ELSEVIER

FEMS Microbiology Letters 160 (1998) 169–176

FEMS MICROBIOLOGY LETTERS

MiniReview

Genetic organization and function of the aflatoxin B1 biosynthetic genes

C.P. Woloshuk *, R. Prieto

Department of Botany and Plant Pathology, Purdue University, 1155 Lilly Hall, West Lafayette, IN 47907, USA

Received 29 April 1997; revised 1 October 1997; accepted 23 December 1997

Abstract

Aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Most of the genes involved in the biosynthesis of aflatoxin are contained within a single cluster in the genome of these filamentous fungi. Studies directed toward understanding the molecular biology of aflatoxin biosynthesis have led to a number of important discoveries. A pair of fatty acid synthase genes were identified that are involved uniquely in aflatoxin biosynthesis. Two genes were also characterized that represent new families of cytochrome P450 monooxygenases. Gene expression is coordinated during aflatoxin production and is under the control of a positive regulatory gene belonging to a family of fungal transcriptional activators associated with various metabolic pathways in fungi. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

Keywords: Aflatoxin; *Aspergillus flavus*; *Aspergillus parasiticus*

1. Introduction

The sudden appearance of turkey-X disease in 1960, which resulted in the death of over 100 000 turkeys in the United Kingdom, led to the discovery of a family of structurally related metabolites called aflatoxins [1,2]. Aflatoxins are secondary metabolites produced by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus*. These toxins are commonly found prior to harvest in oil seed crops such as corn, cotton, peanuts and tree nuts. Aflatoxins greatly impact the food and feed industry because they are highly toxic and carcinogenic in a variety of animal species [3]. Aflatoxin B1 (AFB1) has been shown to produce G-T transversions at codon 249 of the p53 tumor suppressor gene, whose altered sequence has been associated with a number of human cancers [4]. Due to concern for the potential effects of aflatoxins on human health, most countries have expensive monitoring programs and legislation that restrict marketing of aflatoxin-contaminated grain [5].

Neither *A. flavus* nor *A. parasiticus* has a sexual stage, but a parasexual cycle has been elucidated in each [6,7]. Genetic studies using the parasexual cycle are difficult due to the similar size of haploid and diploid conidia which precludes easy and accurate determination of ploidy levels. Despite the obstacles, Papa described 24 distinct aflatoxin non-producing mutants and a number of spore color and auxotrophic mutants of *A. flavus* derived from the common

* Corresponding author. Tel.: +1 (765) 494-3450; Fax: +1 (765) 494 0363; E-mail: woloshuk@btny.purdue.edu

0378-1097/98/$19.00
PII S0378-1097(97)00588-0

parental line, PC-7 [8]. He used parasexual analyses to map over 30 loci to eight linkage groups. The aflatoxin loci are all recessive in diploids, except for the dominant *afl-1* locus. Of the 11 mapped aflatoxin loci, nine are in linkage group VII, one is in linkage group II, and one is in linkage group VIII. In *A. parasiticus*, several anthraquinone-accumulating mutants as well as spore color and auxotrophic mutants are available. Genetic studies on *A. parasiticus* are less extensive and the number of linkage groups reported range from six to ten [8].

The objective of this review is to discuss the genetic organization and function of the genes involved in aflatoxin biosynthesis. The first gene was isolated and described in 1992 [9,10], and since the last review on the molecular biology of aflatoxin biosynthesis [11], rapid and significant progress followed the discovery that genes involved in aflatoxin biosynthesis in both *A. flavus* and *A. parasiticus* are clustered [12,13]. The cluster in *A. flavus* is located on a 4.9 mb chromosome [14]. Prieto et al. [15] have shown by complementation in a cluster-deletion mutant of *A. flavus* that the aflatoxin pathway genes reside in approximately 90 kb of cloned genomic DNA. Yu et al. [16] partially mapped the clusters in *A. flavus* and *A. parasiticus*, and they estimated the cluster size to be 75 kb. The relative position of genes within the clusters and the nucleotide sequence of the genes are similar between the two species, although *A. parasiticus* appears to have an additional copy of several genes indicating that at least part of the cluster is duplicated [17,18].

Our understanding of aflatoxin biosynthesis was significantly advanced by the discovery that in *A. nidulans* the genes involved in sterigmatocystin biosynthesis, which is identical to that portion of the aflatoxin biosynthetic pathway, are also clustered [19,20]. The entire nucleotide sequence of the *A. nidulans* cluster was recently determined, and 25 transcripts were identified whose expressions correlated with sterigmatocystin production [19]. The arrangement of the genes within the cluster is different from the aflatoxin gene cluster, but the deduced amino acid sequence of the gene products are similar. The genetic experiments that are possible with *A. nidulans* should facilitate the discovery of additional information about the regulatory circuit for both sterigmatocystin and aflatoxin.

2. Norsolorinic acid biosynthesis

The polyketide backbone of AFB1 involves the extension of a hexanoate unit by a polyketide synthase (PKS) that assembles seven acetyl units from malonyl CoA without ketoreduction to generate noranthrone [21,22]. The synthesis of norsolorinic acid (NA), the first stable intermediate, is completed after oxidation of the noranthrone by a proposed oxidase (Fig. 1) [11,21]. Support for a specialized PKS and fatty acid synthase (FAS) was provided by the cloning and characterization of several *A. parasiticus* genes including *pksA* (*pksL1*) and *fas1*. Chang et al. [23] identified *pksA* when transforming an NA-accumulating strain of *A. parasiticus* with DNA containing fragments of the aflatoxin gene cluster [23]. Approximately 5% of the transformants lost the ability to produce NA when the transformants were disrupted in the *pksA* gene. A gene, *pksL1* (which is *pksA*), was identified among several cDNA clones isolated by differential screening [24]. Gene disruption of *pksL1* resulted in transformants that did not accumulate aflatoxins or NA suggesting the gene is involved in aflatoxin biosynthesis. The FAS gene *fas1* was identified by complementation in *A. parasiticus* strain UVM8, which produces no aflatoxin or pathway intermediates [25]. Additional gene disruption experiments established that *fas1* functioned prior to the formation of NA and that the gene was not vital for fungal growth. A second FAS gene, *fas2*, has been located adjacent to *fas1* (Fig. 1) and is likely the β-subunit of the functional FAS [26].

Genes from *A. nidulans* encoding PKS (*stcA*) and FAS alpha and beta subunits, *stcJ* and *stcK*, respectively, were localized to the sterigmatocystin gene cluster [27]. Disruption of *stcJ* and *stcK* had no effect on fungal growth, but sterigmatocystin was not produced unless cultures were supplied with exogenous hexanoic acid. These data indicated the specific function of these genes in secondary metabolism. Hexanoic acid feeding data from mutants of both *A. nidulans* and *A. parasiticus* disrupted in the FAS genes suggest that PKS and FAS function cooperatively [26,27].

Analyses of nucleotide sequences indicate that motifs common to known PKS and FAS are conserved in the gene products, namely β-ketoacyl synthase, acyltransferase and acyl carrier protein [12,23–25].

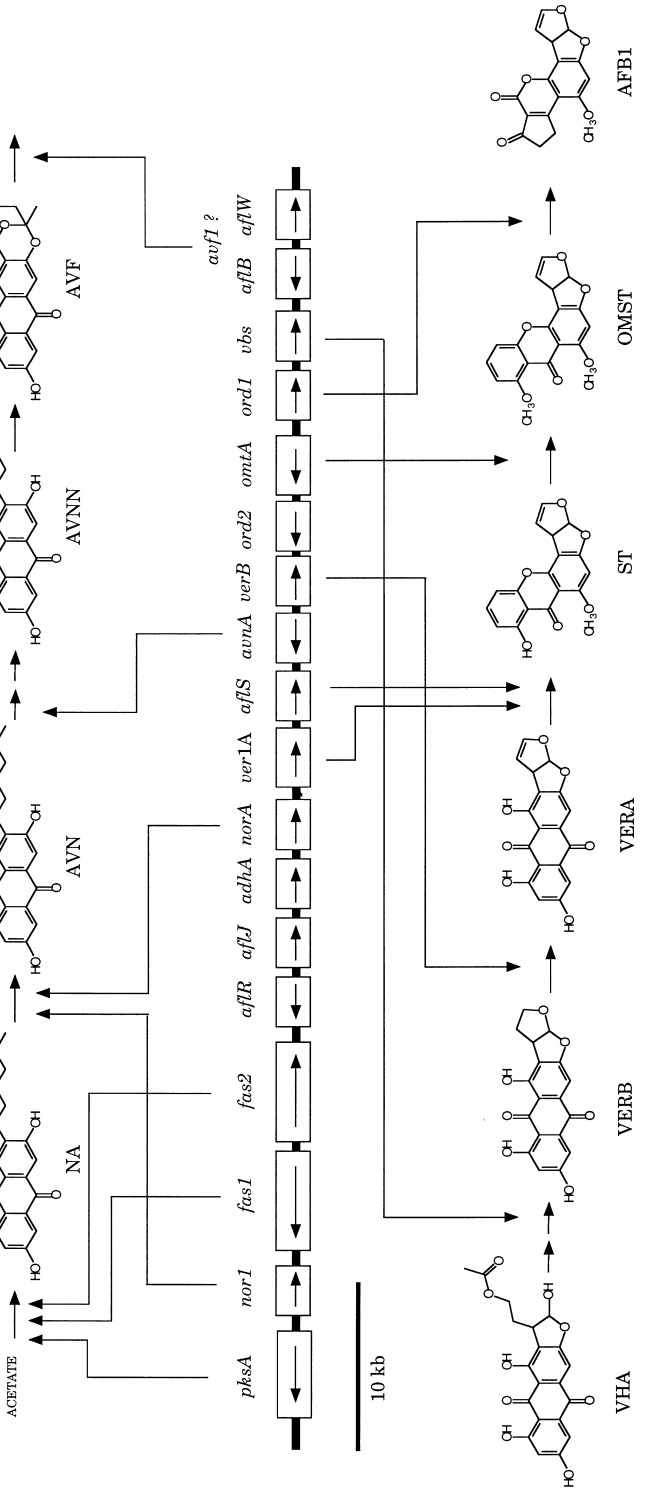

Fig. 1. Biosynthetic pathway of aflatoxin B1, showing the major intermediates and the aflatoxin gene cluster.

The FAS genes also contain conserved domains for β-ketoacyl reductase, enoyl reductase and enoyl hydrase [12,23,25]. The absence of ketoreductase in the PKS sequence is consistent with a lack of a reductive step in the PKS reaction that produces noranthrone.

3. Norsolorinic acid-averatin

Conversion of NA to averatin (AVN) via a ketoreductase has been extensively studied (Fig. 1) [21]. Isolated by complementation in a leaky mutant of *A. parasiticus* that accumulates NA and aflatoxins [9], *nor1* encodes a 29 kDa protein with amino acid similarity to dehydrogenases that have NADPH-binding motifs [28]. Disruption of *nor1* in a wild-type strain resulted in transformants that accumulated NA. Similar to the original NA mutant, these *nor1*-disrupted transformants were not completely blocked in AFB1 biosynthesis. These data suggested the existence of other NA-reductases. In fact, a second reductase capable of converting NA to AVN in vitro was previously purified to homogeneity and shown to be a single subunit protein of about 40 kDa [21,29]. Using monoclonal antibodies against this NA-reductase, Cary et al. [17] identified a cDNA clone corresponding to a gene, *norA*, located in the aflatoxin gene cluster of both *A. parasiticus* and *A. flavus* (Fig. 1). The predicted amino acid sequences of NOR1 and NORA share low identity (22%), but both have motifs indicating a dehydrogenase-type enzyme with adenine nucleotide-binding domains [17,28]. Attempts to disrupt both copies of the *norA* in a wild-type *A. parasiticus* strain failed [17]. In *A. nidulans*, *stcE* and *stcV* have gene products with amino acid sequences similar to NOR1 and NORA, respectively; however, confirmation of the function of these genes is lacking [19].

4. Averatin-versicolorin A

Genes involved in the conversion of AVN to versicolorin A (VER A) are the least defined of the genes in aflatoxin biosynthesis. Through the use of mutant strains, pathway inhibitors, and radioactively labelled intermediates, the major metabolites in this conversion have been determined: AVN → averufanin (AVNN) → averufin (AVF) → 1-hydroxyversicolorone (HVN) → versiconal hemiacetal acetate (VHA) → versiconal (VHOH) → versicolorin B (VER B) → VER A (Fig. 1) [13,21]. Using a cDNA clone, Yu et al. [13] disrupted the corresponding gene in the aflatoxin gene cluster of *A. parasiticus* and recovered transformants that accumulated AVN. The gene, *avnA*, encodes a 56.3 kDa cytochrome P450 monooxygenase, suggesting that the gene is involved in the conversion of AVN to AVNN. A similar gene, *stcF*, has been identified in the sterigmatocystin cluster of *A. nidulans* [13,19].

Prieto et al. [15] transformed a mutant of *A. flavus* lacking the entire aflatoxin gene cluster [30] with two overlapping cosmids (5E6 and 8B9) and recovered transformants that accumulated AVF. The addition of a third overlapping cosmid (13B9) restored aflatoxin biosynthesis in this mutant, suggesting that a gene involved in the conversion of AVF to VHA is located on cosmid 13B9. The putative gene, *avf1*, was mapped to a 7 kb fragment on cosmid 13B9 (Fig. 1) [15]. Sequence analysis indicated that this DNA fragment contains two genes, *aflB* and *aflW* (Fig. 1), which encode proteins with similar amino acid sequences to *stcB* and *stcW*, respectively, in *A. nidulans* but with unknown functions ([19], R. Prieto and C.P. Woloshuk, unpublished).

Conversion of VHOH to VER A is important in aflatoxin biosynthesis because the dihydrobisfuran ring system is formed in this step. The dihydrobisfuran is essential to the mutagenic nature of AFB1 [31]. The double bond at the 2, 3 position in the difuran moiety is the target for renal and hepatic cytochrome P450 enzymes or lipoxygenase that generate a highly reactive epoxide [31,32]. The formation of the dihydrobisfuran is achieved by the dehydrative cyclization enzyme known as versicolorin B synthase (VBS) followed by the oxidative desaturation of VER B to VER A. The gene encoding VBS, *vbs*, was isolated with PCR primers designed based on peptide fragments of the purified enzyme [33]. The deduced *vbs* gene product showed significant identity to many flavin-dependent oxidases and dehydrogenases [33].

In *A. nidulans*, Kelkar et al. [34] recently showed that *stcL* codes for a cytochrome P450 monooxygenase involved in the conversion of VER B to VER A in the sterigmatocystin pathway. Further biochemi-

cal and molecular evidence suggests that the biosynthesis of aflatoxin B2 branches off the pathway at VER B [21,34]. The structure of aflatoxin B2 is identical to AFB1 except it lacks the double bond in the difuran moiety [21]. Recently, the gene *verB* (Fig. 1) was cloned which appears to be the homolog of *stcL* in *A. flavus* and *A. parasiticus* (D. Bhatnagar, unpublished). A mutant of *A. flavus* that accumulates aflatoxin B2 was found to lack *verB* expression.

5. Versicolorin A-sterigmatocystin

Sterigmatocystin is formed from VER A through several enzymatic reactions, including oxidation, ketoreduction, decarboxylation, and methylation. Demethylsterigmatocystin is a known intermediate metabolite in this conversion [21]. Genetic complementation of an *A. parasiticus* mutant that accumulates VER A led to the isolation *ver1A* [10,35], which encodes a NADPH-dependent ketoreductase [10]. A second gene, *ver1B*, was identified in *A. parasiticus* that has 95 percent identity to *ver1A*. *ver1B* is truncated rendering it non-functional and the gene is not located in the gene cluster [18]. Gene-disruption of *stcU*, a homolog of *ver1A*, in a sterigmatocystin-producing strain of *A. nidulans* resulted in VER A accumulation [20]. Disruption of a second *A. nidulans* gene, *stcS*, also resulted in the accumulation of VER A [36]. *StcS* encodes a cytochrome P450 monooxygenase, which has been assigned to a new cytochrome P450 family, CYP59. A gene, *aflS*, similar to *stcS* has been located in the aflatoxin gene cluster (Fig. 1). A third gene, *stcP*, from *A. nidulans* most likely encodes an O-methyltransferase [37]. Mutants with a disrupted *stcP* accumulated demethylsterigmatocystin in the culture medium, indicating that *stcP* is involved in the conversion of this intermediate to sterigmatocystin.

6. Sterigmatocystin to aflatoxin

Sterigmatocystin is converted to O-methylsterigmatocystin (OMST) by a 40 kDa O-methyltransferase (Fig. 1). Antiserum raised against the purified protein from *A. parasiticus* was used to screen a cDNA library and isolate the corresponding gene *omt1* [38]. Amino acid sequence deduced from the cDNA indicated OMT1 contains conserved amino sequence for S-adenosylmethionine-binding [16]. Extracts from *E. coli* expressing *omt1* converted exogenously supplied sterigmatocystin to OMST requiring only the addition of S-adenosylmethionine. Both *A. flavus* and *A. parasiticus* contain single copies of *omt1* that are 97 percent identical [16], but there is no evidence of the gene in *A. nidulans*.

The gene responsible for the conversion of OMST to AFB1 was identified with an *A. flavus* strain containing a deletion of the entire aflatoxin gene cluster [15,39]. Transformants of this mutant containing cosmid 8B9 did not produce aflatoxin or any pathway intermediates but converted exogenously supplied OMST to AFB1 (Fig. 1) [15]. The gene, *ord1*, responsible for this enzyme activity was mapped to a 3.3 kb genomic DNA fragment on cosmid 8B9 [15,40]. The product of *ord1* is a cytochrome P450 monooxygenase which was assigned to a new cytochrome P450 family named CYP64. Yeast expressing *ord1* under the control of the *Saccharomyces cerevisiae* galactose-inducible *gal1* promoter were able to convert OMST to AFB1, indicating that *ord1* is sufficient to accomplish the last step of the aflatoxin biosynthetic pathway [40]. In *A. parasiticus*, *ordA* was identified by chromosome walking, and this gene shares over 90 percent nucleotide sequence identity with *ord1* (D. Bhatnagar, unpublished).

7. Gene regulation

Expression of genes in the aflatoxin biosynthesis cluster is regulated by *aflR*. The gene was isolated from *A. flavus* by complementation in a mutant that did not produce aflatoxin and failed to convert exogenously supplied pathway intermediates to AFB1 [26,41]. In *A. parasiticus*, *aflR* was discovered when investigators observed increased metabolite production in an OMST-accumulating strain transformed with a cosmid containing a large DNA fragment from the aflatoxin gene cluster [42]. The additional copies of *aflR* in the transformants were apparently responsible for an increase in metabolite production. In *A. nidulans*, *aflR* was identified and has been shown to be a functional homolog of the *A. flavus*

and *A. parasiticus* genes [43]. The protein encoded by *aflR* contains a zinc cluster motif (Cys-Xaa2-Cys-Xaa6-Cys-Xaa6-Cys-Xaa2-Cys-Xaa6-Cys) similar to a family of fungal transcriptional activators associated with several metabolic pathways in fungi [39,42]. The zinc cluster motif is involved in the binding of the regulatory protein to the DNA target sequence. Recombinant AFLR was shown to bind a palindrome 120 bp upstream of the *aflR* translation start site, suggesting the autoregulation of *aflR* expression [44]. There are currently no publications describing the binding of AFLR to the promoters of the pathway genes.

The cluster arrangement of the aflatoxin biosynthetic genes is similar to a growing number of clustered genes involved in secondary metabolite and nutrient utilization pathways in filamentous fungi. A recent review indicated that there are no obvious answers to questions concerning the regulatory and evolutionary significance of these gene clusters [45]. In *A. flavus* and *A. parasiticus*, an intact aflatoxin gene cluster is not required for aflatoxin production. Ectopic genomic integration of wild-type genes (both structural genes and *AflR*) into various mutants results in complementation of the mutated genes with no measurable effects on gene expression [10,15,41]. Further, there is little evidence to indicate that gene order within the aflatoxin cluster influences regulation. The evolutionary relatedness between the aflatoxin cluster and the sterigmatocystin cluster is not known. Although clusters contain genes with similar structure and function, the gene order within the cluster and the direction of transcription of some of the corresponding genes are different [45]. Therefore the aflatoxin gene cluster probably does not regulate expression through an interaction with a specific chromatin structure. However, the cluster may stabilize or insure the inheritance of aflatoxin production in a fungal population.

8. Future directions

Progress in the molecular biology of aflatoxin biosynthesis should continue at its current pace. Various laboratories are generating gene-knockout mutants of the remaining genes in the aflatoxin cluster. The phenotypes of these mutants should give insight into the functions of the respective genes. One intriguing gene that has proven difficult to define its function is *aflJ* (Fig. 1) [41]. An *aflJ* disruption mutant of *A. flavus* does not produce aflatoxin or convert pathway intermediates to aflatoxin. Analysis of the nucleotide sequence did not reveal enzymatic domains similar to any known gene (G.A. Payne, unpublished).

New areas of research now focus on the genes that reside outside of the gene cluster. Studies of four *A. flavus* strains from Papa's collection [8] with mutated loci in linkage group VII but not mapped to the gene cluster suggest that the mutations in these strains affect the regulation of *aflR* (M. Weaver and C.P. Woloshuk, unpublished). Another of Papa's *A. flavus* strains with a mutation on linkage group II also appears to affect gene expression prior to *aflR* (M. Brown and G.A. Payne, unpublished). The isolation of these genes by genetic complementation should lead to new information.

Characterization of the genes involved in sterigmatocystin biosynthesis in *A. nidulans* with its classical genetic system will contribute significantly to our understanding of aflatoxin/sterigmatocystin biosynthesis. Several mutations have led to the identification of a set of regulatory genes involved in both fungal development and toxin production (T. Adams, unpublished). These studies should soon reveal information about the signal transduction pathways controlling aflatoxin/sterimatocystin biosynthesis.

Little is known about the gene(s) involved in the biosynthesis of the aflatoxin G-family, which are only produced by *A. parasiticus*. Biochemical evidence suggests that aflatoxin G1 is produced by a separate pathway branching from sterigmatocystin [21,46]. Currently there is no evidence indicating that the genes responsible for the branched pathway are within the gene cluster of *A. parasiticus*. However, the genes at the ends of the cluster have not been thoroughly characterized.

Finally, nomenclature for the aflatoxin biosynthetic genes should be examined to achieve consistency in the criteria for gene symbols. Recognized as a problem by Bennett [8] before the first gene was isolated, no standard convention has been used for naming the genes from *A. flavus* and *A. parasiticus*. As a result of the inconsistency in gene symbols and the frequent changes made in the gene symbols, we

have created confusion even among those who work in the field. In this review, we have referred only to the current symbols for aflatoxin genes. The molecular biology of aflatoxin biosynthesis has reached a point that the current symbols should be changed to comply with the convention used in *A. nidulans*.

Acknowledgments

We thank Deepak Bhatnagar and Larry Dunkle for reviewing this manuscript. We also thank T. Adams, D. Bhatnagar, J. Linz, N. Keller, and G. Payne for sharing their unpublished information. This is Journal Publication No. 15396 of the Purdue University Agricultural Research Station.

References

[1] Blount, W.P. (1961) Turkey "X" disease. J. Brit. Turkey Fed. 9, 52–54.
[2] Hartley, R.D., Nesbitt, B.F. and O'Kelly, J. (1963) Toxic metabolites of *Aspergillus flavus*. Nature 198, 1056–1058.
[3] Roebuck, B.D. and Maxuitenko, Y.Y. (1994) Biochemical mechanisms and biological implications of the toxicity of aflatoxins as related to carcinogenesis. In: The Toxicity of Aflatoxins: Human Health, Veterinary, and Agricultural significance (Eaton, D.L. and Groopman, J.D., Eds.), pp. 22–43. Academic Press, San Diego, CA.
[4] Eaton, D.L. and Gallagher, E.P. (1994) Mechanisms of aflatoxin carcinogenesis. Annu. Rev. Pharm. Tox. 34, 135–172.
[5] Van Egmond, H.P. (1989) Current situation on the regulations for mycotoxins. Overview of tolerances and status of the standard methods of sampling and analysis. Food Add. Contam. 6, 139–188.
[6] Papa, K.E. (1973) The parasexual cycle in *Aspergillus flavus*. Mycologia 65, 1201–1205.
[7] Papa, K.E. (1978) The parasexual cycle in *Aspergillus parasiticus*. Mycologia 70, 766–773.
[8] Bennett, J.W. and Papa, K.E. (1988) The aflatoxigenic *Aspergillus*. In: Genetics of Plant Pathogenic Fungi (G.S. Sidhu, Ed.), pp. 263–278. Academic Press, London.
[9] Chang, P.-K., Skory, C.D. and Linz, J.E. (1992) Cloning of a gene associated with aflatoxin B1 biosynthesis in *Aspergillus parasiticus*. Curr. Genet. 21, 231–233.
[10] Skory, C.D., Chang, P.K. and Linz, J.E. (1992) Isolation and characterization of a gene from *Aspergillus parasiticus* associated with the conversion of versicolorin A to sterigmatocystin in aflatoxin biosynthesis. Appl. Environ. Microbiol. 58, 3527–3537.
[11] Trail, F., Mahanti, N. and Linz, J.E. (1995) Molecular biology of aflatoxin biosynthesis. Microbiology 141, 755–765.
[12] Trail, F., Mahanti, N., Rarick, M., Mehigh, R., Liang, S.-H., Zhou, R. and Linz, J.E. (1995) Physical and transcriptional map of an aflatoxin gene cluster in *Aspergillus parasiticus* and functional disruption of a gene involved early in the aflatoxin pathway. Appl. Environ. Microbiol. 61, 2665–2673.
[13] Yu, J., Chang, P.-K., Cary, J.W., Bhatnagar, D. and Cleveland, T.E. (1997) *avnA*, a gene encoding a cytochrome P-450 monooxygenase, is involved in the conversion of averatin to averufin in aflatoxin biosynthesis in *Aspergillus parasiticus*. Appl. Environ. Microbiol. 63, 1349–1356.
[14] Foutz, K.R., Woloshuk, C.P. and Payne, G.A. (1995) Cloning and assignment of linkage group loci to a karyotype map of the filamentous fungus *Aspergillus flavus*. Mycopathologia 87, 787–794.
[15] Prieto, R., Yousibova, G.L. and Woloshuk, C.P. (1996) Identification of aflatoxin biosynthetic genes by genetic complementation in a mutant of *Aspergillus flavus* lacking the aflatoxin gene cluster. Appl. Environ. Microbiol. 62, 3567–3571.
[16] Yu, J., Chang, P.-K., Cary, J.W., Wright, M., Bhatnagar, D., Cleveland, T.E., Payne, G.A. and Linz, J.E. (1995) Comparative mapping of aflatoxin pathway gene clusters in *Aspergillus flavus* and *Aspergillus parasiticus*. Appl. Environ. Microbiol. 61, 2365–2371.
[17] Cary, J.W., Wright, M., Bhatnagar, D., Lee, R. and Chu, F.S. (1996) Molecular characterization of an *Aspergillus parasiticus* dehydrogenase gene, *norA*, located on the aflatoxin biosynthesis gene cluster. Appl. Environ. Microbiol. 62, 360–366.
[18] Liang, S., Skory, C.D. and Linz, J.E. (1996) Characterization of the function of the *ver-1A* and *ver-1B* genes, involved in aflatoxin biosynthesis in *Aspergillus parasiticus*. Appl. Environ. Microbiol. 62, 4568–4575.
[19] Brown, D.W., Yu, J.H., Kelkar, H.S., Fernandes, M., Nesbitt, T.C., Keller, N.P., Adams, T.H. and Leonard, T.J. (1996) Twenty-five correlated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. Proc. Natl. Acad. Sci. USA 93, 1418–1422.
[20] Keller, N.P., Kantz, N.J. and Adams, T.H. (1994) *Aspergillus nidulans verA* is required for production of the mycotoxin sterigmatocystin. Appl. Environ. Microbiol. 60, 1444–1450.
[21] Bhatnagar, D., Ehrlich, K.C. and Cleveland, T.E. (1992) Oxidation-reduction reactions in biosynthesis of secondary metabolites. In: Handbook of Applied Mycology Vol. 5: Mycotoxins in Ecological Systems (Bhatnagar, D., Lillehoj, E.B. and Arora, D.K., Eds.), pp. 255–286. Marcel Dekker, New York.
[22] Brobst, S.W. and Townsend, C.A. (1994) The potential role of fatty acid initiation in the biosynthesis of the fungal aromatic polyketide aflatoxin B1. Can. J. Chem. 72, 200–207.
[23] Chang, P.-K., Cary, J.W., Yu, J., Bhatnagar, D. and Cleveland, T.E. (1995) The *Aspergillus parasiticus* polyketide synthase gene *pksA*, a homolog of *Aspergillus nidulans wA*, is required for aflatoxin B1 biosynthesis. Mol. Gen. Genet. 248, 270–277.
[24] Feng, G.H. and Leonard, T.J. (1995) Characterization of the polyketide synthase gene (*pksL1*) required for aflatoxin biosynthesis in *Aspergillus parasiticus*. J. Bacteriol. 177, 6246–6254.

[25] Mahanty, N., Bhatnagar, D., Cary, J.W., Joubran, J. and Linz, J.E. (1996) Structure and function of *fas-1A*, a gene encoding a putative fatty acid synthetase directly involved in aflatoxin biosynthesis in *Aspergillus parasiticus.* Appl. Environ. Microbiol. 62, 191–195.

[26] Watanabe, C.M.H., Wilson, D., Linz, J.E. and Townsend, C.A. (1996) Demonstration of the catalytic roles and evidence for the physical association of type I fatty acid synthases and a polyketide synthase in the biosynthesis of aflatoxin. Chem. Biol. 3, 463–469.

[27] Brown, D.W., Adams, T.H. and Keller, N.P. (1996) *Aspergillus* has distinct fatty acid synthases for primary and secondary metabolism. Proc. Natl. Acad. Sci. USA 93, 14873–14877.

[28] Trail, F., Chang, P.K., Cary, J. and Linz, J.E. (1994) Structural and functional analysis of the *nor1* gene involved in the biosynthesis of aflatoxin by *Aspergillus parasiticus*. Appl. Environ. Microbiol. 60, 4078–4085.

[29] Bhatnagar, D. and Cleveland, T.E. (1990) Purification and characterization of a reductase from Aspergillus parasiticus SRRC 2043 involved in aflatoxin biosynthesis. FASEB J. 4, A2164.

[30] Woloshuk, C.P., Yousibova, G.L., Rollins, J.A., Bhatnagar, D. and Payne, G.A. (1995) Molecular characterization of the *afl-1* locus in *Aspergillus flavus*. Appl. Environ. Microbiol. 61, 3019–3023.

[31] Massey, T.E., Stewart, R.K., Daniels, J.M. and Liu, L. (1995) Biochemical and molecular aspects of mammalian susceptibility to aflatoxin B1 carcinogenicity. Proc. Soc. Exp. Biol. Med. 208, 213–227.

[32] Roy, S.K. and Kulkarni, A.P. (1997) Aflatoxin B1 epoxidation catalysed by partially purified human liver lipoxygenase. Xenobiotica 27, 231–241.

[33] Silva, J.C., Minto, R.E., Barry, C.E., Holland, K.A. and Townsend, C.A. (1996) Isolation and characterization of the versicolorin B synthase gene from *Aspergillus parasiticus*. J. Biol. Chem. 271, 13600–13608.

[34] Kelkar, H.S., Skloss, T.W., Haw, J.F., Keller, N.P. and Adams, T.H. (1997) *Aspergillus nidulans stcL* encodes a putative cytochrome P-450 monooxygenase required for bisfuran desaturation during aflatoxin/sterigmatocystin biosynthesis. J. Biol. Chem. 272, 1589–1594.

[35] Skory, C.D., Chang, P.K., Cary, J. and Linz, J.E. (1993) Regulated expression of the *nor-1* and *ver-1* genes associated with aflatoxin biosynthesis. Appl. Environ. Microbiol. 59, 1642–1646.

[36] Keller, N.P., Segner, S., Bhatnagar, D. and Adams, T.H. (1995) *stcS*, a putative monooxygenase is required for the conversion of versicolorin A to sterigmatocystin in *Aspergillus nidulans*. Appl. Environ. Microbiol. 61, 3628–3632.

[37] Kelkar, H.S., Keller, N.P. and Adams, T.H. (1996) *Aspergillus nidulans stcP* encodes an O-methyltransferase that is required for sterigmatocystin biosynthesis. Appl. Environ. Microbiol. 62, 4296–4298.

[38] Yu, J., Cary, J.W., Bhatnagar, D., Cleveland, T.E., Keller, N.P. and Chu, F.S. (1993) Cloning and characterization of a cDNA from *Aspergillus parasiticus* encoding an O-methyltransferase involved in aflatoxin biosynthesis. Appl. Environ. Microbiol. 59, 3564–3571.

[39] Woloshuk, C.P., Foutz, K.R., Brewer, J.F., Bhatnagar, D., Cleveland, T.E. and Payne, G.A. (1994) Molecular characterization of *aflR*, a regulatory gene for aflatoxin biosynthesis. Appl. Environ. Microbiol. 60, 2408–2414.

[40] Prieto, R. and Woloshuk, C.P. (1997) *ord1*, an oxidoreductase gene responsible for the conversion of O-methylsterigmatocystin to aflatoxin in *Aspergillus flavus*. Appl. Environ. Microbiol. 63, 1661–1666.

[41] Payne, G.A., Nystrom, G.J., Bhatnagar, D., Cleveland, T.E. and Woloshuk, C.P. (1993) Cloning of the *afl-2* gene involved in aflatoxin biosynthesis from *Aspergillus flavus*. Appl. Environ. Microbiol. 59, 156–162.

[42] Chang, P.-K., Cary, J.W., Bhatnagar, D., Cleveland, T.E., Bennett, J.W., Linz, J.E., Woloshuk, C.P. and Payne, G.A. (1993) Cloning of the *Aspergillus parasiticus apa-2* gene associated with the regulation of aflatoxin biosynthesis. Appl. Environ. Microbiol. 59, 3273–3279.

[43] Yu, J.-H., Butchko, R.A.E., Fernandes, M., Keller, N.P., Leonard, T.J. and Adams, T.H. (1996) Conservation of structure and function of the aflatoxin regulatory gene *aflR* from *Aspergillus nidulans* and *A. flavus*. Curr. Genet. 29, 549–555.

[44] Chang, P.-K., Ehrlich, K.C., Yu, J., Bhatnagar, D. and Cleveland, T.E. (1995) Increased expression of *Aspergillus parasiticus aflR*, encoding a sequence-specific DNA-binding protein, relieves nitrate inhibition of aflatoxin biosynthesis. Appl. Environ. Microbiol. 61, 2372–2377.

[45] Keller, N.P. and Hohn, T.M. (1997) Metabolic pathway gene clusters in filamentous fungi. Fungal Genet. Biol. 21, 17–29.

[46] Bhatnagar, D., McCormick, S.P., Lee, L.S. and Hill, R.A. (1987) Identification of O-methylsterigmatocystin as an aflatoxin B1 and G1 precursor in *A. parasiticus*. Appl. Environ. Microbiol. 53, 1028–1033.