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REVIEW

Genetic and Molecular Analysis of Aflatoxin Biosynthesis

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Index Descriptors: sterigmatocystin; secondary metabolism; polyketide biosynthesis; *Aspergillus flavus; Aspergillus parasiticus; Aspergillus nidulans;* mycotoxins; Zn(II)2cys6 binuclear cluster DNA binding motif.

The aflatoxin biosynthetic pathway represents one of the best studied pathways of fungal secondary metabolism. Its elucidation is the result of over 30 years of study by scientists in many disciplines. For recent reviews on the chemistry of the pathway see articles by Bhatnagar *et al.* (1992) Minto and Townsend (1997), and Woloshuk and Prieto (1997). Concern over the toxicity and carcinogenicity of aflatoxin has been the prime force driving research in this area. Aflatoxin B1 (AFB1) is the most potent naturally occurring carcinogen known (Squire, 1989), and epidemiological data implicate aflatoxin as a component of liver cancer in humans in certain parts of the world (Hall and Wild, 1994). Although aflatoxins are not extremely toxic, consumption of aflatoxin contaminated food by animals can lead to decreased weight gain, hemorrhaging, and suppression of the immune system (Miller and Wilson, 1994).

Aflatoxins are produced by four species of *Aspergillus: A. flavus, A. parasiticus, A. nomius,* and *A. tamarii* (Payne, 1998; Kurtzman *et al.,* 1986; Goto *et al.,* 1996). From an agricultural standpoint, the two most significant fungi are *A. flavus* and *A. parasiticus.* These two fungi have only limited parasitic abilities, yet they can successfully colonize and produce aflatoxin in developing host tissue in the field. *A. flavus* is the more aggressive and by far the more predominant of the two species on corn, peanuts, cottonseed, and tree nuts (Payne, 1992, 1998). Although aflatoxin contamination can be a problem in storage, preharvest contamination is the major concern.

Certainly one goal for studying aflatoxin biosynthesis is to obtain information needed to devise novel control strategies to prevent aflatoxin contamination of food. Fortunately, these studies also have led to a better understanding of fungal secondary metabolism. The well-characterized biosynthetic scheme for aflatoxin biosynthesis (Fig. 1) and genetically characterized strains of the fungus have been important tools for understanding the molecular biology of the pathway. Recent studies with *A. nidulans* have aided our understanding of aflatoxin biosynthesis and the link between secondary metabolism and development. *A. nidulans* does not produce aflatoxin, but it and several other fungal species produce the penultimate compound, sterigmatocystin (Cole and Cox, 1981; Barnes *et al.,* 1994). Current evidence shows that the sterigmatocystin pathway (ST) in *A. nidulans* and the aflatoxin pathway (AF) share a common biosynthetic scheme (Brown *et al.,* 1996a; Keller and Hohn, 1997). Studies on both of these pathways have allowed us to elucidate the complexity of the biosynthesis of these related polyketides. Therefore, the term AF/ST will be used to represent our knowledge of both these pathways.

The object of this review is to cover recent data on the genetics and molecular biology of AF/ST biosynthesis. To put the research in perspective, however, the review also

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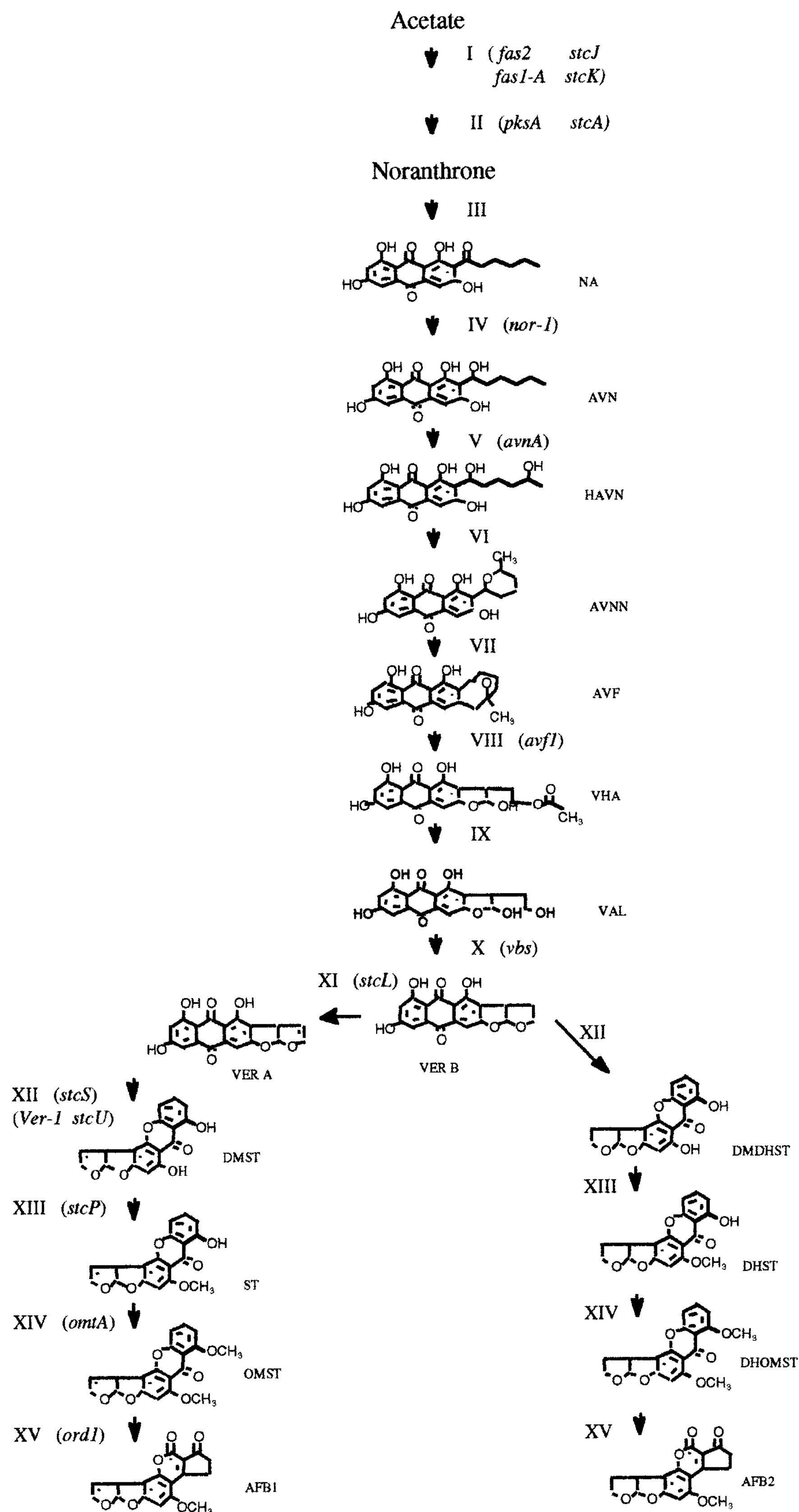

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will cover some of the early studies on the genetics of *A. flavus* and *A. parasiticus.* Studies with these species provided the first information on the pathway, and they likely will become more important as we begin to understand the fine regulation of aflatoxin biosynthesis. Although *A. nidulans* provides a powerful genetic system to study the AF/ST pathway, there is some evidence that the two pathways may be regulated differently (Feng and Leonard, 1998). Fine details on the regulation of aflatoxin biosynthesis and its possible role in the ecology of *A. flavus* and *A. parasiticus* will require molecular and classical genetic analysis of these fungi.

I. MAPPING OF THE AFLATOXIN CLUSTER

A. Classical Genetic Analysis

Aspergillus flavus and *A. parasiticus* are anamorphic fungi with no known sexual stage. This, along with the presence of multinucleate conidia, has hampered genetic analyses within these fungi. Despite these drawbacks, significant information has been derived from genetic analyses of these organisms. Both *A. flavus* (Papa, 1973) and *A. parasiticus* (Papa, 1978; Bennett *et al.,* 1980a) have a well characterized parasexual cycle, and a prodigious amount of research has led to the mapping of several genes in these fungi (for reviews see Bennett and Papa, 1988; Keller *et al.,* 1992a). Bennett and Papa (1988) defined three types of mutants in *A. parasiticus:* (1) anthraquinone-accumulating mutants blocked in the aflatoxin pathway; (2) spore color and auxotrophic mutants used as markers in elucidating the parasexual cycle and generating linkage maps; and (3) two classes of morphological mutants named fan and fluff with either attenuated or complete loss of aflatoxin biosynthesis. The latter class of mutants will be discussed later in the section on regulation. Probably the most important contribution of the *A. parasiticus* genetic studies was the development of the anthraquinone-accumulating mutants. Mutants in *A. parasiticus* that accumulate norsolorinic acid (NA), averantin (AVN), averufin (AVF), hydroxyversicolorone (HVN), and versicolorin A (VERA) have been used in elucidating steps in the aflatoxin biosynthetic pathway (Bennett and Christensen, 1983; Bennett and Papa, 1988; Bennett *et al.,* 1980b; Bhatnager *et al.,* 1992; Billington and Hsieh, 1989; Steyn, 1980; Townsend *et al.,* 1988). Further, two of these mutants, *nor-1* and *ver-1,* were used to clone aflatoxin biosynthetic genes by genetic complementation (Chang *et al.,* 1992; Skory *et al.,* 1992).

The formal genetics of *A. flavus* is more developed than that of *A. parasiticus* and over 36 genes have been mapped to eight linkage groups. Papa (1976) generated 23 strains with auxotrophic and conidial color mutations and 23 nonallelic aflatoxin biosynthesis variants (Papa, 1979, 1984). Ten aflatoxin nonproducing mutations map to linkage group (LG) VII: *afl-1, afl-15, afl-16, afl-17, afl-19, afl-20/afl-22, afl-21, afl-25, nor-1,* and *aflR*(*afl-2*) (Papa, 1984). Two genes, *aflR* (Payne *et al.,* 1993; Woloshuk *et al.,* 1994) and *nor-1* were isolated from *A. flavus* by genetic complementation of the respective mutants. Two other mutations, *afl-4* and *aflB2* map to LG II and VIII, respectively. All of the mutations reported by Papa are recessive except for *afl-1* (Papa, 1980). Woloshuk *et al.* (1995) characterized this mutation in strain 649 as a deletion of over 120 kb, including the AF cluster, and proposed a *trans*-sensing mechanism for the dominant nature of this mutation. Although parasexual analysis is laborious, it has been an important method for characterizing mutants of *A. flavus* and is still being used to determine gene action (Woloshuk *et al.,* 1995) and to move selectable markers among strains (Payne *et al.,* 1993).

Because *A. nidulans* has a well-developed classical and molecular genetics system, Wunch and Bennett (1992) proposed that *A. nidulans* may be a useful model system to study the biosynthesis of aflatoxin. Based on findings of Hajjar *et al.* (1989), who showed that the Glasglow strain of *A. nidulans* produced ST, Wunch and Bennett (1992) mutagenized *A. nidulans* and looked for anthraquinone-accumulating mutants. They were able to isolate an averufin-accumulating strain, KW38, which produced no

FIG. 1. Proposed biosynthetic pathway for aflatoxin B1 and B2. Intermediates include: norsolorinic acid (NA), averantin (AVN), 5′hydroxyaverantin (HAVN), averufanin (AVNN), averufin (AVF), versiconal hemiacetal acetate (VHA), versiconal (VAL), versicolorin B (VERB), and versicolorin A (VERA). The aflatoxin B1 (AFB1) pathway includes demethylsterigmatocystin (DMST), sterigmatocystin (ST), and *O*-methylsterigmatocystin (OMST). The aflatoxin B2 (AFB2) branch includes demethyldihydrosterigmatocystin (DMDHST), dihydrosterigmatocystin (DHST), dihydrosterigmatocystin (DHST), and dihydro-*O*-methylsterigmatocystin (DHOMST).

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detectable levels of ST. Averufin (AVF) production by this strain was found to be comparable to, but less than that of an AVF-accumulating strain of *A. parasiticus.* The use of *A. nidulans* developmental mutants to study aflatoxin biosynthesis will be discussed later.

B. Pulsed-Field Gel Electrophoresis of the Chromosomes with the Aflatoxin Pathway Genes

Electrophoretic karyotypes have been established for both *A. flavus* and *A. parasiticus* (Foutz *et al.,* 1995; Keller *et al.,* 1992b). Keller *et al.* (1992b) were able to resolve 5–8 chromosomes in *A. flavus* and 5–7 chromosomes in *A. parasiticus.* With the exception of small chromosomes in some isolates, the chromosomes of the two fungi ranged in size from 3.0 to approximately 7.0 Mb. The respective sizes of the chromosomes separated from *A. flavus* strain SRRC 285 were 7.0, 6.5, 5.0, 4.8, 4.3, 4.0, 3.1, and <1.5 Mb (Fig. 2). These sizes are very similar to those found by Foutz *et al.* (1995) for *A. flavus* strain 656-2 (Fig. 2). Both Keller *et al.* (1992b) and Foutz *et al.* (1995) assigned genetic markers to chromosomes in *Aspergillus* species. Keller *et al.* (1992b) assigned markers to five chromosomes each in

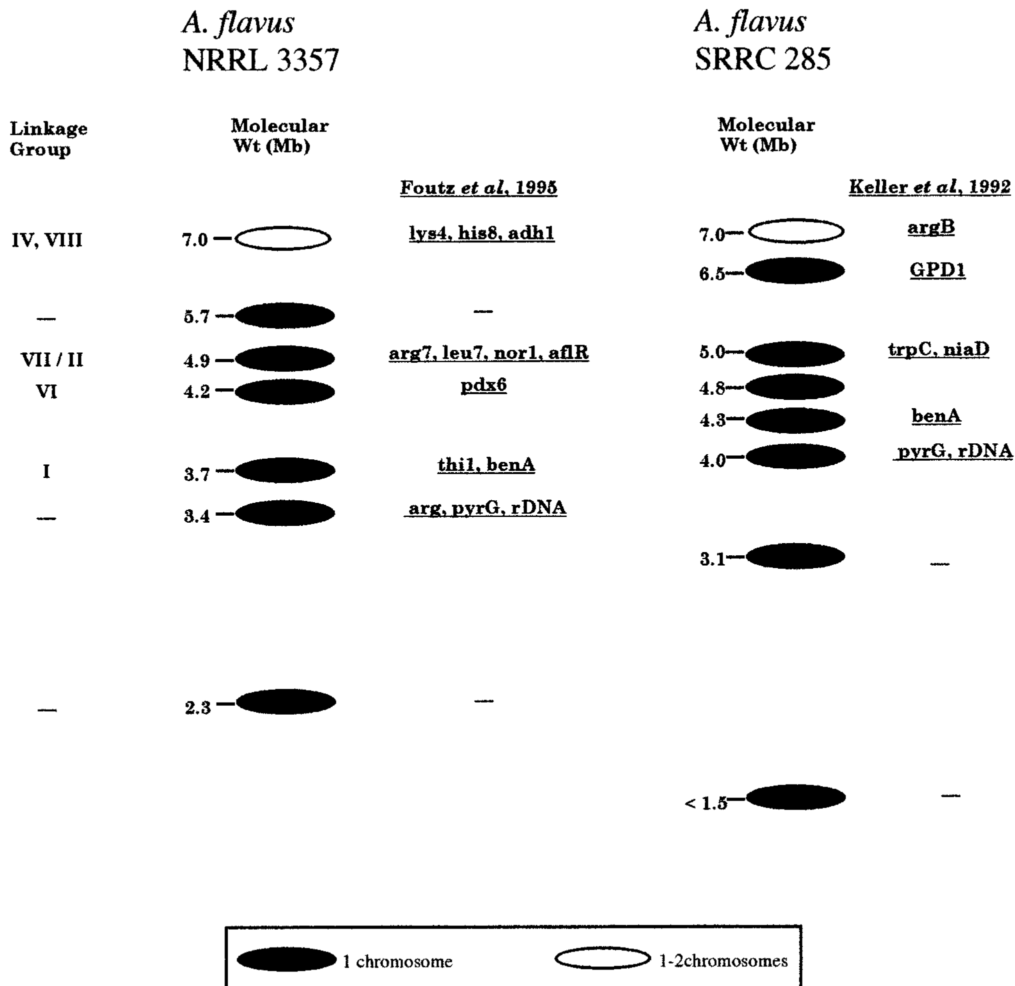

FIG. 2. Diagram of karyotypes of two *A. flavus* strains comparing chromosome sizes determined by CHEF separation. The chromosomal location of linkage group makers (Bold) and unmapped genes (underlined) is presented. The data for strain NRRL 3357 is modified from Foutz *et al.* (1995), while the data from strain SRRC 285 is modified from Keller *et al.* (1992). The unmapped genes used for localization include the *A. nidulans argB* and rDNA genes, the *A. flavus benA*, and *adh1* genes, the *A. parasiticus trpC, niaD,* and *pyrG* genes, and the *Cochliobolus heterostrohus GPD1* gene. The linkage group specific markers for *A. flavus* include *thi1, arg2, lys4, pdx6, arg7, leu7,* and *his8.* The aflatoxin biosynthetic cluster-specific *nor-1* and *aflR* genes were also used and were previously mapped to linkage group VII.

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A. flavus and *A. parasiticus.* Foutz *et al.* (1995) used linkage group-specific probes to assign six linkage groups to five chromosomes in *A. flavus.* The aflatoxin biosynthetic genes, *nor-1* and *aflR,* hybridized to the 4.9 Mb chromosome along with two LG VII markers identified by K. E. Papa. Thus, aflatoxin biosynthetic genes in *A. flavus* reside on a 4.9-Mb chromosome and are clustered within LG VII. The 7.0-Mb chromosome of *A. flavus* hybridizes with genes mapped to both LG IV and LG VIII, suggesting that the 7.0-Mb band is composed of two chromosomes. Assuming that the 7.0-Mb band is two chromosomes, the estimated genome size for *A. flavus* is 36 Mb (Foutz *et al.,* 1995; Keller *et al.,* 1992b).

II. MOLECULAR ELUCIDATION OF THE AFLATOXIN PATHWAY

A. Cloning Strategies

A variety of cloning strategies was employed to identify and clone genes from the AF/ST pathways. These include: genetic complementation, DNA sequence homology, reverse genetics, and physical and transcript mapping. Other genes not described in this section were obtained by a combination of these strategies. The cloning of genes catalyzing 12 enzymatic conversions required for AF biosynthesis has provided insights into the chemistry of this complex pathway and made this one of the best characterized secondary metabolic pathways.

1. Genetic complementation. The first two genes characterized from the pathway were identified by genetic complementation of well-characterized *A. parasiticus* mutants blocked in aflatoxin production (Table 1). The *nor-1* gene (Chang *et al.,* 1992) was identified by complementation of the norsolorinic acid (NA) accumulating *A. parasiticus* mutant B62 (Table 1). The *ver-1* gene (Skory *et al.,* 1992) was likewise identified by complementation of the VERA accumulating strain CS10. The roles of these genes in AF biosynthesis were subsequently confirmed by recombinational inactivation (*nor-1,* Trail *et al.,* 1994; *ver-1,* Liang and Linz, 1994).

Genetic complementation of the loss of function mutation in *A. flavus* strain 650-33 led to the identification of the pathway-specific transcriptional regulator for the AF biosynthetic pathway (Payne *et al.,* 1993; Woloshuk *et al.,*

TABLE 1
Aspergillus sp. Strains Blocked in Aflatoxin/Sterigmatocystin Biosynthesis

Aspergillus species	Strain	Metabolite accumulated	Gene mutated	Reference
A.p.	ATCC 24690	NA	*nor-1*	Watanabe *et al.,* 1996
	B62 (niaD)	NA	*nor-1*	Chang *et al.,* 1992
A.f.	ATCC 60041	NA		Papa, 1982
A.p.	ATCC 56774	AVN	*avnA*	Yu *et al.,* 1997
	NIAH-204	AVN		Yabe *et al.,* 1988
A.p.	ATCC 24551	AVF	*avf-1*	Bhatnagar *et al.,* 1992
A.n.	KW38	AVF		Wunch *et al.,* 1992
A.p.	ATCC 36537	VERA	*ver-1*	Skory *et al.,* 1992
	CS10 (pyrG)	VERA	*ver-1*	Skory *et al.,* 1992
	NIAH-9	VERA		Yabe *et al.,* 1988
A.p.	WE-47	HVN	*hvn-1*	Townsend *et al.,* 1988
A.p.	SRRC 2043	OMST	*ord-1*	Bhatnagar *et al.,* 1992
A.f.	SRRC 141	AFB2	*stcL?*	Papa, 1977
A.f.	FGSC A1010	None	*aflR*	Flaherty and Payne, 1997
	649	None	Deletion	Papa, 1980
A.p.	LY3–4	None	Deletion	Yu and Leonard, 1995
	UVM8	None	*fas-1A, nor-1*	Mahanti *et al.,* 1996
	NIAH-26	None		Yabe *et al.,* 1988
	NIAH-1	None		Yabe *et al.,* 1988

Note. Aspergillus species include *A. parasiticus* (A.p); *A. flavus* (A.f.); and *A. nidulans* (A.n.). The deletion in strain 649 encompasses the aflatoxin biosynthetic cluster and is estimated to be greater than 120 kb. The deletion in strain LY3–4 is estimated to be approximately 55 kb in size and include several cluster genes.

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1994). Chang *et al.* (1993) cloned a gene from *A. parasiticus, apa-2,* that when transformed into *A. parasiticus* toxin producing strains resulted in overproduction of pathway intermediates. This gene is 95% identical to the *afl-2* gene (Chang *et al.,* 1993) and complements the *afl-2* mutation in strain 650-33 (Woloshuk *et al.,* 1994). This led to the renaming of these two genes to *aflR,* to reflect the regulatory nature of these two functional homologues. When a similar gene was cloned from the *A. nidulans* ST cluster, it too retained the *aflR* designation (Yu *et al.,* 1996).

The availability of an *A. flavus* strain with an AF cluster deletion led to a novel strategy for identifying and cloning pathway genes using genetic complementation. Strain 649 contains such a deletion, and transformation of this strain with the overlapping cosmids 5E6, 8B9 or 20B11, and 13B9 restores AF production (Prieto *et al.,* 1996). Transformants of strain 649 containing cosmids 5E6 and 20B11 accumulated AVNN, while transformants containing cosmids 5E6 and 8B9 accumulated AVF. In this way, the *avf-1* gene required for the conversion of AVF to VHA was localized to the 13B9 cosmid. The *avf-1* gene was subsequently subcloned to a 7-kb fragment of the 13B9 cosmid. This work, in conjunction with the characterization of *vbs* (Silva *et al.,* 1996a), expanded the predicted size of the AF cluster to greater than 75-kb. Transformants of 649 containing the 8B9 cosmid are capable of converting OMST to AF, indicating that the oxidoreductase gene required for this conversion was localized to the 8B9 cosmid. The *ord-1* gene was subsequently subcloned to a 3.3-kb 8B9 fragment (Prieto *et al.,* 1996) and characterized as a novel P-450 monooxygenase (Prieto *et al.,* 1997).

2. Sequence homology. Several genes in the pathway have been cloned from cDNA or genomic DNA libraries using probes based on conserved regions of the candidate genes. The *verA* gene from *A. nidulans* was cloned from a genomic cosmid library in this manner, using as a probe DNA from the *ver-1* gene of *A. parasiticus* (Keller *et al.,* 1994). The *verA* gene was subsequently renamed *stcU* upon its localization within the ST cluster (Brown *et al.,* 1996b). The *pksL1* gene from *A. parasiticus* was identified using primers for conserved regions of the ACP and KS domains of polyketide synthases (Feng and Leonard, 1995). The gene was identified from a group of 19 clones that were positively correlated with aflatoxin biosynthesis by differential screening (Feng *et al.,* 1992). A similar technique was also used to clone the *stcJ* and *stcK* FAS subunits from *A. nidulans* (Brown *et al.,* 1996b).

3. Reverse genetics. The *omtA* and *vbs* genes from *A. parasiticus* were cloned by reverse genetics. Polyclonal antibodies made to the *O*-methyl transferase enzyme were used to isolate the *omtA* gene from a cDNA expression library constructed from RNA isolated from aflatoxin producing mycelium (Yu *et al.,* 1993). The *vbs* gene was cloned using probes made from nondegenerate primers derived from amino acid sequence obtained from peptide fragments of the purified enzyme (Silva *et al.,* 1996a). At least two other pathway enzymes have been purified to homogeneity, the NA reductase (*nor-1;* Bhatnagar and Cleveland, 1990; Chuturgoon and Dutton, 1991), and two versiconal hemiacetal acetate (VHA) reductase genes (Matsushima *et al.,* 1994). The successful cloning of *omtA* and *vbs* using strategies based on enzyme purification, suggests that a similar approach for identifying the VHA reductase genes should be successful. It should be noted however, that Brown *et al.* (1996a) proposed *stcI* as the pathway VHA reductase gene based on the presence of esterase motifs in the predicted gene product. Gene disruption experiments on this gene are ongoing.

4. Physical and transcript mapping. Once the AF and ST pathways were localized to gene clusters (Payne *et al.,* 1995; Brown *et al.,* 1996a), a new strategy for cloning was developed. Regions adjacent to known pathway genes were sequenced and transcriptionally mapped. Putative open reading frames were characterized as to gene function based on the analysis of the predicted primary amino acid sequence. These regions were disrupted, and the identification of accumulating metabolites determined. In this manner *pksA, fas-1A, avnA,* and *aflJ* were confirmed to be essential for AF biosynthesis and *stcA, stcL, stcS,* and *stcP* for ST production in *A. nidulans.* This strategy was greatly aided by the physical and transcriptional mapping of the AF and ST clusters (Trail *et al.,* 1995a,b; Yu *et al.,* 1995; and Brown *et al.,* 1996a).

B. Aflatoxin Biosynthetic Pathway

It is estimated that at least 19 gene products are required for aflatoxin biosynthesis. Since 1992, 17 genes catalyzing 12 enzymatic steps in the pathway have been cloned and characterized. Additionally, the *aflR* and *aflJ* genes are known to be required. Each of these genes and the conversions they catalyze are reviewed.

1. Acetate to noranthrone (Fig. 1 (I, II)). Fatty acid synthases (FASs) and polyketide synthases (PKSs) are enzymes that contain similar catalytic domains catalyzing condensation reactions, but produce structurally dissimilar molecules. Both have been demonstrated to be involved in AF/ST biosynthesis in *Aspergillus* species. FASs incorporate acetate starter units with malonate extender units by

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successive decarboxylative Claisen condensation reactions (Watanabe *et al.,* 1996). The β-keto group formed is fully reduced to an alkyl group by keto reduction, dehydration, and enoyl reduction prior to subsequent condensation reactions. All fungal FASs characterized to date contain an α-subunit with acyl carrier protein (ACP), keto reductase (KR), and ketosynthase (KS) domains, and a β-subunit with acetyltransferase (AT), enoyl reductase (ER), dehydratase (DH), and malonyl/palmityl transferase (MPT) domains (Fig. 3A). Polyketide synthases (PKSs) can use primers other than acetate and can catalyze extensions with malonate and a variety of α-alkylated derivatives. All fungal PKSs contain the KS, AT, and ACP domains required for polyketide chain extension (Fig. 3B). However, only PKS1 from *Cochliobolus heterostrophus* contains all the domains required for complete processing of β-carbons (Fig. 3B) (Yang *et al.,* 1996). Incomplete processing of β-carbons contributes to the complexity and diversity of fungal polyketides.

(a) Acetate to hexanoic acid: fatty acid synthase (Fig. 1 (I)). The first stable intermediate in the biosynthesis of the polyketide aflatoxin is the decaketide norsolorinic acid (NA) (Dutton, 1988). Norsolorinic acid was originally thought to be produced by reiterative condensation of an acetyl CoA starter unit and nine malonyl-CoA extender units catalyzed by a polyketide synthase (PKS) via 10 condensation and two reduction cycles (Bhatnagar *et al.,* 1992). This hypothesis visualized the aflatoxin PKS as analogous to an FAS and may have been influenced by the knowledge that some fungal polyketide synthases, such as the 6-methylsalicylic acid synthase required for the biosynthesis of the antibiotic tetraketide patulin by *Penicillium patulum* (Beck *et al.,* 1990), catalyze all the initial reactions in the biosynthetic pathway. Since all fungal PKSs characterized to date are type I multifunctional enzymes, conservation of the previously described polyketide biosynthetic scheme was hypothesized. However, Townsend *et al.* (1984) identified an aliphatic moiety adjacent to a keto group in the tail of NA (Fig. 4) and proposed the production of a 6-carbon hexanoyl-CoA starter unit by an FAS. This starter unit would theoretically be extended by a PKS to generate the decaketide noranthrone (Fig. 4). This hypothesis is supported by the observation that exogenously supplied labeled C6 hexanoic acid can be incorporated intact into pathway intermediates (Brobst and Townsend, 1994).

Further evidence for the role of a novel short-chained fatty acid starter unit in aflatoxin biosynthesis came from the work of Mahanti *et al.* (1996). These workers were able

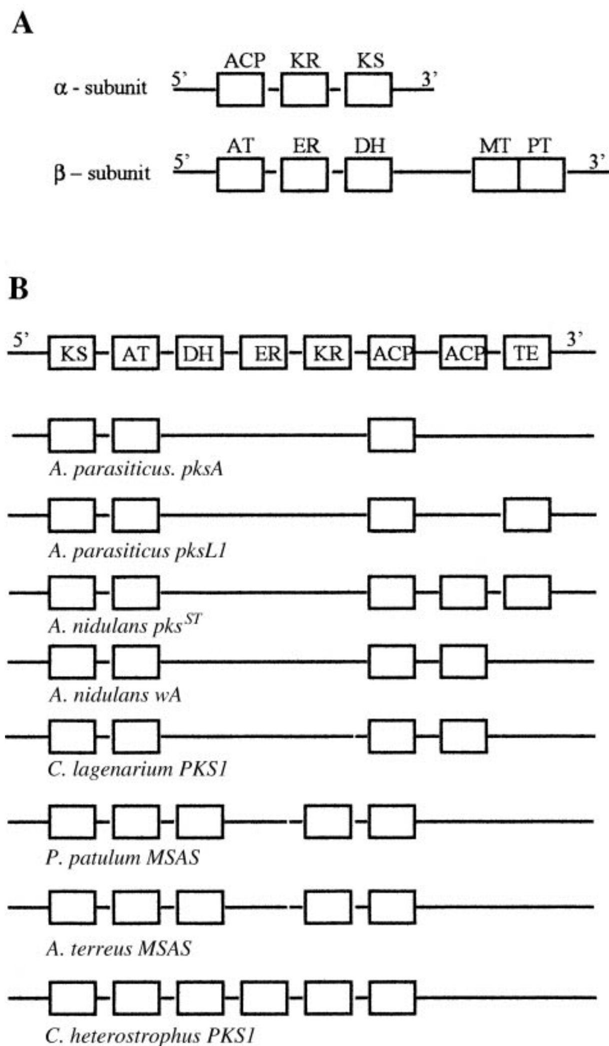

FIG. 3. Organization of fungal fatty acid synthase (FAS) and polyketide (PKS) genes. (A) The colinear order of domains comprising the FAS α and β subunits found conserved in all characterized filamentous fungi. These domains include: acyl carrier protein (ACP), keto reductase (KR), Keto synthase (KS), acetyltransferase (AT), enoyl reductase (ER), dehydratase (DH), and malonyl/palmityl transferases (MT/PT). (B) The order of domains in fungal PKS genes. The top diagram represents a theoretical PKS containing all described PKS domains, including the thioesterase (TE) domain. The remaining diagrams represent the order of domains for published PKSs. Polyketide chain extension requires the KS, AT, and ACP domains, while complete processing of β-carbons requires the DH, ER, and KR domains. (B) Modified from Yang *et al.* (1996).

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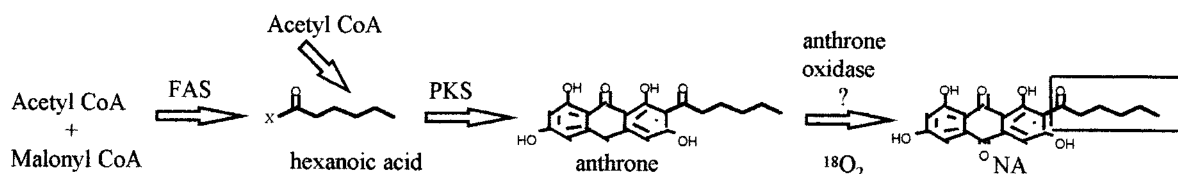

FIG. 4. Biosynthesis of the first stable intermediate in the aflatoxin pathway, norsolorinic acid (NA). It has been demonstrated that an FAS is required for biosynthesis of the hexanoyl starter unit, which is then extended with malonlyl units by a PKS to produce noranthrone. Biosynthesis of NA has been proposed via oxidation either by a putative noranthrone oxidase or by a nonenzymatic process.

to demonstrate the requirement for a putative FAS in aflatoxin biosynthesis at a point prior to the synthesis of NA. The putative FAS β-subunit, *fas-1A,* was cloned and shown to restore NA biosynthesis in *Aspergillus parasiticus* strain UVM8, which is blocked in both *fas-1A* and *nor-1* function. Further, gene disruption of *fas-1A* in the NA accumulating strain B62, resulted in loss of NA biosynthesis. Two extensive regions of *fas-1A* demonstrated significant levels of predicted amino acid sequence similarity to the enoyl reductase and the malonyl/palmityl transferase domains of the FAS β-subunit encoded by FAS1 of *Saccharomyces cerevisiae* (Mahanti *et al.,* 1996). The *fas-1A* gene had previously been shown to reside in the aflatoxin biosynthesis cluster (Yu *et al.,* 1995), and encode a 7.5-kb transcript (Trail *et al.,* 1995b).

Biochemical evidence for the physical association of type I FAS and PKS in the biosynthesis of aflatoxin was provided by Watanabe *et al.* (1996) who demonstrated that the *N*-acetylcysteamine thioester of hexanoic acid could be incorporated into NA biosynthesis in *fas-1* disruptants. The low rate of incorporation was suggested to be due to inefficient transfer of the hexanoate starter unit from the mutated FAS to the functional PKS.

The role of an FAS in ST biosynthesis was confirmed by Brown *et al.* (1996b) who demonstrated the presence of two distinctive FAS complexes in *A. nidulans,* one for primary fatty acid metabolism and another required for metabolism of the secondary polyketide ST. Four genes were identified by PCR amplification of conserved FAS α and β subunit domains, and subsequent screening of an *A. nidulans* cosmid library. Two of these genes, *stcJ* and *stcK,* have been shown to reside in the ST cluster and encode polypeptides with homologies to fatty acid synthases (Brown *et al.,* 1996a). Transcripts for these two genes have been shown to be induced under ST producing conditions and follow the ST biosynthetic profile (Brown *et al.,* 1996a). Disruption of *stcJ* or *stcK* results in morphologically normal strains capable of growth without the addition of long chain fatty acids, but deficient in ST biosynthesis. Biosynthesis of ST is restored in these strains by the addition of hexanoic acid, although at a 20-fold reduction in quantity. Disruption of the *fasA* or *fasB* genes results in strains requiring the addition of long chain fatty acids for growth. When grown under permissive conditions, these strains are capable of ST production. The incorporation of acetate into NA in *Aspergillus* species utilizes a novel biosynthetic pathway whereby an FAS derived short chain fatty acid primes a Type I multienzyme PKS. This may not, however, be unique as a FAS with significant identities to FAS1 is required for production of HC toxin by *Cochliobolus carbonum* (Ahn and Walton, 1997; Nikolskaya *et al.,* 1995).

(b) Hexanyl CoA to noranthrone: Polyketide synthase (Fig. 1 (II)). In type I PKSs, the condensation reaction is catalyzed by a single multienzyme polypeptide. The first fungal PKS described was 6-MSAS, which catalyzes the biosynthesis of 6-methylsalicylic acid from one acetyl CoA and three malonyl CoAs in *Penicillium patulum* (Beck *et al.,* 1990). The next PKS characterized was the wA gene from *A. nidulans* that is required for synthesis of an uncharacterized green pigment present in the walls of mature conidia (Mayorga and Timberlake, 1992). The *wA* gene contains conserved motifs for β-ketoacyl synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) (Fig. 3B). Unlike the previously characterized MSASs from *Penicillium* species, *wA* does not contain a β-ketoacyl reductase (KR) domain, suggesting that the starter unit for conidial pigment biosynthesis is extended by *wA* without further ketoreduction to generate the naphthapyranone parasperone A (Brown *et al.,* 1993). This biosynthetic scheme is similar to that proposed for aflatoxin, and the first stable intermediates in these two polyketide pathways are chemically similar (Brown *et al.,* 1993). Such observations have led to speculation of a common ancestral polyketide pathway between aflatoxin and the green conidial pigments of *Aspergilli.* The *wA* gene also contains two ACP motifs, but the significance of this is unclear (Fig. 3B). The expression of *wA* has some interesting features.

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First, the gene is under very strict developmental control, most directly by the *wetA* regulatory gene (Marshall and Timberlake, 1991). Second, *wA* is proposed to be expressed within the phialide cells, and the pigment intermediate is proposed to be secreted into the nascent conidial wall (Mayorga and Timberlake, 1992). These observations are intriguing, because it has been proposed that intermediates in the aflatoxin pathway may be produced both in the cytosol and in organelles (Miller *et al.,* 1996), as has been demonstrated for penicillin biosynthesis in *Penicillium chrysogenum* (Muller *et al.,* 1991; Lendenfeld *et al.,* 1993). This would require an organized movement of precursors, perhaps in a similar fashion to the movement of conidial pigment precursors from phialide cells to conidia. The first isolated PKS associated with aflatoxin biosynthesis, *pksA,* was identified by physical mapping and confirmed by gene disruption in *A. parasiticus* (Chang *et al.,* 1995b). This gene contains conserved KS, AT, and ACP domains. The green pigmented, nontoxigenic transformants containing disrupted copies of *pksA,* produced no aflatoxin intermediates, but were capable of converting exogenously supplied NA to aflatoxin. The *pksA* gene is localized to the 5′-terminal region of the identified aflatoxin biosynthetic cluster and is adjacent to *nor-1* (Yu *et al.,* 1995). Feng and Leonard (1995) isolated a PKS from *A. parasiticus* by sequence homology, and gene disruption of the gene *pksL1* resulted in transformants, which did not produce any aflatoxin intermediates. In contrast to *pksA,* the primary sequence of the putative *pksL1* polypeptide revealed four conserved domains; KS, AT, ACP, and TE. The expression pattern of the 6.6-kb *pksL1* transcript was consistent with aflatoxin biosynthesis. Further, this transcript was undetected in tissue grown under aflatoxin nonconducive temperature and media conditions. Yu and Leonard (1995) isolated a PKS from *A. nidulans* by physical and transcript mapping, and a 7.2-kb transcript was identified for the *pks ST* gene. This gene contains sequential motifs for KS, AT, two ACPs, and TE. The *pks ST* gene was subsequently renamed *stcA* upon its localization within the ST cluster (Brown *et al.,* 1996b). The presence of two ACP domains in *wA* and *pksA* suggests a common ancestor for the PKSs from *A. nidulans.* It would be intriguing to determine if these duplicate copies of ACP are both functional, and if so, if they provide this enzyme greater tolerance to the ACP-specific inhibitor cerulenin (Ohno *et al.,* 1975).

2. Noranthrone to norsolorinic acid (NA): Noranthrone oxidase (Fig. 1. (III)). Vederas *et al.* (1980) proposed the oxidation of noranthrone to NA by a putative noranthrone oxidase (Fig. 4). No noranthrone oxidase has been reported from *A. flavus, A. parasiticus,* or *A. nidulans.* However, the *stcC* gene in the sequenced *A. nidulans* ST cluster contains motifs consistent with oxidase activity and shares 29% identity with a chloroperoxidase from *Caldariomyces fumago,* including conservation of a heme binding site (Brown *et al.,* 1996a). Bhatnagar *et al.* (1992) proposed that a monooxygenase would be required to oxidize noranthrone to NA. Four P-450 monooxygenases and a putative FAD monooxygenase have been reported from the *A. nidulans* ST cluster. Of these monooxygenases, only *stcB* and *stcW* have not been placed in the pathway.

3. Alternate pathways of NA to averantin: Reductase nor-1 and norA (Fig. 1 (IV)). Norsolorinic acid is bright orange, therefore, mutants blocked in the conversion of NA are readily identifiable. NA-accumulating mutants have been identified in both *A. flavus* (Papa, 1979, 1982; Payne *et al.,* 1993) and in *A. parasiticus* (Detroy *et al.,* 1973; Bennett, 1979). These mutants are not completely blocked in aflatoxin biosynthesis, but in all cases produce less aflatoxin than wild-type strains. The lack of a complete block in aflatoxin biosynthesis in NA-accumulating mutants suggests that there may be more than one enzyme involved in the conversion of NA.

Yabe *et al.* (1991) identified three enzymatic steps involved in the conversion of NA to the pathway intermediate AVF: (1) a dehydrogenase catalyzed step converting NA to AVN; (2) a monooxygenase step converting AVN to HAVN; and (3) a second dehydrogenase activity converting HAVN to AVF. Chuturgoon and Dutton (1991) purified a 140-kDa dehydrogenase from cell free extracts of a VERA accumulating mutant of *A. parasiticus* capable of converting NA to AVN. Yabe *et al.* (1991) determined this conversion to be reversible and that NADP(H) is the preferred nicotinamide nucleotide cofactor. A second reductase, with a molecular mass of 40 kDa, capable of converting NA to AVF was purified by Bhatnagar and Cleveland (1990).

Chang *et al.* (1992) isolated the gene that complements the *nor-1* mutation in *A. parasiticus.* The gene, *nor-1,* was the first aflatoxin biosynthetic gene cloned. The putative peptide encoded by this gene has a predicted molecular mass of 29 kDa. Thus, this gene does not appear to encode the enzymes isolated by Bhatnagar and Cleveland (1990) or Chuturgoon and Dutton (1991). The protein encoded by *nor-1* has not been purified, but database analysis of the predicted peptide suggests that it functions as a ketoreductase (Trail *et al.,* 1994).

Another enzyme with NA reductase activity is NorA.

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This 43-kDa protein was purified from *A. parasiticus* and shown to be capable of converting NA to AVN (Cary *et al.,* 1996). The norA cDNA was isolated and monoclonal antibodies were raised against NorA. These antisera significantly inhibit the enzymatic conversion of NA to AVN, but do not react with the 29-kDa product of *nor-1.* Sequence analysis of norA predict a putative protein of 43.7 kDa with a high degree of homology to an aryl alcohol dehydrogenase of *Phanerochaete chrysosporium.* The norA gene resides in the AF cluster, just upstream of *ver-1,* and is located a significant distance from *nor-1.* The actual function of this gene in aflatoxin biosynthesis is unknown. However, it has been suggested from these data that more than one step is involved in the conversion of NA to AVF in aflatoxin biosynthesis (Cary *et al.,* 1996).

4. Averantin to 5′-hydroxaverantin: Monooxygenase avnA (Fig. 1. (V)). Bennett *et al.* (1980a) isolated an averantin (AVN) accumulating mutant of *A. parasiticus* and utilized radiotracer experiments to demonstrate that [^{14}C]averantin was incorporated into aflatoxin B1 biosynthesis after NA and before AVF. McCormick *et al.* (1987) used similar radiotracer experiments to place AVNN between AVN and AVF. The gene associated with the conversion of averantin to HAVN, has been designated *avnA* (Yu *et al.,* 1997). Disruption of this gene results in a loss of aflatoxin production. Aflatoxin production is restored in disrupted strains fed exogenous HAVN and AVNN, but not AVN, indicating that *avnA* is involved in the conversion of AVN to HAVN. This gene resides in the aflatoxin cluster adjacent to *omtA* and was originally assigned the name *ord-1* (Yu *et al.,* 1993). The designation *ord-1* is now used for the gene involved in the conversion of OMST to AFB1 (Prieto *et al.,* 1996). Gene *avnA* has high sequence similarity to the *stcF* gene from the *A. nidulans* ST cluster. The predicted peptide sequence also has high homology to eukaryotic cytochrome P-450 monooxygenases.

5. Versiconal to versicolorinB: Cyclase vbs (Fig. 1. (X)). The versicolorin B synthase catalyzes the side chain cyclodehydration of racemic VHA to optically active VERB (McGuire *et al.,* 1996). This enzyme is responsible for providing the absolute stereochemistry of the bisfuran ring form that is present in all members of the aflatoxin family of mycotoxins. This synthase was purified from cell-free extracts and peptide fragments of the enzyme were sequenced. The protein sequence has similarity to flavin-dependent oxidases and dehydrogenases. Knowledge of the protein sequence provided probes for cloning the *vbs* gene from the cDNA and genomic libraries from *A. parasiticus.* The gene resides within the aflatoxin gene cluster (Silva *et al.,* 1996a; Yu *et al.,* 1995), approximately 3.3 kb upstream of the *omtA* gene, separated from *omtA* by an open reading frame encoding an additional putative cytochrome P450 monooxygenase of unknown function. Silva *et al.* (1996b) further characterized the versicolorin B synthase by overexpression of the enzyme in yeast and confirmed its role in converting VHA to VERB.

6. Versicolorin B to versicolorin A: Monooxygenase stcL (Fig. 1. (XI)). Versicolorin B is situated at a branch point in aflatoxin biosynthesis. The conversion of VERB to ST leads to the production of AFB1, while the conversion of VERB to DHST leads to the production of AFB2. The conversion of VERB to VERA is proposed to require a desaturation of the bisfuran ring, presumably by an oxidation step (Yabe *et al.,* 1991). The ST biosynthetic cluster from *A. nidulans* contains four genes with homology to cytochrome P450 monooxygenases (Brown *et al.,* 1996a). Kelkar *et al.* (1997) disrupted a predicted P450, the *stcL* gene from *A. nidulans,* and found that disruptants failed to produce ST and accumulated DHST. Disruptants fed VERA were restored in ST production, suggesting that the ST pathway may branch at VERB, as reported for the AF pathway. Disruptants fed with NA produced only DHST, indicating that the block in the pathway occurs at the conversion of VERB to VERA.

7. Versicolorin A to DMST/versicolorin B to DH-DMST (Fig. 1 (XII)). The conversion of VERA to DMST has been proposed to require oxidation, ketoreduction, and decarboxylation steps. This is an excellent example of the complexity of the aflatoxin biosynthetic pathway, as at least two gene products have now been identified to be required for these modifications. Complementation of the VERA accumulating mutation in *A. parasiticus* showed that the *ver-1* is required for this conversion (Skory *et al.,* 1992). The *ver-1* gene has greatest homology to the *Streptomyces coelicolor actIII* gene that encodes a ketoreductase required for the biosynthesis of the polyketide actinorhodin (Hallam *et al.,* 1988). A motif for an adenine nucleotide binding domain was also identified for *ver-1* (Skory *et al.,* 1992). Liang *et al.* (1996) proposed that *ver-1* deoxygenated VERA, and that an additional dehydratase activity of *ver-1* yielded a 6-deoxyversicolorin A intermediate. Keller *et al.* (1994) identified an *A. nidulans ver-1* homolog, *verA.* Analysis of the *verA* sequence demonstrated close sequence similarity to *A. parasiticus ver-1* and homology to several genes encoding NADPH-dependent reductase activities involved in polyketide biosynthesis. When the ST biosynthetic cluster was identified (Brown *et al.,* 1996a), *verA* was renamed

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stcU. Adjacent to *stcU* is the gene *stcS,* which has sequence homology to P-450 monooxygenases (Keller *et al.,* 1995). Disruption of this gene also resulted in the accumulation of VERA, confirming that both keto-reduction (*stcU*) and oxidation (*stcS*) are required for the conversion of VERA to DMST.

8. DMST to ST/DMDHST TO DHST: Methyltransferase stcP (Fig. 1 (XIII)). Yabe *et al.* (1989) identified a requirement for two distinct *O*-methyltransferase activities in aflatoxin biosynthesis. The first methyltransferase activity was associated with the conversion of DMST and DHDMST to ST and DHST, respectively (*O*-methyltransferase I). The second activity, presumably absent in *A. nidulans,* was associated with the conversion of ST to OMST and DHST to DHOMST (*O*-methyltransferase II) (Yabe *et al.,* 1989). The *stcP* gene from the ST cluster is predicted to be a putative *O*-methyltransferase (Brown *et al.,* 1996a). The predicted amino acid sequence of the *stcP* gene product is only 30% identical to the previously characterized *O*-methyltransferase II *omtA* from *A. parasiticus;* however, the putative *S*-adenosylmethionine binding site is conserved in both methyltransferases. Disruptants of *stcP* accumulate DMST, confirming that *stcP* encodes an *O*-methyltransferase I (Kelkar *et al.,* 1996).

9. ST to OMST/DHST to DHOMST: O-Methyltransferase omtA (Fig. 1 (XIV)). Jeenah and Dutton (1983) first reported the conversion of ST to OMST in *A. parasiticus.* Cleveland *et al.* (1987) were able to resolve a postmicrosomal fraction catalyzing the conversion of ST to OMST and a microsomal fraction catalyzing the conversion of OMST to AFB1 from cell-free extracts of *A. parasiticus.* They postulated that a methyltransferase was responsible for the ST to OMST conversion and an oxido-reductase was responsible for the OMST to AFB1 conversion. Several *O*-methyltransferases have subsequently been reported to convert ST to OMST (Bhatnagar *et al.,* 1988; Keller *et al.,* 1993; Yabe *et al.,* 1989). The prevailing evidence is that the 40-kDa *O*-methyltransferase purified by Keller *et al.* (1993) is required for these two conversions (Fig. 1 (XIV)). Antiserum prepared against the 40-kDa methyltransferase was used to identify a clone from a cDNA expression library for *A. parasiticus* (Yu *et al.,* 1993). This clone was used to isolate the *omtA* gene; however, this gene has not been disrupted.

10. OMST to AFB1/DHOMST to AFB2: Oxidoreductase ord1 (Fig. 1 (XV)). Complementation of the AF cluster deletion in *A. flavus* strain 649 requires the addition of three sequential overlapping cosmids, 5E6, 8B9, and 13B9 (Prieto *et al.,* 1996). However, the addition of only the 8B9 cosmid allows this strain to convert exogenous OMST to AFB1. The oxidoreductase required for this conversion was subcloned from the 8B9 cosmid. The putative *ord-1* peptide has homology to cytochrome P-450 monooxygenases (Prieto and Woloshuk, 1997). Expression of the *ord-1* gene in *S. cerevisiae* conveyed the ability to convert OMST to AFB1 in yeast (Prieto and Woloshuk, 1997).

11. Other genes required for AF/ST biosynthesis. The only gene product other than the preceding enzymatic and regulatory polypeptides known to be required for AF/ST biosynthesis is AflJ from *A. flavus* (Meyers *et al.,* 1998). The *aflJ* gene lies adjacent to *aflR,* but is divergently transcribed from a shared 737-bp promoter region. Disruption of this gene abolishes aflatoxin production, but has no impact on the transcription of several pathway genes. The gene encodes no known enzymatic or regulatory domains, but contains regions predicted to encode 3 membrane spanning regions. The function of the *aflJ* gene product is unknown at this time.

12. Other pathway conversions. A number of cloning strategies have been utilized to clone genes from the AF/ST biosynthetic pathways. Genes required for 12 enzymatic steps in AF/ST biosynthesis have been cloned and characterized. In addition, the regulatory gene *aflR* and the presumably nonenzymatic gene product of *aflJ* have been shown to be required for AF biosynthesis. However, no genes have been identified for five additional putative conversions: anthrone to NA, HAVN to AVNN, AVNN to AVF, VHA to VAL and the predicted decarboxylation function associated with the conversion of VERA to DMST and VERB to DHDMST. Additionally, a number of genes have been implicated in these biosynthetic pathways due to their sequence homology with predicted pathway enzymatic functions and their preferential expression under pathway inductive conditions. These include *adh1, adhA,* and *norA* from *A. parasiticus* and *A. flavus,* and *stcB, stcC, stcG, stcI, stcN, stcV,* and *stcW* from *A. nidulans.* Thus, it appears that in excess of 20 genes will be required for the conversion of acetate to AF, and the pathway-specific regulation of this pathway.

III. REGULATION OF AFLATOXIN BIOSYNTHESIS

Aflatoxin biosynthesis is inducible, and in culture its biosynthesis is induced by simple sugars (Buchanan and Stahl, 1984). Induction likely involves a complex interac-

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tion of several factors including transcriptional regulatory factors and physiological cues within the fungus. Transcriptional regulation of the genes involved in aflatoxin biosynthesis by *A. flavus* and *A. parasiticus* and sterigmatocystin biosynthesis in *A. nidulans* appears to be controlled by *aflR* As evidence, *A. flavus* mutant 650, which contains a mutated copy of *aflR* does not accumulate aflatoxin pathway intermediates and will not convert exogenously supplied intermediates to aflatoxin. Transformation of this strain with a functional copy of *aflR* restores aflatoxin biosynthesis and pathway gene transcription (Payne *et al.,* 1992). Additional copies of *aflR* in *A. parasiticus* has been shown to lead to increased aflatoxin production and elevated transcript accumulation of *nor-1, ver-1,* and *pksA* (Chang *et al.,* 1995b). Flaherty and Payne (1997) demonstrated that altered and elevated transcription of *aflR* leads to elevated and altered transcription of the pathway genes, as well as higher levels of aflatoxin production. Yu *et al.* (1996) demonstrated that the *A. nidulans aflR* is required for the transcription of the sterigmatocystin pathway genes in *A. nidulans* and ST production. Further, forced expression of *A. flavus aflR* in *A. nidulans* induced expression of *verA* (stcU) transcript (Yu *et al.,* 1996), indicating that the pathway regulatory gene for *A. flavus* is functional in *A. nidulans.* Finally, the expression of *aflR* and a 3.3-kb DNA fragment containing the *ord-1* gene in *A. flavus* strain 649 was sufficient to enable conversion of exogenous OMST to AF by a strain deficient in the aflatoxin biosynthetic cluster (Prieto and Woloshuk, 1997).

A. Pathway-Specific Regulation

The putative 47-kDa protein encoded by *aflR* is a zinc binucleate cluster DNA-binding protein (Chang *et al.,* 1993, Woloshuk *et al.,* 1994; Yu *et al.,* 1996). This class of regulatory proteins has been placed in a single protein family designated Zn(II)2Cys6 (Todd and Andrianopoulos, 1997). This motif has been identified in more than 80 proteins and these proteins appear to be unique to fungi (Todd and Andrianopoulos, 1997). Such proteins are known to be involved in a diverse array of cellular processes, including primary and secondary metabolism, drug resistance, and meiotic development. The type protein for this class is the Gal4 gene from *S. cereviseae.*

The *aflR* genes of *A. flavus* and *A. parasiticus* share greater than 95% DNA homology, and the *apa-2* gene complements the *afl-2* mutation in *A. flavus* strain 650-33. In contrast, the *A. nidulans aflR* is only 33% identical to the *A. flavus aflR.* In the zinc cluster region, however, *A. nidulans aflR* is 71% identical to *aflR* in *A. flavus* and *A. parasiticus,* and two regions near the C-terminus also show high identity between the three species (Yu *et al.,* 1996. The *A. flavus aflR* is functional in *A. nidulans* (Yu *et al.,* 1996).

Zn(II)2Cys6 proteins are known to have specific cognitive binding sites. No AflR binding sites have been identified from the promoter region of AF pathway genes. Zn(II)2Cys6 binding sites consists of conserved terminal trinucleotides, usually in a symmetrical configuration, spaced by an internal variable sequence of defined length (Todd and Andrianopoulos, 1997). GAL4, for example, binds to $CGGN_{11}CCG$, while the *Uay* of *A. nidulans* binds to CGG_6XCCG (Suarez *et al.,* 1995). Recently, Fernandez *et al.* (1998) found a binding site for *A. nidulans* AflR in the promoter of the *stcU* gene. The binding site they reported is $TCGN_5CGA$. By deletion analysis, we have located a similar sequence that is required for the regulated transcription of the *nor-1* gene in *A. flavus* (Brown-Jenco and Payne, unpublished data).

Chang *et al.* (1995a) reported binding of *AflR* to a TTAGGCCTAA palindromic sequence in the promoter of the *A. parasiticus aflR.* Such autoregulation has been reported for some members of this gene family. A similar sequence to the one in *A. parasiticus* is present in *A. flavus.* However, such a binding site is completely absent in the promoter of the *A. nidulans aflR,* indicating that either the binding site differs in *A. nidulans* or there is some degenerate binding.

B. Interaction of Aflatoxin Biosynthesis and Development

Aflatoxin biosynthesis and fungal development appear to share regulatory elements or their regulatory elements interact with one another. Bennett and her coworkers (Bennett, 1981; Bennett and Papa, 1988; Bennett *et al.,* 1986) observed that serial transfers of macerated mycelium of *A. flavus* or *A. parasiticus* led to the appearance of morphological variants which also lacked the ability to produce aflatoxin. Kale *et al.* (1994, 1996) further examined morphological variants of *A. parasiticus.* They repeated the continuous culturing experiments of Bennett *et al.* (1986) and observed morphologies, which included an abundance of vegetative mycelia, reduced numbers of condiophores and conidia, and abnormal metulae. These mutants lacked transcripts for *aflR, nor-1,* and *omtA,* and failed to accumulate any of the pathway intermediates. Guzman-de-Pena and Ruiz-Herrera (1997) also associated the loss of sporulation with loss of aflatoxin production. They demonstrated that the addition of diaminobutanone

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(DAB) to cultures of *A. parasiticus* prior to 30 h of culture inhibited both sporulation and AF biosynthesis. The addition of putrescine partially reversed the effect of DAB on AF biosynthesis and accumulation.

The genetics of development and sporulation in *A. nidulans* is much better characterized than that for the aflatoxin producing fungi. Timberlake (1990) observed similar morphological mutants in *A. nidulans* to those described above by Bennett and Kale. A series of mutations resulting in a similar fluffy phenotype to that previously described in *A. parasiticus* was recently described by Wieser *et al.* (1997). Recessive mutations in the genes known to regulate sporulation in *A. nidulans,* such as in *fluG, flbA, flbB, flbC, flbD,* and *flbE* result in a fluffy phenotype. However, fluffy phenotypes also accompany dominant mutations in *fadA* and a dominant interfering mutation in the sporulation transcriptional regulator, *brlA.* The availability of well-characterized developmental mutants in *A. nidulans* allowed Hicks *et al.* (1997) to study the interrelationship between development and biosynthesis of ST. Strains with mutations in the early activators of asexual sporulation such as *fluG* and *flbA,* failed to accumulate ST, leading Hicks *et al.* (1997) to propose that growth, development, and ST production in *A. nidulans* and aflatoxin production in *A. parasiticus* is regulated by a heterotrimeric G-protein-mediated signaling pathway. The hypothesis they have proposed is that vegetative growth is mediated by the Gαi-protein *fadA,* and that sporulation and ST production require the partial inactivation of *fadA.* In this model, *fadA* is proposed to be partially inactivated by the product of *flbA,* which presumably interferes with the GTPase activity of one of the heterotrimeric subunits. However, *flbA* must also have some other function as overexpression of *flbA* in a *fadA* deletion mutant also activates ST production and sporulation.

C. Other Regulatory Elements

From the work of Prieto and Woloshuk (1997) it is clear that *aflR* is the only gene in the biosynthetic cluster required for transcriptional activation of pathway genes. However, evidence for additional regulatory controls was clearly established in the work of Flaherty and Payne (1997). They found constitutive expression of *aflR,* leading to constitutive expression of the pathway genes, did not change the profile of aflatoxin accumulation. That is, after resuspension of the *aflR* overexpression strain into conducive media, aflatoxin first appeared after 6 h and peaked at 18 h, just as in a wild-type strain. Further, several researchers have detected *aflR* or pathway gene transcripts in advance of aflatoxin biosynthesis (Feng and Leonard, 1995; Liu and Chu, 1996). Thus, transcription of pathway genes is required but not sufficient for aflatoxin biosynthesis, suggesting that there may be physiological conditions controlling the onset of aflatoxin biosynthesis. Given the large number of physiological parameters previously demonstrated to impact the timing and level of aflatoxin biosynthesis (Joffe and Lisker, 1969; Bhatnagar *et al.,* 1986a,b; Wiseman and Buchanan, 1987; Buchanan *et al.,* 1987), these physiological controls may be quite subtle. Previous attempts to discern the physiological determinants of aflatoxin biosynthesis, and their genetic controls, have been thwarted by the complexity of fungal responses to changes in media composition (see Maggon *et al.,* 1997, and Luchese and Harrigan, 1993, for general review of the physiology of aflatoxin biosynthesis). However, specific physiological inducers which impact aflatoxin biosynthesis have been identified (Payne and Brown, 1998). These include simple sugars as carbon source (Wiseman and Buchanan, 1987), exogenous NADPH levels (Tyagi and Venkitasubramanian, 1981), nitrate as sole nitrogen source (Kachholz and Demain, 1983), and external pH (Maggon *et al.,* 1977). An additional important factor, adenylate concentration within subcellular compartments (Buchanan *et al.,* 1987), now can be manipulated experimentally using nystatin to selectively permeabilize the plasma membranes (Roos *et al.,* 1997). This technique has been used to study the effects of directed and subtle changes in cytosolic adenylate charges on vacuolar amino acid uptake and efflux. So where does this lead us? With current genomics technology, it should be possible to identify the genes responsible for subtle physiological control of secondary metabolism. For example, using DNA array technology (Marshall and Hodgson, 1998), the transcript levels of the predicted 6200 genes of *Saccharomyces cerevisea* can be determined simultaneously (Brown *et al.,* 1998). Using total RNA from control cultures of *S. cerevisea* or cultures perturbed by the presence of experimental compounds, genes, which are upregulated or down regulated due to the presence of an active compound can be determined. Such transcript profiling is currently being utilized to identify new targets for drug discovery. Given the knowledge of exogenously supplied media additives which effect aflatoxin biosynthesis, could we not artificially perturb the biosynthesis of aflatoxin using these compounds and use similar techniques to look for novel genes which are altered in their regulation? There is some evidence that such a protocol will work. Keller *et al.* (1997) demonstrated that external pH effected expression of both the global pH

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regulator *PacC* and the expression of ST pathway gene *stcU.* What genes might demonstrate altered transcript levels due solely to the addition of exogenous 100 mM NADPH to a growing culture, or 300 nmol of nitrate to an ammonia depleting culture? To address such questions a sufficient library representing the fungal genome will be required. Currently, an EST library for *A. nidulans* is available; however, the availability of an ordered ORF DNA library would be optimal. A similar library for *A. flavus* is also warranted, due to the possible differences in the physiological control of these two pathways (Payne and Brown, 1998). Using these types of experiments, subtle physiological controls of aflatoxin could be elucidated, initiating a new era in the study of aflatoxin biosynthesis, such that the synergy of genomics technology and the vast wealth of physiological data leads to the identification of additional levels of genetic control of secondary metabolism.

IV. CONCLUSIONS

The aflatoxin biosynthetic pathway represents one of the best-characterized pathways of secondary metabolism in fungi. Its elucidation has resulted from the combined efforts of researchers in many disciplines. Early seminal research on the chemistry of the pathway and the placement of intermediates in the biosynthetic scheme allowed for the ensuing genetic analysis of the pathway. It is interesting that most of the knowledge of this pathway and the molecular biology of aflatoxin biosynthesis has been derived from studies on two asexual fungi, *A. flavus* and *A. parasiticus.* Studies with these fungi led to the isolation of 10 genes in the aflatoxin pathway and the elucidation or confirmation of nine enzymatic conversions. Studies with *A. flavus* and *A. parasiticus* provided the genetic material to identify the sterigmatocystin biosynthetic cluster in *A. nidulans.* The well-characterized genetics of *A. nidulans* has clearly allowed the field to advance at a more rapid pace. Research from several labs working on all three fungi has led to the identification of 17 genes and 12 enzymatic conversions in less than 10 years.

Given the similarities between the AF and ST pathways to date, one would predict similar regulator circuits for the two pathways. However, if these two compounds serve differ roles, there may be significant differences in the regulation of each pathway. At this point we have little evidence for the role of either AF or ST in the ecology of the fungus. The production of these two compounds is very energy dependent and comes at a cost to the fungus. Because ST is found in several genera of fungi, but AF is known to be produced by only four species within the Aspergillus Section Flavi, one could speculate that the role of aflatoxin may be more specific than that of sterigmatocystin. If this is true one may predict that the timing of production or the localization of the two compounds within the fungi may differ. Indeed, there is some evidence that aflatoxin biosynthesis may be more tightly regulated than ST biosynthesis (Feng and Leonard, 1998). New DNA technologies should allow for further characterization of these two pathways, help elucidate their interaction with development, and provide clues as to their importance to the producing organisms.

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