Two fatty acid synthases (FAS) and a polyketide synthase (NR-PKS, PksA) are involved in the synthesis of the polyketide from a hexanoyl starter unit. Seven iterative, malonyl-derived ketide extensions are required to produce norsolorinic acid anthrone (noranthrone) (Wilson, 1989; Trail et al., 1995a; Trail et al., 1995b; Brown et al., 1996a; Brown et al., 1996b; Watanabe et al., 1996; Watanabe & Townsend, 2002; Yabe & Nakajima, 2004; Crawford et al., 2006; Crawford et al., 2008a;

Crawford et al., 2008b). Mahanti et al. (Mahanti et al., 1996) cloned, by genetic complementation, a 7.5-kb large transcript which is required for NOR formation in a blocked *A. parasiticus* mutant. Its protein has high degree of similarity (67%) and identity (48%) to the beta-subunit of FASs (FAS1) of *Saccharomyces cerevisiae* and *Yarrowia lipolytica*. Metabolite feeding and gene disruption experiments further confirmed that *uvm8* encodes a subunit of a novel fatty acid synthase (FAS) directly involved in the backbone formation of the polyketide precursor of NOR during aflatoxin biosynthesis, therefore, on the basis of its function, the *uvm8* gene was renamed *fas-1A*. In the revised naming scheme, the *fas-1A* gene was renamed as *fas-1*, it encodes fatty acid synthase-1 in the aflatoxin biosynthetic pathway gene cluster (Figure 1). Another large transcript (*fas-2A*) which encodes an alpha-subunit of fatty acid synthase in the aflatoxin gene cluster was reported (Mahanti et al., 1996). The gene *fas-1A* and *fas-2A* were renamed *fas-1* and *fas-2*. They encode two fatty acid synthases (FAS α and FAS β) (Payne, 1998). In *A. nidulans* the involvement of FASs in sterigmatocystin (ST) biosynthesis was also confirmed and were named *stcJ* and *stcK* in the ST cluster (Brown et al., 1996a; Brown et al., 1996b). The biochemical evidence for the role of a fatty acid synthase and a polyketide synthase (PKS) in the biosynthesis of aflatoxin was demonstrated (Watanabe & Townsend, 1996). Further details on the early stage of aflatoxin biosynthesis involving fatty acid synthases and polyketide synthases were reported (Watanabe et al., 1996; Hitchman et al., 2001; Watanabe & Townsend, 2002; Crawford et al., 2006). The N-acetylcysteamine thioester of hexanoic acid was incorporated into NOR in a *fas-1* disrupted transformant. A polyketide synthase gene (*pksA*) in *A. parasiticus* was demonstrated by gene disruption to be required for aflatoxin biosynthesis (Chang et al., 1995a). The predicted amino acid sequences of these PKSs contain the typical four conserved domains commonly found in other known PKS proteins: β -ketoacyl synthase (KS), acyltransferase (AT), acyl carrier protein (ACP), and thioesterase (TE) (Chang et al., 1995a). Townsend's group has dissected the functional domains of the PKS for aflatoxin biosynthesis (Crawford et al., 2008a; Crawford et al., 2008b; Crawford et al., 2008c). These include domains for the starter unit acyl transferase (SAT) which recognizes hexanoyl CoA and the N-acetylcysteamine thioester of hexanoic acid, the acyl carrier protein (ACP), ketosynthase (KS), malonyl-CoA:ACP transacylase (MAT), product template (PT) allowing the iterative steps in forming the polyketide, and a thioesterase/Claisen-like cyclase (TE/CLC) (Crawford et al., 2008a). The predicted product converted by PksA is noranthrone. The conversion of noranthrone to NOR, the first stable intermediate in the pathway (Bennett et al., 1971; Lee et al., 1971; Papa, 1979; Bennett et al., 1981; Papa, 1982; Bennett et al., 1994; Bennett et al., 1997), is poorly defined, but it has been proposed to be catalyzed by a noranthrone oxidase, a monooxygenase, or to occur spontaneously (Dutton, 1988). Sequence analysis and enzymatic studies supports the contention that the *hypC* (a gene in the intergenic region of *pksA* and *nor-1*) gene product is the required noranthrone oxidase involved in the catalysis of the oxidation of norsolorinic acid anthrone to NOR (Ehrlich, 2009). The *fas-1*, *fas-2*, and *pksA* genes were renamed as *aflA*, *aflB* and *aflC* respectively (Wilson, 1989; Yu et al., 2004a; Yu et al., 2004c) (Figure 1). The *aflA*, *aflB* and *aflC* gene homologues in *A. nidulans* are *stcJ*, *stcK*, and *stcA*, respectively (Brown et al., 1996b).

3.2 Norsolorinic acid (NOR) to averantin (AVN)

The first stable AF intermediate was identified as NOR produced in *A. parasiticus* uv-generated disruption mutants (Bennett et al., 1971; Lee et al., 1971; Detroy et al., 1973;

Bennett et al., 1981) and in *A. flavus* (Papa, 1979; Papa, 1982). The NOR-accumulating mutants are leaky mutants whose aflatoxin biosynthesis is not completely blocked. By genetic complementation, the gene, *aflD* (*nor-1*), encoding a reductase was cloned (Chang et al., 1992). A recombinant Nor-1 protein expressed in *E. coli* catalyzed the reduction of NOR. Therefore, *aflD* (*nor-1*) encodes the ketoreductase needed for the conversion of the 1'-keto group in NOR to the 1'-hydroxyl group of AVN (Zhou & Linz, 1999). Disruption of the *aflD* (*nor-1*) gene also confirmed its involvement in conversion of NOR to AVN in aflatoxin biosynthesis (Trail et al., 1994). The *aflD* (*nor-1*) homologous gene in *A. nidulans* is *stcE* (Brown et al., 1996b). Genes homologous to *aflD* (*nor-1*), in the AF cluster, such as *aflE* (*norA*) and *aflF* (*norB*) are predicted to encode short chain aryl alcohol dehydrogenases. These proteins may also be able to catalyze the reduction of NOR to AVN depending on the reductive environment of the cell and may explain the leakiness of the *nor-1* mutation if they are able to complement Nor-1's function (Cary et al., 1996),

3.3 Averantin (AVN) to 5'-hydroxyaverantin (HAVN)

Radioisotope incorporation experiments established the earliest evidence for the conversion of AVN to HAVN (Bennett et al., 1980; McCormick et al., 1987). In these studies, three enzymatic steps can account for the conversion of NOR to averufin (AVF) (Yabe et al., 1991a). They are (i) NOR to AVN catalyzed by a reductase, (ii) NOR to HAVN catalyzed by a monooxygenase, and (iii) HAVN to AVF catalyzed by a second dehydrogenase. It was also proposed that the oxidation reactions are reversible and that NADPH was the preferred cofactor (Yabe et al., 1991b). The gene previously named *ord-1* encoding a P-450 monooxygenase was cloned and disrupted (Yu et al., 1997). Substrate feeding studies of the *ord-1* mutant confirmed that HAVN is the intermediate in the conversion of AVN to AVF. The *ord-1* gene, which has a high degree of sequence similarity to *A. nidulans* *stcF* (Brown et al., 1996b), was renamed *aflG* (*avnA*).

3.4 5'-Hydroxyaverantin (HAVN) to oxoaverantin (OAVN), and averufin (AVF)

Numerous studies have established averufin as one of the key intermediates in aflatoxin formation (Lee et al., 1971; Hsieh, 1973; Lin & Hsieh, 1973; Lin et al., 1973; Fitzell et al., 1975; Singh & Hsieh, 1977; Keller et al., 2000). Several intermediates were reported to be involved in the conversion from AVN to AVF (Lin & Hsieh, 1973; Bhatnagar et al., 1992). One of these averufanin (AVNN), based on later studies was considered a shunt metabolite and not a genuine aflatoxin intermediate (Sakuno et al., 2003; Yabe & Nakajima, 2004). Chang et al. (Lee et al., 1971; Lin & Hsieh, 1973; Chang et al., 2000) characterized the cluster gene *aflH* (*adhA*) in *A. parasiticus* which encodes an alcohol dehydrogenase. It was showed that *adhA* deletion mutants accumulated predominantly HAVN and after prolonged growth the mutants were able to produce small amounts of AVNN consistent with AVNN being a shunt metabolite. Thus, HAVN might be converted directly to AVF or indirectly to AVF by an additional cytosolic enzyme. Sakuno et al. (Sakuno et al., 2003) characterized two cytosolic enzymes and a new aflatoxin intermediate named 5'-oxoaverantin (OAVN) as an intermediate between HAVN and AVF. The enzyme for the conversion from HAVN to OAVN is encoded by the *aflH* (*adhA*) gene. The *adhA* gene deletion mutant is leaky indicating that additional enzyme(s) or gene(s) may be involved in the conversion from OAVN to AVF. The enzymatic steps for aflatoxin biosynthesis and the possible involvement of additional enzymes have also been described (Townsend, 1997; Ehrlich, 2009; Ehrlich et

al., 2010). Woloshuk and Payne (Woloshuk & Payne, 1994) identified an alcohol dehydrogenase gene, *adh1*, in *A. flavus*, expressed concurrently with aflatoxin pathway genes. No further report is made on the role of *A. flavus adh1* gene in aflatoxin synthesis. The *aflH* (*adhA*) gene in *A. flavus* and the *adhA* gene in *A. parasiticus* share no significant homology at either the DNA or the amino acid level.

3.5 Averufin (AVF) to versiconal hemiacetal acetate (VHA)

VHA was identified as an aflatoxin precursor formed by oxidation of AVF (Fitzell et al., 1977). The conversion of AVF to VHA involves the cytochrome P450 monooxidase, CypX, and another gene, *aflI* (*avfA*). Although *aflI* is required for the conversion, its oxidative role is unclear (Yu et al., 2000b). *A. nidulans* also has an *aflI* gene homolog (*stcO*) (Brown et al., 1996b; Yu et al., 2000b). Complementation of an averufin-accumulating mutant, *A. parasiticus* SRRC 165, with the *aflI* gene of *A. flavus* restored the strain's ability to convert AVF to VHA and to produce aflatoxins (Yu et al., 2000b). It is likely that the *aflI* (*avfA*) encoded protein along with CypX gene product is involved in the ring-closure step in the formation of hydroxyversicolorone. It is possible that the *avfA* gene product is associated with the P450 monooxygenase to carry out the conversion as no additional intermediates other than AVF result from the disruption of either gene.

3.6 Versiconal hemiacetal acetate (VHA) to versiconal (VHOH, also abbreviated as VAL)

Several research groups have demonstrated that an esterase is involved in the conversion of VHA to VHOH (VAL) (Schroeder et al., 1974; Yao & Hsieh, 1974; Bennett et al., 1976b; Fitzell et al., 1977; Hsieh et al., 1989; Yabe et al., 1991a; Yabe et al., 1991b; Kusumoto & Hsieh, 1996). The esterase was purified in *A. parasiticus* (Hsieh et al., 1989; Kusumoto & Hsieh, 1996). An esterase gene, *aflJ* (*estA*), in the aflatoxin gene cluster was identified (Yu et al., 2002). The homologous gene in the *A. nidulans* ST biosynthetic gene cluster is *stcI*. In the *A. parasiticus aflJ* (*estA*) deletion mutants, the accumulated metabolites were mainly VHA and versicolorin A (VERA) (Chang et al., 2004). A small amount of versiconol acetate (VOAc) and other downstream aflatoxin intermediates, including VHOH and versicolorin B also accumulated. A metabolic grid containing VHA, VOAc, VHOH, and versiconol (VOH) was previously described and it was suggested that the reactions from VHA to VHOH and from VOAc to VOH are catalyzed by the same esterase (Yabe et al., 1991a). Later, another metabolic grid containing versicolorone (VONE), VOAc, and VHA was identified (Yabe et al., 2003). Indeed, it has now been proven that the *estA*-encoded esterase catalyzes the conversion of both VHA to VHOH and VOAc to VOH during aflatoxin biosynthesis (Chang et al., 2004).

3.7 Versiconal (VHOH) to versicolorin B (VER B)

The enzymatic evidence that VHOH is converted to VERB by a cyclase was first provided by Lin and Anderson (Lin & Anderson, 1992). This enzyme was identified as versicolorin B synthase and was studied intensively by Townsend's laboratory (Zuckerman et al., 1967; Hsieh, 1973; McGuire et al., 1996; Silva et al., 1996; Silva & Townsend, 1997). The gene was cloned and named *vbs* (Zuckerman et al., 1967; Hsieh, 1973; Silva et al., 1996). The expected cyclase activity was demonstrated by the expressed recombinant protein of the *vbs* gene (Silva et al., 1996; Silva & Townsend, 1997). The VHOH cyclase (Lin & Anderson, 1992) and

VER B synthase (McGuire et al., 1996) were independently isolated from *A. parasiticus*. The enzyme catalyzes the side chain cyclodehydration of racemic VHA to VER B. This is another key step in aflatoxin formation since it closes the bisfuran ring of aflatoxin, the moiety ultimately responsible for aflatoxin's toxicity and carcinogenicity. The *vbs* gene was renamed *aflK (vbs)* a (Yu et al., 2004c). The homologous gene in the *A. nidulans* ST biosynthetic gene cluster is *stcN*.

3.8 Versicolorin B (VER B) to versicolorin A (VER A)

VER B is a critical branch point leading to the formation of either AFB₁/AFG₁ or AFB₂/AFG₂. Similar to AFB₂/AFG₂, VER B contains a tetrahydrobisfuran ring and, like AFB₁/AFG₁, VERA contains a dihydrobisfuran ring. The conversion of VER B to VER A requires desaturation of the bisfuran ring of VER B by an unstable microsomal enzyme that requires NADPH (Yabe et al., 1993). Disruption of *stcL* in *A. nidulans* (Kelkar et al., 1997) abolished ST synthesis and resulted in the accumulation of VER B. The *stcL* gene encodes a cytochrome P-450 monooxygenase. The homologue, *aflL (verB)*, is present in the aflatoxin gene cluster of *A. parasiticus* and *A. flavus* strains. Cultural conditions appear to markedly affect the activity of VER B desaturase and thereby, the final ratio of AFB₁ to AFB₂ and AFG₁ to AFG₂ (Yabe & Nakajima, 2004).

3.9 Versicolorin A (VER A) to demethylsterigmatocystin (DMST) and versicolorin B (VER B) to demethyldihydrosterigmatocystin (DMDHST)

The formation of DMST and the biochemical conversion steps from VERA to DMST (and VerB to DHDHST) have been described in great detail (Henry & Townsend, 2005). The *aflM (ver-1)* gene (Skory et al., 1992), cloned by genetic complementation of VER A-accumulating *A. parasiticus* CS10, was shown to be responsible for the conversion of VER A to an intermediate that has not been isolated. The *aflM (ver-1)* gene was predicted to encode a ketoreductase, similar Nor-1. The *ver-1* homologue, *stcU*, (previously named *verA*) was identified in *A. nidulans* (Keller et al., 1994). Double mutation of *stcU* and *stcL* resulted in accumulation of only VER A (Keller et al., 1994). The *stcS* gene (previously named *verB*), another cytochrome P-450 monooxygenase gene, was also identified and studies showed that it is also involved in the conversion of VER A to an intermediate in the formation of DMST (possibly the first intermediate, which is then acted upon by Ver-1). Disruption of *stcS* resulted in the accumulation of VER A as did disruption of Ver-1 (Keller et al., 1995). Thus, both *stcU* and *stcS* are required for the conversion of VER A to DMST. The *stcS* homologue in *A. parasiticus*, named *aflN (verA)*, has also been identified (Yu et al., 2004a; Yu et al., 2004c). A third enzyme is required for the conversion: *hypA (aflY)*. This gene is predicted to encode a Baeyer-Villiger monooxygenase. Disruption of this gene also led to accumulation of VERA suggesting that, like VER-1, it acts as part of an enzyme complex without allowing the formation of an intermediate. A fourth enzyme, *OrdB* has also been implicated in the conversion, and like *AvfA*, its homolog, may be a helper protein for the monooxygenase, *CypX*.

3.10 Demethylsterigmatocystin (DMST) to sterigmatocystin (ST) and demethyldihydrosterigmatocystin (DMDHST) to dihydrosterigmatocystin (DHST)

Enzyme purification studies revealed that two *O*-methyltransferases, I and II, are involved in aflatoxin biosynthesis (Yabe et al., 1989). *O*-methyltransferase I catalyzes the transfer of

the methyl from S-adenosylmethionine (SAM) to the hydroxyls of DMST and DHDMST to produce ST and DHST, respectively. This 43-kDa enzyme was purified from *A. parasiticus* and characterized (Yabe et al., 1998; Yabe et al., 1999). The corresponding gene, *dmtA*, was isolated from *A. parasiticus* based on a partial amino acid sequence of the purified enzyme (Motomura et al., 1999). Yu et al. (Yu et al., 2000b) concurrently isolated the same gene but named it *aflO* (*omtB*) (for O-methyltransferase B) from *A. parasiticus*, *A. flavus* and *A. sojae*. The predicted *dmtA*-encoded protein contains a consensus SAM-binding motif (Motomura et al., 1999). The *aflO* (*omtB*) homolog in *A. nidulans* was identified as *stcP*. This gene is required for the conversion of DMST to ST in *A. nidulans* as shown by gene disruption (Kelkar et al., 1996).

3.11 Sterigmatocystin (ST) to O-methylsterigmatocystin (OMST) and demethylsterigmatocystin (DMST) to dihydro-O-methylsterigmatocystin (DHOMST)

The gene for O-methyltransferase required for the conversion of ST to OMST and DHST to DHOMST was first cloned (Yu et al., 1993) from *A. parasiticus* by reverse genetics using antibodies raised against the purified *A. parasiticus* O-methyltransferase A (Keller et al., 1993). This gene was initially named *omt-1*, then *omtA* and finally renamed *aflP* (*omtA*) (Yu et al., 1993). The recombinant enzyme was expressed in *E. coli* and its activity to convert ST to OMST was demonstrated by substrate feeding studies (Yu et al., 1993). O-methyltransferase A has strict substrate-specificity and cannot methylate DMST or DHDMST. Thus, the O-methyltransferase A encoded by *aflP* (*omtA*) is the enzyme responsible for the conversion of ST to OMST and DMST to DHOMST. The genomic DNA sequence of this gene (*omtA*) was cloned from *A. parasiticus* and *A. flavus* (Yu et al., 1995b). This *aflP* (*omtA*) gene homologue was also detected in other aflatoxigenic and non-aflatoxigenic *Aspergillus* species (Klich et al., 1995). The absence of the *aflP* orthologue in *A. nidulans* is the reason that *A. nidulans* produces ST as the end product instead of aflatoxins.

3.12 O-methylsterigmatocystin (OMST) to aflatoxin B₁ (AFB₁) and aflatoxin G₁ (AFG₁) and demethyldihydrosterigmatocystin (DMDHST) to aflatoxin B₂ (AFB₂) and aflatoxin G₂ (AFG₂)

The relationship between B-group and G-group aflatoxin formation was proposed based on feeding experiments (Yabe et al., 1988). A P-450 monooxygenase gene in *A. flavus* named *ord-1* was shown to be necessary for this reaction (Prieto et al., 1996; Prieto & Woloshuk, 1997). This P-450 monooxygenase gene, *aflQ* (*ordA*), was cloned in *A. parasiticus* and demonstrated in a yeast system that it is involved in the conversion of OMST to AFB₁/AFG₁, and DHOMST to AFB₂/AFG₂ (Yu et al., 1998). Whether *aflQ* (*ordA*) gene product, OrdA, catalyzes two successive monooxygenase reactions in the later steps of aflatoxin biosynthesis is not clear. Studies (Yu et al., 1998) suggested that additional enzyme(s) is required for the synthesis of G-group aflatoxins. After the cloning and characterization of the *cypA* gene, it is clear that *cypA* encoded a cytochrome P450 monooxygenase for the formation of G-group aflatoxins (Ehrlich et al., 2004). Most recently, the *nadA* gene, which was shown, by gene profiling studies using microarray, to be a member of the aflatoxin gene cluster (Price et al., 2006; Yu et al., 2011) rather than belonging to the adjoining sugar utilization cluster as originally proposed (Yu et al., 2000a), was found to play a role in AFG₁/AFG₂ formation. Yabe's group recently disrupted the *nadA* gene and reported that NadA is a cytosolic enzyme for the conversion from a new aflatoxin intermediate named NADA, which is between OMST and AFG₁, to

AFG₁ (Cai et al., 2008). The *aflE* (*norA*) gene was initially believed to be involved in the conversion of NOR due to certain degree of sequence similarity to the *aflD* (*nor-1*) gene (Cary et al., 1996). However, recent studies support the hypothesis that the *aflE* (*norA*) is involved in the final two steps in AFB₁ formation (Ehrlich, 2009). In the same report, the transcript, *hypB*, a homolog of *hypC*, may be involved in one of the oxidation steps in the conversion of OMST to aflatoxins. *A. flavus* produces only AFB₁ and AFB₂, whereas *A. parasiticus* produces all four major aflatoxins, AFB₁, AFB₂, AFG₁, and AFG₂. Coincidentally, only the G-group aflatoxin producer, *A. parasiticus*, has intact *nadA* and *norB* genes. Preliminary data suggests that *norB* encodes another enzyme predominantly involved in AFG₁/AFG₂ formation (Ehrlich et al., 2008).

4. Regulation of aflatoxin biosynthesis

The aflatoxin pathway genes are found to be clustered in the genome of *A. flavus* and *A. parasiticus* (Yu et al., 1995a; Woloshuk & Prieto, 1998; Yu et al., 2004a; Yu et al., 2004c). These genes are expressed concurrently except for the regulatory gene *aflR*. In this gene cluster, a positive-acting regulatory gene, *aflR*, is located in the middle of the gene cluster. Adjacent to *aflR* a divergently transcribed gene, *aflS* (*aflJ*), was also found to be involved in the regulation of transcription (Meyers et al., 1998; Chang, 2004). Other physically unrelated genes, such as *laeA* and *veA*, also have been shown to exhibit a “global” regulatory role on aflatoxin biosynthesis (Kato et al., 2003; Bok & Keller, 2004; Calvo et al., 2004; Perrin et al., 2007).

4.1 Regulation by *aflR*

The *aflR* gene, encoding a 47 kDa sequence-specific zinc-finger DNA-binding protein is required for transcriptional activation of most, if not all, the structural genes of the aflatoxin gene cluster (Chang et al., 1993; Payne et al., 1993; Woloshuk et al., 1994; Chang et al., 1995b; Yu et al., 1996a; Yu et al., 1996b; Flaherty & Payne, 1997; Ehrlich et al., 1998; Chang et al., 1999a; Chang et al., 1999b). Like other Gal4-type regulatory proteins that bind to palindromic sequences, functional AflR probably binds as a dimer. It binds to the palindromic sequence 5'-TCGN5CGR-3' in the promoter regions of the structural genes (Ehrlich et al., 1999a; Ehrlich et al., 1999b). The AflR-binding motifs are found to be located from -80 to -600 bp, with the majority at the -100 to -200 bp, relative to the translation start site. AflR binds, in some cases, to a deviated sequence rather than the typical motif such as in the case of *aflG* (*avnA*). When there is more than one binding motif, only one of them is the preferred binding site such as in the case of *aflC* (*pksA*) (Ehrlich et al., 1999a; Ehrlich et al., 1999b). The more upstream motif is found to belong to another gene for turning on the expression of *hypC* (Ehrlich, unpublished observation). Deletion of *aflR* in *A. parasiticus* abolishes the expression of other aflatoxin pathway genes (Cary et al., 2000). Overexpression of *aflR* in *A. flavus* up-regulates aflatoxin pathway gene transcription and aflatoxin accumulation (Flaherty & Payne, 1997) in a fashion similar to that reported for *A. parasiticus* (Chang et al., 1995b). These results demonstrate that AflR is specifically involved in the regulation of aflatoxin biosynthesis. Indeed, all 23 upregulated genes, identified by transcription profiling using DNA microarray assays comparing wild-type and *aflR*-deleted *A. parasiticus* strains, have the consensus AflR binding motif in their promoter regions (Meyers et al., 1998; Price et al., 2006; Wilkinson et al., 2007a; Wilkinson et al., 2007b).

4.2 Regulation by *aflS* (*aflJ*)

The *aflS* (*aflJ*) gene, although not demonstrating significant homology with any other encoded proteins found in databases, is necessary for aflatoxin formation. In the *A. parasiticus* *aflR* transformants, the production of aflatoxin pathway intermediates was significantly enhanced in transformants that contained an additional *aflR* plus *aflS* (Chang et al., 1995b). Quantitative PCR showed that in the *aflS* knockout mutants, the lack of *aflS* transcript is associated with 5- to 20-fold reduction of expression of some aflatoxin pathway genes such as *aflC* (*pksA*), *aflD* (*nor-1*), *aflM* (*ver-1*), and *aflP* (*omtA*). The mutants lost the ability to synthesize aflatoxin intermediates and no aflatoxins were produced (Meyers et al., 1998). However, deletion of *aflS* (*aflJ*) did not have a discernible effect on *aflR* transcription, and vice versa. Du et al. (Du et al., 2007) showed that overexpression of *A. flavus* *aflS* (*aflJ*) did not result in elevated transcription of *aflM* (*ver-1*), *aflP* (*omtA*), or *aflR*, but it appears to have some effect on *aflC* (*pksA*), *aflD* (*nor-1*), *aflA* (*fas-1*), and *aflB* (*fas-2*) (Du et al., 2007), which are required for the biosynthesis of the early aflatoxin pathway intermediate, averantin. The mechanism(s) by which *aflS* modulates transcription of these pathway genes in concert with *aflR* is under investigation by gene profiling analysis using microarray technology.

4.3 Regulation by *laeA*

The novel global regulatory gene, *laeA* (for lack of *aflR* expression), was first identified from *A. nidulans* (Bok & Keller, 2004). This gene is well conserved in fungi as shown by its presence in the genomes of all fungi so far sequenced. LaeA is a nuclear protein which contains an S-adenosylmethionine (SAM) binding motif and activates transcription of several other secondary metabolism gene clusters in addition to the AF cluster. Examples include the sterigmatocystin and penicillin clusters in *A. nidulans*, the gliotoxin cluster in *A. fumigatus*, and aflatoxin cluster in *A. flavus* (Bok & Keller, 2004; Bouhired et al., 2007). It also regulates genes required for virulence of *A. fumigatus* (Sugui et al., 2007). Perrin et al. (Perrin et al., 2007) carried out a whole-genome comparison of the transcriptional profiles of wild-type and *laeA*-deleted *A. fumigatus* strains and found that LaeA positively controls the expression of 20% to 40% of major classes of secondary metabolite biosynthesis genes. It also regulates some genes not associated with secondary metabolite clusters. Similar results were confirmed in gene expression profiling in *A. flavus* using microarrays to study the genetic mechanism of sclerotia formation (Yu, personal communication). The exact mechanism of how LaeA regulates secondary metabolism gene clusters is not yet known. Interestingly, when an unrelated gene such as *argB* was placed within the boundary of the ST gene cluster, it was co-regulated with other genes in the cluster. But, when a gene in the cluster, such as *aflR* was placed elsewhere in the genome, its regulation was not affected by LaeA (Bok et al., 2006). One proposed regulatory mechanism is that LaeA differentially methylates histone protein and it alters the chromatin structure for gene expression. Unlike the mentioned signaling factors, the primary role of LaeA is to regulate metabolic gene clusters, not sporulation, because *laeA*-deleted strains produced wild-type levels of conidia (Bok & Keller, 2004). Most recent analyses of nonaflatoxigenic *A. parasiticus* *sec*- (for secondary metabolism negative) variants generated through serial transfer of mycelia of the *sec*+ parents show that *laeA* was expressed in both *sec*+ and *sec*- strains (Kale et al., 2007). This result suggests that LaeA only exerts its effect on aflatoxin biosynthesis at a certain level and is independent of other regulatory pathways that are involved in fungal development.

4.4 Regulation by *veA*

The *veA* gene in *A. nidulans* (Mooney & Yager, 1990) is a gene initially found to be crucial for light-dependent conidiation. The light dependence is abolished by a mutation (*veA1*) which allows conidiation of *A. nidulans* to occur in the dark. A comparison of the light effect on sterigmatocystin production by *A. nidulans veA+* and *veA1* strains showed that both strains produced sterigmatocystin but the highest amount was produced by the *veA+* strain grown in darkness. However, *veA*-deleted *A. flavus* and *A. parasiticus* strains completely lost the ability to produce aflatoxin regardless of the illumination conditions (Duran et al., 2007; Stinnett et al., 2007). Under normal growth conditions, some *A. flavus* and all *A. parasiticus* strains produce conidia in both dark and light conditions. Stinnett et al. (Stinnett et al., 2007) showed that VeA contains a bipartite nuclear localization signal (NLS) motif and its migration to the nucleus is light-dependent and requires the importin α carrier protein. In the dark VeA is located mainly in the nucleus; under light it is located both in cytoplasm and nucleus. VeA has no recognizable DNA-binding sequences and likely exerts its effect on sterigmatocystin and aflatoxin production through protein-protein interactions with other regulatory factors. Post-translational modifications such as phosphorylation and dephosphorylation may modulate its activity. Lack of VeA production in the *veA*-deleted *A. flavus* and *A. parasiticus* strains consequently abolishes aflatoxin production because a threshold concentration of nuclear VeA might be necessary to initiate aflatoxin biosynthesis.

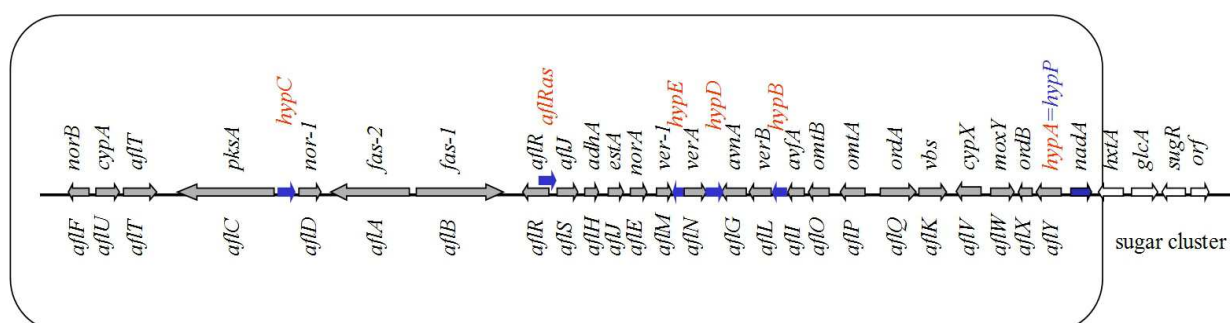


Fig. 1. Aflatoxin pathway gene cluster in *A. flavus*. This figure shows the order and location of the 30 aflatoxin pathway genes plus an *aflR* antisense gene clustered together in about 80 kb DNA region. The old gene names are labeled on top of the line and the new gene names systematically renamed according to gene convention are labeled below the line (Yu et al., 2004c). The transcripts of *hypA*, *hypB*, *hypC*, *hypD*, *hypE* and *aflRas* are identified through *Aspergillus flavus* EST. Arrows indicate the direction of gene transcription.

5. Conclusions

Aflatoxins are toxic and carcinogenic secondary metabolites produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* that contaminate preharvest crops and post harvest grains. Scientists worldwide have extensively studied biosynthesis of aflatoxins for more than 50 years. Aflatoxin biosynthesis is a complex process involving many intermediates and enzymes. Regulation of aflatoxin gene expression occurs at multiple levels and by multiple regulatory components. There are genetic factors, biotic and abiotic elements that affect aflatoxin formation. Recent studies have shed more light on the functions of the enzymes involved in each of the steps of aflatoxin biosynthesis, the genes encoding those enzymes,

and the regulatory mechanisms of aflatoxin formation. Better understanding of the mechanisms of aflatoxin biosynthesis helps to identify natural inhibitors of fungal growth aflatoxin formation, and eventually will allow design of effective strategies to can reduce or eliminate aflatoxin contamination of food and feed commodities.

6. References

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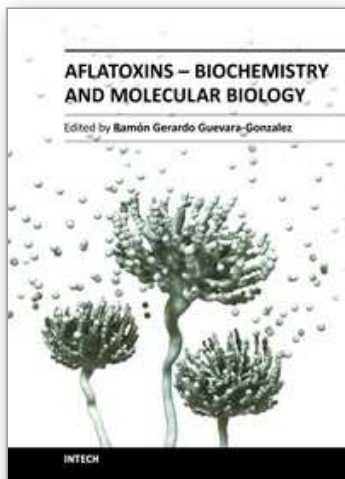
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Aflatoxins – Biochemistry and Molecular Biology is a book that has been thought to present the most significant advances in these disciplines focused on the knowledge of such toxins. All authors, who supported the excellent work showed in every chapter of this book, are placed at the frontier of knowledge on this subject, thus, this book will be obligated reference to issue upon its publication. Finally, this book has been published in an attempt to present a written forum for researchers and teachers interested in the subject, having a current picture in this field of research about these interesting and intriguing toxins.

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