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Isolation and Characterization of a Gene from *Aspergillus parasiticus* associated with the Conversion of Versicolorin A to Sterigmatocystin in Aflatoxin Biosynthesis

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ISOLATION AND CHARACTERIZATION OF A GENE FROM *ASPERGILLUS PARASITICUS* ASSOCIATED WITH THE CONVERSION OF VERSICOLORIN A TO STERIGMATOCYSTIN IN AFLATOXIN BIOSYNTHESIS

By

Christopher David Skory

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF A GENE FROM *ASPERGILLUS PARASITICUS* ASSOCIATED WITH THE CONVERSION OF VERSICOLORIN A TO STERIGMATOCYSTIN IN AFLATOXIN BIOSYNTHESIS

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A transformation system with efficiencies of 30 - 50 stable transformants per μg DNA was developed for *Aspergillus parasiticus* utilizing the homologous *pyrG* gene which encodes orotidine-monophosphate (OMP) decarboxylase. The *pyrG* gene from *A. parasiticus* was isolated by *in situ* plaque-hybridization of a lambda genomic DNA library using the heterologous *A. nidulans pyrG* gene as a probe. Uridine auxotrophs of *A. parasiticus* ATCC 36537 (*ver*-1), a mutant deficient in the conversion of the aflatoxin biosynthetic intermediate versicolorin A to sterigmatocystin, were isolated by selection on 5-fluoro-orotic acid following nitrosoguanidine mutagenesis. Fungal isolates with mutations in the *pyrG* gene resulting in elimination of OMP decarboxylase activity were detected by assaying cell free extracts for their ability to convert ^{14}C labelled OMP to [^{14}C]- uridine-monophosphate (UMP). Transformation of *A. parasiticus* CS10 (*ver*-1, *pyrG*) protoplasts with the homologous *pyrG* gene restored the fungal cells to prototrophy.

DNA isolated from the wild-type aflatoxin producing (Afl^+) fungus *Aspergillus parasiticus* NRRL 5862 was then used to construct a cosmid genomic DNA library employing the homologous *pyrG* gene for selection of fungal transformants. The cosmid library was transformed into *A. parasiticus* CS10 (*ver*-1, *pyrG*). One $pyrG^+$, Afl^+ transformant was identified and used for marker rescue technique. Transformation of rescued DNA fragments into *A. parasiticus* CS10 resulted in production of wild type levels of aflatoxin and abundant formation of sclerotia. The gene responsible for this complementation was identified by Northern RNA analyses and transformation with subcloned DNA fragments. The approximate location of transcription initiation and polyadenylation sites of *ver*-1 were determined by RNase protection assay and cDNA sequence analysis. The predicted amino acid sequence revealed striking similarity with *Streptomyces* ketoreductases involved in polyketide biosynthesis. Appearance of *ver*-1 RNA transcripts was not readily apparent until near the end of trophophase (growth phase) and this accumulation was followed approximately 8 hrs later by idiophase when aflatoxins are produced. Additionally, the presence of this transcript continued well beyond that which was observed with a gene such as *pyrG* that is involved in primary metabolism. The *ver*-1 transcript did not accumulate when grown under non-aflatoxin supporting conditions, but was detectable 4-7 hrs after transfer to a glucose containing medium which does support aflatoxin biosynthesis.

Dedicated to my parents and to all of my family. I owe my success to their love and support throughout my education.

ACKNOWLEDGMENTS

I thank Dr. Jyh-Song Horng who is primarily responsible for allowing the idea of isolating aflatoxin biosynthetic genes to become a reality. Thanks are also due to Dr. Perng-Kuang Chang who assisted Dr. Horng in the development of the *nia*D transformation system and for his help in the isolation of the *nor*-1 gene. I am also grateful for the assistance of Reuvan Rasooly in sequencing the *ver*-1 gene and thank Dr. Jeff Cary for sequencing the *ver*-1 cDNA.

I am especially indebted to the many people in the Food Microbiology lab for their assistance and support throughout my Doctoral project. Also, I appreciate the time and advice from Dr. Bill Helferich, Dr. Pat Oriel, and Dr. Pat Hart who have graciously served on my guidance committee.

Most importantly, I want to express my extreme gratitude to Dr. James J. Pestka and Dr. John E. Linz. It was through their own success and their confidence in me that I found the motivation to succeed with this thesis.

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INTRODUCTION AND RATIONALE

Aflatoxins are a group of low molecular weight secondary metabolites produced by the fungi *Aspergillus parasiticus* and *A. flavus*. These compounds are known to be potent animal hepatocarcinogens and are suspected to be carcinogens of humans (Chu, 1991). Frequent contamination of domestic agricultural commodities such as corn, peanuts, and cottonseed presents a potential threat to the health of animals and humans (Jelinek *et al*, 1989). There has been considerable effort, since their discovery in the early 1960's, to study the biosynthesis of aflatoxins. This work has allowed the proposal of a generally accepted, but incomplete biosynthetic pathway scheme.

Currently, there is a limited understanding of the details of the molecular mechanisms which regulate aflatoxin production. Investigation into the regulation of this biosynthetic pathway at the molecular level has proceeded primarily by three different strategies: (1) identification and purification of proteins involved in conversion steps leading to aflatoxins, (2) differential hybridization analysis for isolation of genes involved in aflatoxin biosynthesis, and, (3) complementation analysis using a genomic DNA library to transform fungal mutants deficient in aflatoxin biosynthesis.

The efforts of this project were initially directed at developing a genetic transformation system in *A. parasiticus* that would permit efficient manipulation of DNA in the fungus. This work was performed to provide a means of identifying and characterizing one of the genes (*ver*-1) involved in the conversion of the aflatoxin precursor versicolorin A to sterigmatocystin. The isolation of the *ver*-1 gene could then proceed by way of genetic complementation of *A. parasiticus* *ver*-1 mutants that were unable to carry out this biotransformation step.

The longterm goal of this research was to provide information that would ultimately contribute to the eventual elimination of aflatoxin contamination in food and feed. The *ver*-1 gene is being used in studies of the expression of genes associated with aflatoxin biosynthesis. Understanding the control of aflatoxin biosynthesis should allow critical regulatory points in the pathway to be defined. These regions may then serve as inhibitory sites to be used for management of aflatoxin contamination. This information could facilitate the development of agents or genetically engineered plants which are capable of inhibiting toxin production. Another potential use for aflatoxin biosynthetic genes is the construction, by methods of gene disruption, of genetically stable atoxigenic fungal strains. These could then be used for competitive exclusion of aflatoxin producing strains in the field. Biocompetition of this type has proven to be quite successful in laboratory and field tests (Cole *et al*, 1991;

Cotty, 1990; Cotty and Daigle, 1991; Ehrlich, 1987)

LITERATURE REVIEW

Natural Occurrence of aflatoxins

Aflatoxins are produced primarily by the fungi *A. parasiticus* and *A. flavus*. The ubiquitous nature of these organisms presents a worldwide dilemma of preventing contamination by these toxin producers. However, the mere presence of these strains does not necessarily result in toxin contamination since not all strains of *A. flavus* produce aflatoxin. There are also numerous other factors such as substrate availability, temperature, humidity, and competing microflora which can affect the biosynthesis of aflatoxins (Heathcote, 1978).

Many different types of food commodities throughout the world have increased susceptibility to aflatoxin contamination. These include corn, peanuts, brazil nuts, pistachio nuts, cottonseed, rice, and copra (Jelinek *et al*, 1989). Aflatoxin contamination is known to vary regionally as a result climate differences. Preharvest fungal growth in corn and peanuts is favored by warm temperatures and prolonged drought conditions which can lead to crop damage. Postharvest

aflatoxin production is typically seen with warm temperatures and high humidity. In the United States, corn is most frequently contaminated with aflatoxins in the southeastern regions of the United States as a result of these types of weather conditions. The midwestern regions typically have not encountered such problems with the exception of severe drought conditions in 1983 and 1988 which resulted in a widespread aflatoxin dilemma (CAST, 1989; Chu, 1989; Ellis *et al*, 1991; Pestka and Casale, 1990).

Aflatoxins can be potentially controlled by preventing fungal contamination or growth, precluding toxin production, or by detoxification of contaminated commodities. Ammoniation has been proposed as a means of lowering concentrations of aflatoxin in contaminated feeds (Park *et al*, 1988). The effectiveness of this technique for ensuring the breakdown of aflatoxin and its mutagenic and toxic residues is still questioned. To date, the FDA has not approved ammoniation as a method of detoxifying foods and feeds. Thus, prevention of aflatoxin contamination by preventing fungal growth or toxin production appears to be the most feasible approach.

Biosynthesis of aflatoxins.

Aflatoxins are secondary metabolites which are produced by the imperfect fungi *Aspergillus parasiticus* and *A. flavus*. Both species produce aflatoxin B_1 and B_2, however, only *A.*

parasiticus is known to also produce aflatoxin G_1 and G_2. Biotransformation studies with blocked mutants using radiolabeled precursors and pathway intermediates have allowed an aflatoxin biosynthetic pathway to be proposed (Figure 1). Most efforts have focused on the production of aflatoxin B_1 since this compound is the most abundant and most biologically active of the aflatoxins.

The conversion of acetate to aflatoxin B_1 (AFB_1) through the polyketide pathway was first suggested by Biollaz *et al* (1970). It was determined using radiolabelled [1-^{14}C]-acetate and [2-^{14}C]-acetate that the carbon skeleton of AFB_1 was completely derived from acetate subunits. The condensation of these acetate molecules is believed to be catalyzed by the enzyme polyketide synthase (PKS) which is very similar in both structure and function to the eukaryotic enzyme fatty acid synthase (Dondadia *et al*, 1991; Hopwood and Sherman, 1990) (Figure 2).

Polyketide synthesis in aflatoxin biosynthesis typically consists of sequential condensation or chain elongation of malonyl subunits followed by modification of the growing chain. The source of the malonyl subunit is believed to be derived most often from the carboxylation of acetyl CoA at the expense of one ATP (Dutton, 1988; Bhatnagar *et al*, 1992) (Figure 3). Condensation of malonyl CoA to the growing carbon chain, which is bound to the acyl carrier protein (ACP), is catalyzed by β-ketoacyl synthase. Acetyl transferase (or CoA-

Figure 1. Proposed pathway for the biosynthesis of aflatoxin B_1. *A. parasiticus* mutants impaired in specific bioconversion steps are represented next to an arrow disrupted by a double line. The arrow disrupted by a single line represents inhibition of conversion by the insecticide dichlorvos.

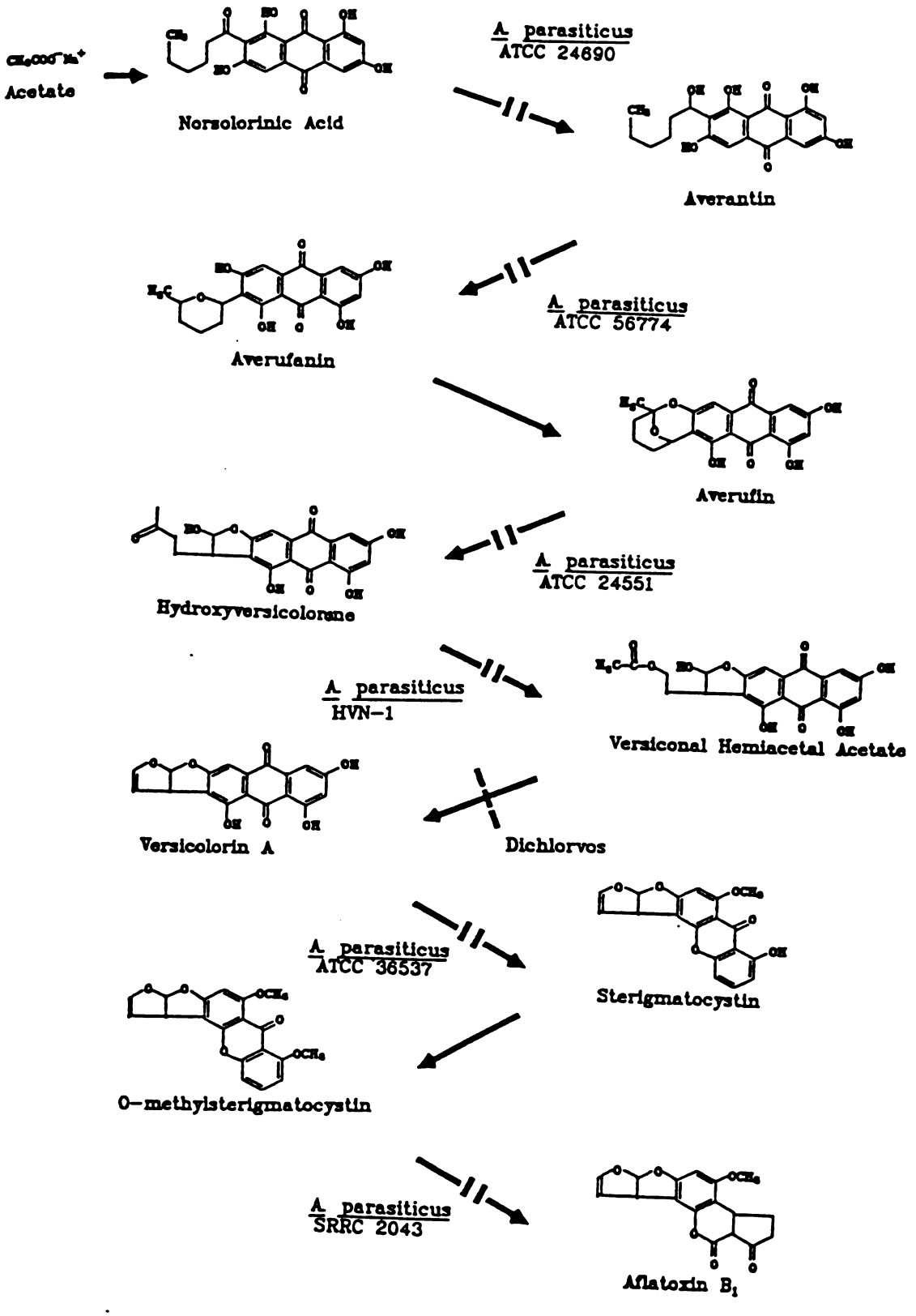

Acetate
Norsolorinic Acid
A. parasiticus
ATCC 24690
Averantin
A. parasiticus
ATCC 56774
Averufanin
Averufin
A. parasiticus
ATCC 24551
Hydroxyversicolorone
A. parasiticus
HVN-1
Versiconal Hemiacetal Acetate
Versicolorin A
Dichlorvos
A. parasiticus
ATCC 36537
Sterigmatocystin
O-methylsterigmatocystin
A. parasiticus
SRRC 2043
Aflatoxin B1

Figure 2. Proposed pathway for the production of norsolorinic acid through polyketide biosynthesis. The oval structure represents the polyketide synthase containing an acyl carrier protein (■) and a β-ketoacyl synthase (○). Thiol groups are represented as -SH.

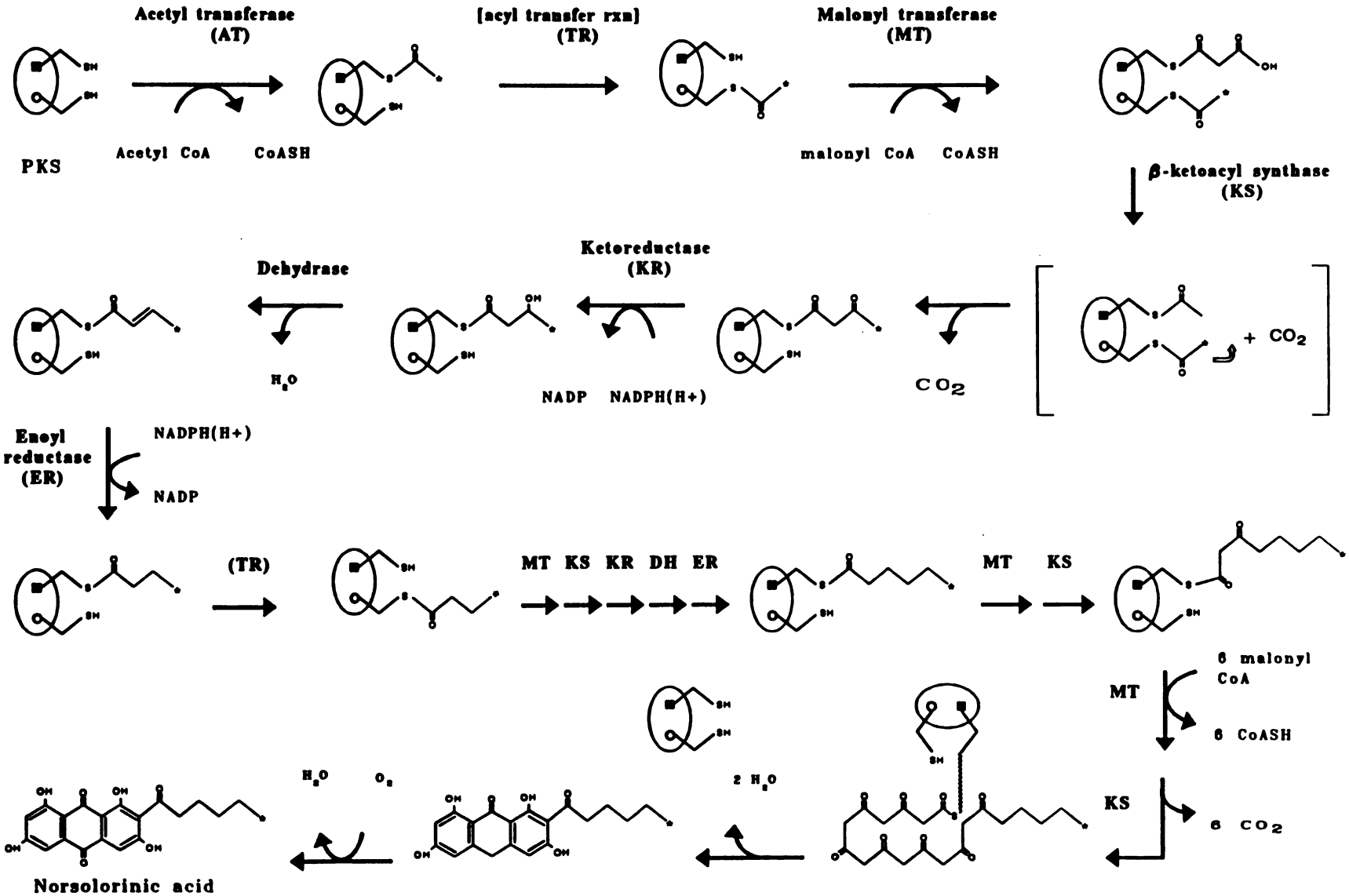
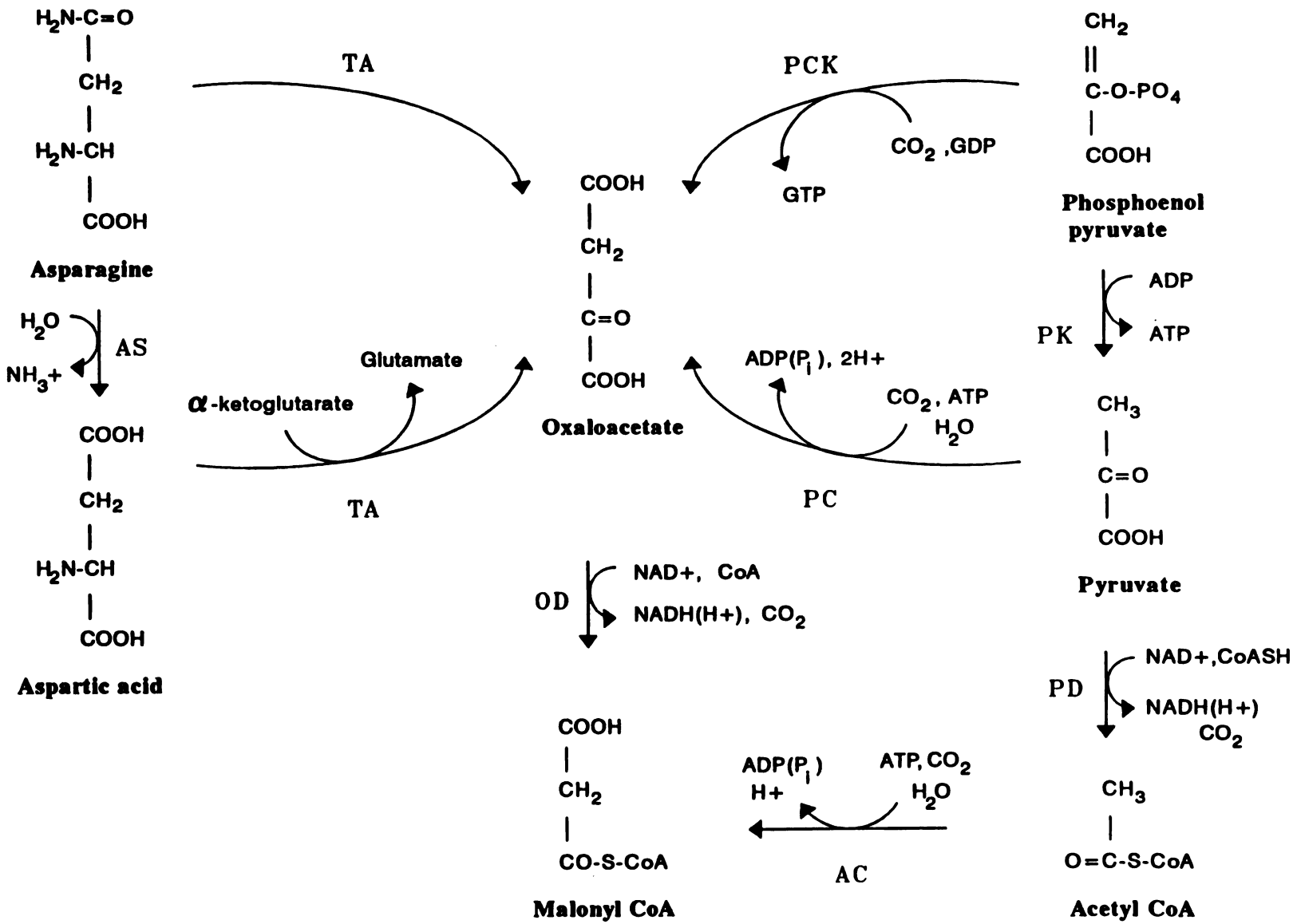

Acetyl transferase
(AT)
PKS
Acetyl CoA
CoASH
[acyl transfer rxn]
(TR)
Malonyl transferase
(MT)
malonyl CoA
CoASH
β-ketoacyl synthase
(KS)
+ CO2
CO2
Ketoreductase
(KR)
NADP
NADPH(H+)
Dehydrase
H2O
Enoyl
reductase
(ER)
NADPH(H+)
NADP
(TR)
MT KS KR DH ER
MT
KS
6 malonyl
CoA
MT
6 CoASH
KS
6 CO2
2 H2O
H2O
O2
Norsolorinic acid

Figure 3. Potential sources of malonyl CoA and acetyl CoA for the incorporation into polyketide synthesis. TA, transamination; PCK, phosphoenolpyruvate carboxykinase; PC, pyruvate carboxylase; AC, acetyl CoA carboxylase; PD, pyruvate dehydrogenase; PK, pyruvate kinase; AS, asparaginase; OD, oxaloacetate dehydrogenase.


H2N-C=O
CH2
H2N-CH
COOH
Asparagine
TA
H2O
AS
NH3+
COOH
CH2
H2N-CH
COOH
Aspartic acid
α-ketoglutarate
Glutamate
TA
COOH
CH2
C=O
COOH
Oxaloacetate
PCK
CO2 ,GDP
GTP
CH2
C-O-PO4
COOH
Phosphoenol pyruvate
ADP
PK
ATP
CH3
C=O
COOH
Pyruvate
ADP(Pi), 2H+
CO2, ATP
H2O
PC
OD
NAD+, CoA
NADH(H+), CO2
PD
NAD+,CoASH
NADH(H+)
CO2
COOH
CH2
CO-S-CoA
Malonyl CoA
ADP(Pi)
H+
ATP, CO2
H2O
AC
CH3
O=C-S-CoA
Acetyl CoA

ACP transacylase) and malonyl transferase (or malonyl CoA-ACP transacylase) are responsible for catalyzing the binding of acetyl and malonyl groups, respectively, to the acyl carrier protein. Initially, chain elongation proceeds analogous to fatty acid biosynthesis for the production of the six carbon carboxylic acid, hexanoate (or caproic acid) . This occurs by the sequential condensation of two units of malonate to an acetate precursor. Prior to each condensation step, β-ketoacyl synthase converts each malonyl subunit to acetate by the removal of one CO_2. Each added malonyl subunit is then modified by a ketoreductase, a dehydrase, and then an enoyl reductase to form hexanoic acid. The remaining seven malonyl subunits are added without any subsequent reduction or dehydration. The resultant polyketide is converted to an anthrone structure by dehydration and finally to norsolorinic acid by dehydration and aerobic oxidation (Zaika and Buchanan, 1987; Dutton, 1988; Buchanan *et al*, 1992; Hopwood and Sherman, 1990; Beck *et al*, 1990).

Norsolorinic acid (NA) is the earliest stable decaketide anthraquinone pigment yet isolated in the pathway. Using ultraviolet mutagenesis, Lee *et al* (1971) obtained an *A. parasiticus* mutant which accumulated significant quantities of norsolorinic acid within its mycelium. Buildup of this bright orange-red pigment was found to be the result of impaired conversion of norsolorinic acid to averantin. This conversion step is presumably catalyzed by a dehydrogenase which reduces the carbonyl side chain to form averantin (Dutton, 1988). It

should be noted that the *nor*-1 mutant, *A. parasiticus* ATCC 24690 is a leaky mutant that is still capable of producing some aflatoxin (Table I).

Identification of averantin as a pathway intermediate was possible by using an *A. parasiticus* Afl$^-$ mutant which accumulated this intermediate (Bennett *et al*, 1980). Radiolabelled precursor studies revealed that this compound could be incorporated into AFB_1. The conversion of averantin to averufin was originally thought to occur through the intermediate averufanin (McCormick *et al*, 1987). However, Dutton *et al* (1988) proposed that averufanin was not an intermediate in the aflatoxin biosynthetic pathway, but acted as a side shunt at conversion of the hypothetical 5'-hydroxyaverantin (HAVN) to averufin by a dehydrogenase. It was not until recently that Yabe *et al* (1991) were able to identify and isolate the compound 5'-hydroxyaverantin from a related fungus and demonstrate that it was capable of being converted to AFB_1. This evidence thus suggests the conversion sequence: Norsolorinic acid (NA) → Averantin (AVN) → 5'-hydroxyaverantin (HAVN) → averufin (AVF).

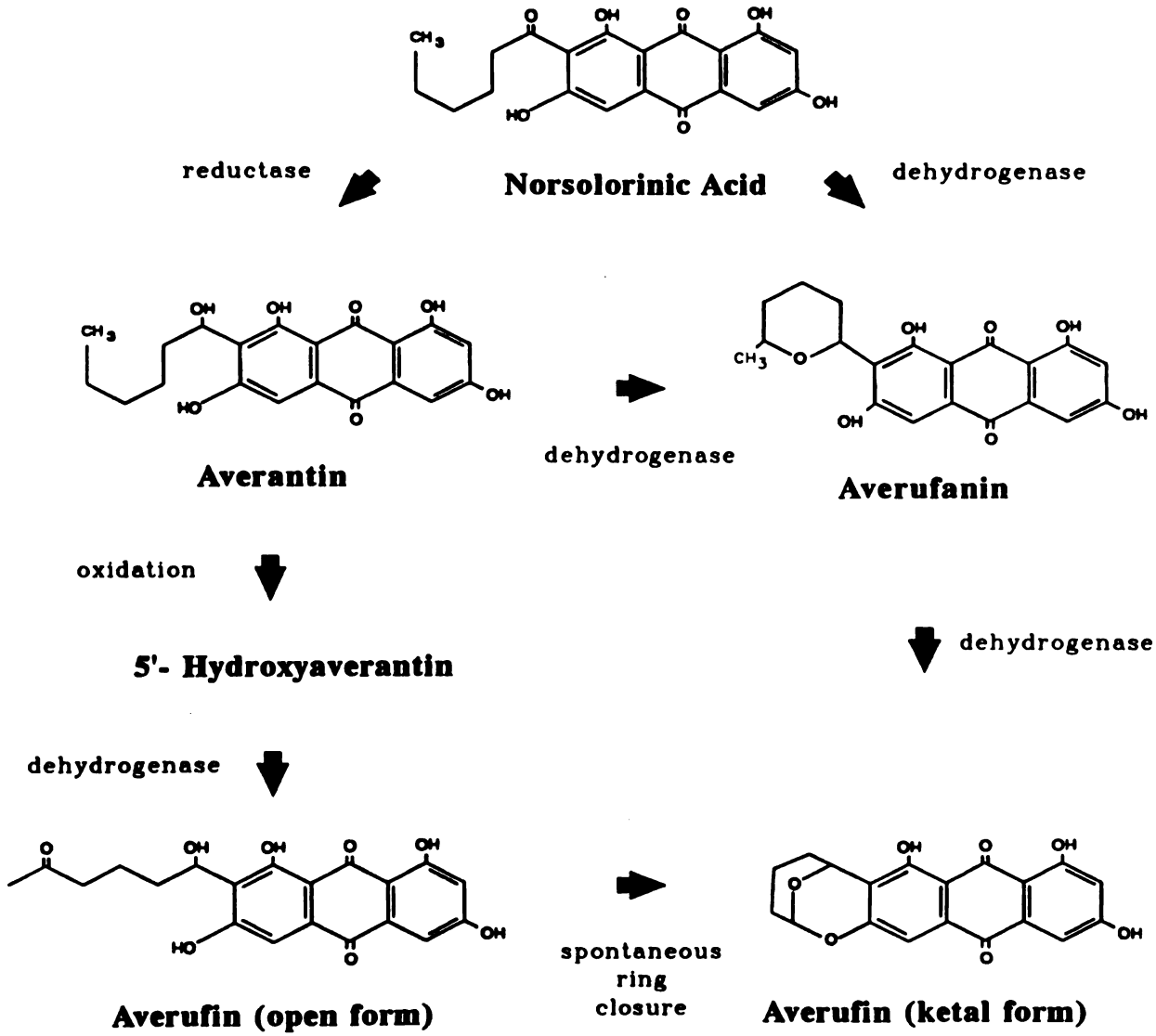
Bhatnagar *et al* (1992) have proposed that the production of averufin from norsolorinic acid could involve alternative routes of biosynthesis (Figure 4). Two reductases that are associated with the biotransformation of NA to averantin were purified by two different laboratories (Chuturgoon *et al*, 1990; Bhatnagar *et al*, 1990). Purification of one of these dehydrogenase activities was performed by coupling NA to a

Table I. Production of aflatoxin by Afl⁻ mutants of *A. parasiticus*

A. parasiticus strain	Metabolite accumulated	Aflatoxin produced[a] (ppb)			
		B_1	B_2	G_1	G_2
Wild-type SRRC 143	aflatoxins	575,172	134,508	363,301	33,652
ATCC 24690 (*nor*-1)	norsolorinic acid	64,432	34,419	12,643	tr
ATCC 56774 (*avn*-1,*nor*-1)	averantin, versicolorin A	n.d.	n.d.	n.d.	n.d.
ATCC 24551 (*avf*-1)	averufin	967	11,209	tr	tr
ATCC 36537 (*ver*-1)	versicolorin A	n.d.	n.d.	n.d.	n.d.
SRRC 2043 (*oms*-1)	O-methyl sterigmatocystin	n.d.	n.d.	n.d.	n.d.

[a]Cultures were grown for 7 days as described in previous studies (Bhatnagar *et al* 1987) and analyzed for aflatoxin content. Genotypes of fungal strains are indicated in parentheses under strain designation. Tr, trace (<100 ppb); n.d., none detected. This table was adapted and reproduced from Bhatnagar *et al* 1992.

Figure 4. Proposed conversion of norsolorinic acid to averufin.


Norsolorinic Acid
reductase
dehydrogenase
Averantin
dehydrogenase
Averufanin
oxidation
5'- Hydroxyaverantin
dehydrogenase
dehydrogenase
Averufin (open form)
spontaneous ring closure
Averufin (ketal form)

solid column matrix and passing a crude cell free homogenate containing the enzyme through the affinity column (Chuturgoon *et al*, 1990). This purified enzyme has been found to be capable of catalyzing the conversion of NA to averantin and has a molecular mass of approximately 140 KDa (Chuturgoon and Dutton, 1991). Bhatnagar *et al* (1990) were able to purify a NA reductase using a five step sequential purification scheme. It was determined with SDS-PAGE that two major protein bands of 38 KDa and 48 KDa were present in this purified sample. Polyclonal antibodies against this protein mix were capable of blocking the NA acid reductase activity. However, it is still questionable whether only one enzyme activity is present is this sample. Without knowing what bioconversions normally occur with NA, we cannot rule out the possibility that Bhatnagar *et al* are observing activity of two or more enzymes which are capable of converting NA to averantin by a different route than the proposed single step conversion. However, finding two supposedly different enzymes that apparently bind and catalyze the conversion of NA does seem to support the hypothesis of alternative pathway steps in the biotransformation of NA as suggested by Bhatnagar *et al* (1992). Additionally, it is also possible that *A. parasiticus* ATCC 24690 (*nor*-1) may be leaky as a result of only one of the proposed bioconversion routes being impaired.

Recently, Chang *et al* (1992) have isolated a gene that is capable of restoring aflatoxin biosynthesis to the described NA accumulating mutant through genetic complementation.

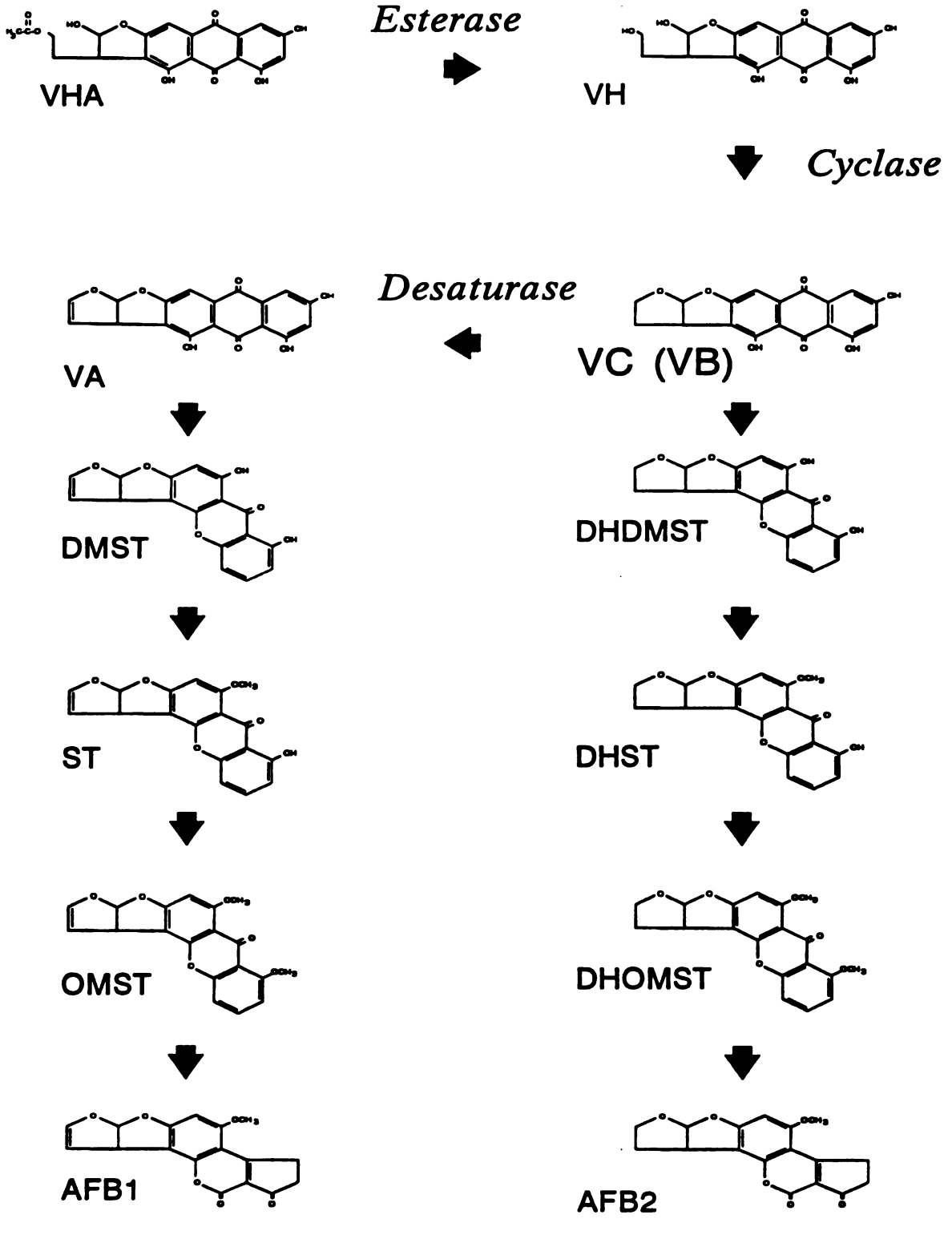
Sequence analysis of this gene suggests that it most likely encodes a dehydrogenase with a predicted molecular size of approximately 29 Kda (Chang *et al*, unpublished). It is believed that this dehydrogenase is involved in reduction of norsolorinic acid, since NA accumulates in the absence of this activity. However, the discrepancy in size from the 110 Kda and 38/48 kDa dehydrogenases previously isolated indicates either a flaw in the proposed pathway or an alternative catalytic activity for the enzyme encoded by the *nor*-1 gene. If the intermediate 5-hydroxyaverantin is included into Bhatnagar's proposed pathway, it is possible that the *nor*-1 dehydrogenase is instead involved in the conversion of 5-hydroxyaverantin to averantin. Successful gene disruption of the *nor*-1 gene in an aflatoxin producing *A. parasiticus* strain should help identify the function of this gene and determine whether alternative conversion steps exist.

It is generally accepted that averufin is converted to 1-hydroxyversicolorone and then eventually to vericonal hemiacetal acetate (VHA). Mutant strains are available that are impaired in each of these bioconversion steps (Donkersloot *et al*, 1972; Townsend *et al*, 1988). The pathway intermediate versiconal hemiacetal acetate was identified by Shroeder *et al* (1974) when it was noted that an orange pigment accumulated when the fungus was grown in the presence of the insecticide dichlorvos which is an acetylcholine esterase inhibitor. It was later determined that this accumulation was the result of enzymatic inhibition of the conversion of versiconal

hemiacetal acetate to versicolorin A (Yao and Hsieh, 1974; Bennett *et al*, 1976). Dichlorvos presumably prevents the removal of the esterified acetate on versiconal hemiacetal acetate which is required for the closure of the terminal furan ring (Bennett *et al*, 1976).

It has recently been proposed that the conversion of VHA to versicolorin A (VA) involves several steps that include a point of branching for the production of AFB_1 and AFB_2 (Lin and Anderson, 1992; Yabe *et al*, 1991; Yabe *et al*, 1991; Bhatnagar *et al*, 1991) (Figure 5). Hsieh *et al* (1989) first identified the enzymatic activities of aflatoxin producing strains that were capable of converting VHA to a versiconal hemiacetal alcohol (VH) and then eventually to versicolorin C (VC). These enzymatic steps were later confirmed to be catalyzed by an esterase and a cyclase respectively (Anderson and Chung, 1990). Lin and Anderson (1992) were able to purify the versiconal cyclase to homogeneity and determine that it contains two identical subunits of a molecular size of 72 KDa each. It should be noted that VC and versicolorin B (VB) are often used interchangeably because VC is a racemic mixture of VB (VB and VB^{*}) and can't be separated by conventional chromatographic techniques. Versicolorin C (or VB) can be converted to either VA by way of a desaturase or to dihydrodemethylsterigmatocystin (DHDMST) by means not yet established. Because of substrate similarities, the enzymes involved in the conversion of VA to demethylsterigmatocystin (DMST) most likely also catalyze the conversion of VC to

Figure 5. Proposed conversion of versiconal hemiacetal acetate to aflatoxin B_1 and aflatoxin B_2. VHA, versiconal hemiacetal acetate; VH, versiconal hemiacetal alcohol; VA, versicolorin A; VC, versicolorin C; DMST, demethylsterigmatocystin; DHDMST, dihydrodemethylsterigmatocystin; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, O-methylsterigmatocystin; DHOMST, dihydro-O-methylsterigmatocystin; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2.


Esterase
VHA
VH
Cyclase
Desaturase
VA
VC (VB)
DMST
DHDMST
ST
DHST
OMST
DHOMST
AFB1
AFB2

DHDMST.

A mutant deficient in the conversion of versicolorin A to sterigmatocystin was isolated by Bennett and Goldblatt (1973) using ultraviolet mutagenesis. This isolate was unable to produce aflatoxins and produced significant quantities of four bright yellow pigments with the predominant compound designated as versicolorin A (Lee *et al*, 1975). Singh and Hsieh (1977) were able to confirm the position of VA in the biosynthetic pathway through the use of radiolabeled precursor studies. Little is yet known regarding the conversion of this anthraquinone containing intermediate to the xanthone based compound, sterigmatocystin (ST). A gene has been isolated (described in this thesis) which is capable of restoring aflatoxin production to a strain derived from the VA accumulating mutant isolated by Bennett and Goldblatt (1973). Sequence analysis strongly suggests that the product of this gene is a reductase. However, it should be mentioned that multiple steps are most likely involved in the conversion of VA to ST and would not be expected to be catalyzed only by a single reductase.

Sterigmatocystin was suggested to be a pathway intermediate by Hsieh *et al* (1973) and later confirmed by Sing and Hsieh (1976). Bhatnagar *et al* (1988) demonstrated that sterigmatocystin was converted to O-methylsterigmatocystin (OMST) by a methyltransferase and finally to AFB_1 by an oxidoreductase (Bhatnagar *et al*, 1991). There is strong evidence that an immediate precursor to sterigmatocystin (ST)

is a compound, demethylsterigmatocystin (DMST), that lacks methylation on both the C-7-OH and C-6-OH groups (Yabe *et al*, 1989). Methylation of DMST and ST are believed to be catalyzed by different methylases which also appear to catalyze the methylation of dihydrodemethylsterigmatocystin (DHDMST) and dihydrosterigmatocystin (DHST), respectively (Yabe *et al*, 1989). Additionally, the methoxy methyl group of AFB_1 was found to be indicative of methionine origin. A methyltransferase that appears to be involved in the methylation of ST and DHST has been purified to homogeneity and determined to contain subunits of a molecular mass of 110 KDa and 58 KDa (Bhatnagar *et al*, 1989). A second unique methyltransferase has recently been isolated by Keller *et al* (1991) and shown to also be involved in synthesis of aflatoxins. This methyltransferase is believed to be involved in methylation of DMST and DHDMST.

Regulation of aflatoxin biosynthesis.

Aflatoxins are considered to be secondary metabolites because they do not seem to be essential for growth and reproduction, and their synthesis seems to be unique from that of primary metabolism. However, regulation of secondary metabolism can not be examined without considering primary metabolism since the products of primary pathways serve as precursors for secondary metabolites (Drew and Demain, 1977).

Additionally, it is believed that primary metabolites may often be involved in the induction of enzymes of secondary metabolism.

Buchanan *et al* (1985) demonstrated that aflatoxin biosynthesis occurred during a period of repressed tricarboxylic acid cycle (TCA) activity. It was shown that the activities of glucose 6-phosphate dehydrogenase, mannitol dehydrogenase and malate dehydrogenase decreased shortly after the addition of glucose and just prior to the induction of aflatoxins (Buchanan *et al*, 1984). He suggested that aflatoxin biosynthesis may be regulated by a carbon catabolite repression of NADPH-generating and TCA cycle enzyme activities. The overall energy status of the fungal cell does not appear to significantly change as a result of these alterations in the glycolytic and TCA cycle activities. However, findings that concurrent inactivation of mitochondria occurs with glucose induced aflatoxin biosynthesis may suggest that control is partly regulated by the energy status of specific subcellular compartments (Buchanan *et al*, 1987).

Additional evidence of the relationship between aflatoxin biosynthesis and levels of NADPH was provided indirectly by the ability of zinc to induce aflatoxin biosynthesis (Coupland and Niehaus, 1987; Niehaus and Failla, 1984). Foreman and Niehaus (1985) revealed that zinc ions inhibit the enzyme activities of mannitol dehydrogenase, mannitol 1-phosphate dehydrogenase, glucose 6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase. It is theorized that

inhibition of enzymes of the pentose phosphate pathway (also called phosphogluconate pathway) and NADPH generating enzymes of the mannitol cycle result in a lowering of the cellular NADPH/NADP ratio and an increased activity of aflatoxin biosynthetic enzymes. Additional evidence to support this notion was provided by determining that nitrate induces synthesis of the enzymes in the pentose phosphate pathway and the mannitol cycle, thereby resulting in an inhibition of aflatoxin biosynthesis (Neihaus and Jiang, 1989).

The precursors for aflatoxin biosynthesis rely on many metabolites of primary metabolism. A correlation between aflatoxigenic isolates and high pyruvate kinase activity was noted by Tyagi and Venkitasubramanian (1981). This relationship is believed to be a result of additional pyruvate being made available for conversion into malonyl CoA (Figure 3). Malonyl CoA, as previously described, can then be converted to acetate and incorporated into AFB_1. Asparagine and aspartate can also stimulate aflatoxin biosynthesis presumably by providing malonyl CoA through transamination and oxidation (Dutton, 1988; Reddy *et al*, 1971).

It is generally believed that filamentous fungi usually control gene expression at the transcriptional level (Gurr *et al*, 1989). The work of Buchanan *et al* (1987) provided evidence that aflatoxin biosynthesis is at least partially regulated at the transcriptional level. This was demonstrated using transcription (actinomycin D) and translation (cycloheximide) inhibitors after culture

replacement from a medium unable to support aflatoxin production to an aflatoxin inducing and supporting medium. It was established that transcriptional and translational processes associated with aflatoxin biosynthesis occurred 3-6 hrs and 6-10 hrs respectively following transfer to the aflatoxin supporting medium. Cleveland and Bhatnagar (1990) found that the methyltransferase enzyme was produced *de novo* slightly before the onset of aflatoxin appearance.

Mutagenicity and carcinogenicity of aflatoxin and its intermediates.

All precursors prior to versicolorin A are believed to lack significant mutagenic potential. Wong *et al* (1977) concluded that the mutagenicity, as determined by the Ames Test, of versicolorin A resides in the bisfuran ring rather than the anthraquinone moiety. Sterigmatocystin, which has a xanthone structure in place of the anthraquinone moiety demonstrated a two-fold increase in mutagenicity. Conversion of sterigmatocystin to the coumarin containing AFB_1 resulted in approximately a 10 fold increase in mutagenicity. Hendricks *et al* (1980) chose to use the embryo exposure technique as a means of predicting the carcinogenic potential of versicolorin A and sterigmatocystin. It was established that the relative hepatocarcinogenicity of AFB_1 to versicolorin A is between sixteen and fifty times more potent.

Sterigmatocystin was estimated to be approximately one-fourth the carcinogenic potency of AFB_1.

It is now believed that the biological activity of the intermediates and AFB_1 is generated by the epoxidation of the 2,3-vinyl ether double bond in the terminal furan ring and its subsequent covalent binding to nucleic acid (Essigman *et al*, 1982). This bioactivation of AFB_1 in mammalian systems is believed to occur by way of the mixed function oxidase cytochrome P450 (Shimada and Guengerich, 1989; Kitada *et al*, 1990). The resultant epoxide is capable of forming adducts in DNA, primarily at nitrogen-7 of guanine, as well as reacting with RNA and protein (Stark, 1980). DNA adducts following exposure to AFB_1 are found primarily in the liver although they can also be found in other tissues (Wild *et al*, 1990). AFB_1-DNA adducts are believed to inhibit RNA synthesis (Yu *et al*, 1990) and induce mutagenic effects (e.g. frameshifts) as well as several other genotoxic effects (Chu, 1991).

The toxic effects of aflatoxins were first realized in 1960 after the death of approximately 100,000 young turkeys in England, which had consumed aflatoxin contaminated feed (Nesbitt *et al*, 1962). Since then, numerous studies have been performed to confirm the powerful toxic and carcinogenic potential of aflatoxins in animal models (Heathcote, 1978). Acute aflatoxicosis in humans has been observed in parts of Taiwan, Thailand, Uganda, and Kenya. Consumption of foods highly contaminated with aflatoxin has resulted in varied symptoms that include loss of appetite, vomiting, abdominal

pain, pulmonary edema, necrosis of the liver, signs which resemble Reye's syndrome, and death (CAST, 1989). Aflatoxins have also been shown to have immunosuppressive effects in mammals. Recognizing aflatoxins as the root cause of an infectious disease is difficult since the symptoms of aflatoxicosis will likely be masked by the manifestation of the infection itself (Pestka and Bondy, 1990; Cusumano *et al*, 1990).

Identifying aflatoxins as a causative agent in human cancers becomes exceeding difficult because of the reliance on epidemiological studies. There does appear to be a geographical correlation between incidence of human liver cancer and prevalence of aflatoxins (Stoloff, 1976). Liver cancer is the most common malignancy in males in parts of South-East Asia and sub-Saharan Africa where dietary exposure to aflatoxins is high (Parkin *et al*, 1980). Wild *et al* (1990) were able to detect aflatoxin-albumin adducts in the sera of 12-100% of subjects tested throughout parts of Africa. This contrasts with no detection of any such adducts from sera of those from France or Poland where exposure to aflatoxin is much lower. It can then be inferred that the high incidence of primary liver cancer could be a result of aflatoxin ingestion and metabolism to the mutagenic forms. However, studies are complicated by the fact that hepatitis B virus is usually endemic in many of regions where aflatoxins present a potential threat of inducing primary liver cancer. This interfering element of complexity with the lack of any other

strong epidemiological evidence has persuaded many researchers to argue that aflatoxins are not a *probable human carcinogen* (Stoloff, 1989; Campbell *et al*, 1990; Campbell *et al*, 1991). Whether aflatoxins act synergistically with hepatitis B virus to result in liver cancer is a strong possibility that is currently being investigated (Chu, 1991). An interesting observation is the common association of primary liver carcinomas believed to be a result of aflatoxins and/or hepatitis B virus and mutations in the third position of codon 249 of the p53 gene. Studies with aflatoxin induced tumors in nonhuman primate models suggest that mutations in the p53 gene are not prerequisite for primary liver cancer (Fujimoto *et al*, 1992). Further studies will be needed to determine the relationship of this specific mutation and aflatoxin/hepatitis B exposure. While additional studies are needed to confirm the association between aflatoxins and primary liver cancer, there does appear to be ample evidence that aflatoxin pose a potential health risk. Acute toxicity in animals and humans should be reason enough to warrant aggressive research into the prevention of this common food contaminant.

MATERIALS AND METHODS

Strains and culture conditions.

Escherichia coli HB101 [*hsd*S20 ($r_B^-m_b^-$), *rec*A13, *ara*-14, *pro*A2, *lac*Y1, *gal*K2, *rps*L20, Sm^r, *xyl*-5 *mtl*-1, *sup*E44] was used for propagating plasmids. *E. coli* LE392 [F^-e14$^-$(*mcr*A$^-$) *hsd*R514 ($r_k^-m_k^+$)*sup*E44, *sup*F58, *lac*Y1, *gal*K2, *gal*T22, *met*B1, *try*R55] was the recipient strain for packaged Lambda phage. *Aspergillus parasiticus* NRRL 5862 (SU-1) served as the aflatoxin producing wild type strain. *A. parasiticus* CS10 (*ver*-1, *wh*-1, *pyr*G) (Skory *et al* 1990), derived from *A. parasiticus* ATCC 36537 (*ver*-1, *wh*-1) (Bennett and Goldblatt, 1973), was used in complementation studies for the isolation of the *ver*-1 gene . *A. parasiticus* ATCC 36537 was obtained by ultraviolet mutagenesis of *A. parasiticus* NRRL 5862. This "blocked mutant" is unable to convert VA to ST. Although the exact nature of the *ver*-1 mutation is not known, neither *A. parasiticus* ATCC 36537 (Bennett and Goldblatt, 1973; Bhatnagar *et al*, 1992) nor *A. parasiticus* CS10 produce detectable levels of aflatoxins in liquid or solid growth media. Additionally, neither strain has been observed or reported to revert back to Afl^+.

Fungal strains were maintained on potato dextrose agar (PDA) or Czapek-Dox agar (CZA) that served as a defined

medium. Coconut agar medium (CAM) was used for screening fungal strains for aflatoxin accumulation by visualization of blue fluorescence under ultraviolet light (Davis *et al*, 1987). Cultures were incubated at 30°C unless otherwise indicated.

Chemicals.

Chemicals, unless specifically referenced in the text were purchased from Sigma Chemical Company. Agarose was purchased from Bethesda Research Laboratories (Gaithersburg, MD). PDA and CZA were purchased from Difco Laboratories (Detroit, MI).

Development of a genetic transformation system

Isolation of *pyr* mutant strains.

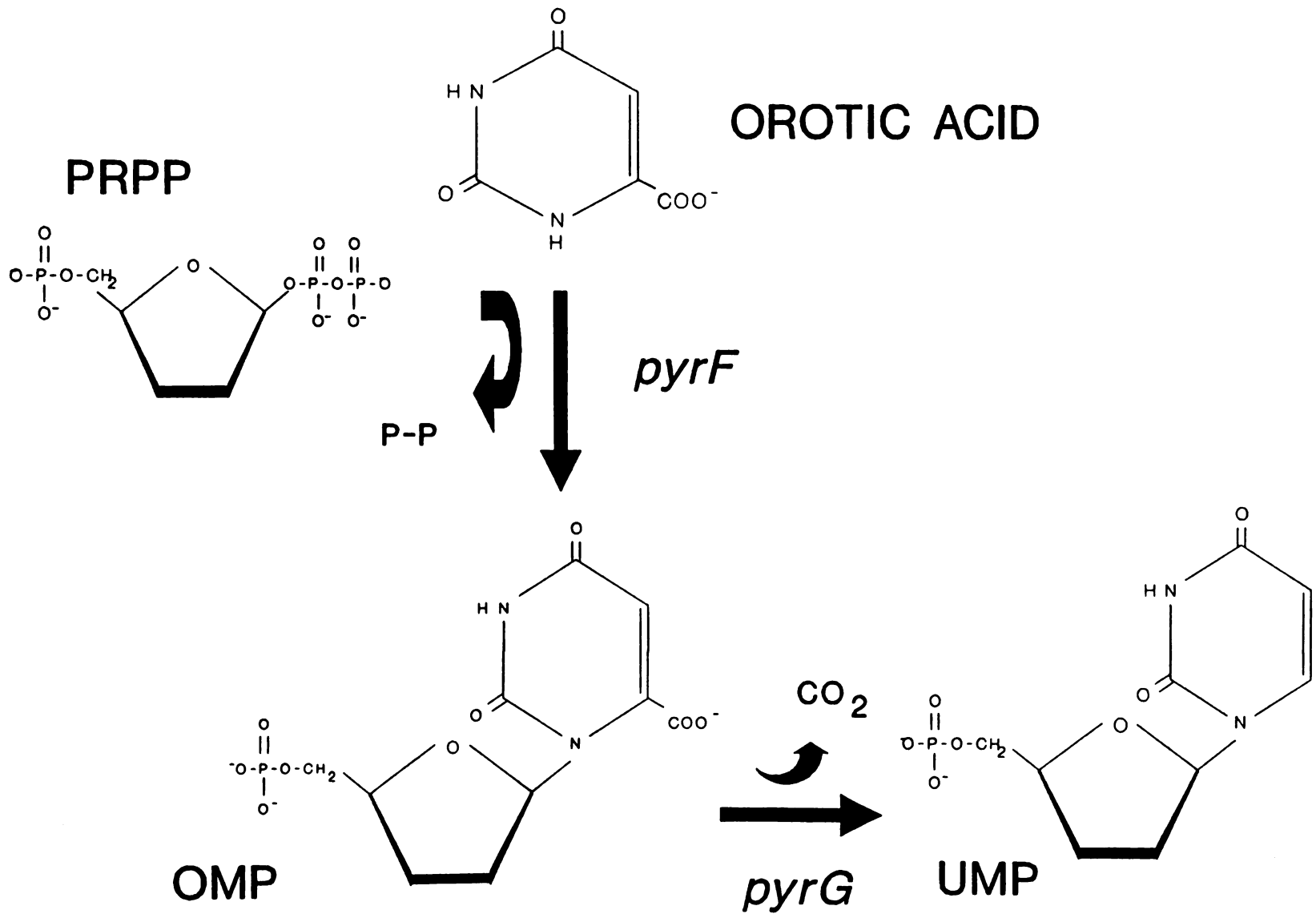
Conidiospores (2×10^8) from the versicolorin A accumulating strain *A. parasiticus* ATCC 36537 were resuspended in 1 ml of Tris-maleate buffer (50 mM Tris-maleate [pH 7.3], 35 mM $NaNO_3$, mM KCl, 4 mM $MgSO_4$, 65 μM $FeSO_4$) and treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) at a concentration of 170 ng/ml for 1 hr 45 min at room temperature. Conidia were washed and resuspended in PD broth (2.4 mg/ml uridine)

and grown in stationary culture at 30° C for two hrs before inoculating onto PDA containing 5-fluoro-orotic acid (FOA) (2 mg/ml) and high concentrations of uridine (2.4 mg/ml). FOA is lethal to uridine prototrophs of *A. parasiticus* presumably due to the enzymatic synthesis of the toxic intermediate 5-fluoro-UMP (Boeke *et al*, 1984). The enzymes orotate phosphoribosyl transferase and orotidine monophosphate decarboxylase are involved in the conversion of orotate to orotidine monophosphate (OMP) and OMP to uridine monophosphate (UMP) respectively (Figure 6). Mutations in either the *pyr*F (orotate phosphoribosyl transferase) or the *pyr*G (orotidine monophosphate decarboxylase) gene resulting in loss of enzymatic activity will confer resistance to FOA by preventing the production of 5-fluoro-UMP. Colonies resistant to FOA were further tested for stable uridine auxotrophy by serial replica-plating onto CZ agar with and without uridine.

Enzymatic analysis of *pyr* mutants.

Since FOA selects for either *pyr*F or *pyr*G mutant strains, enzyme analysis with radiolabelled substrates was used to determine the ability of auxotrophic isolates to convert orotic acid to OMP (*pyr*F) and the ability to convert OMP to UMP (*pyr*G), thereby differentiating between the two mutant types (Figure 6). The protocol employed was a modification of that used by Diez *et al* (1987). Conidia (1×10^7) from

Figure 6. Synthesis of uridine 5'-monophosphate. Phosphoribosyl transferase is encoded by the gene *pyr*F, while OMP-decarboxylase is encoded by the gene *pyr*G. PRPP, phosphoribosyl pyrophophate; P-P, pyrophosphate; OMP, orotidine 5'-monophosphate; UMP, uridine 5'-monophosphate.


PRPP
OROTIC ACID
P-P
pyrF
OMP
CO2
pyrG
UMP

uridine auxotrophs were inoculated into 100 ml of PD broth containing uridine and grown 48 hrs with vigorous shaking at 30° C. Mycelium was harvested by filtration through cheesecloth, frozen in liquid nitrogen, and ground to a fine powder in a precooled mortar and pestle. Powdered mycelium was resuspended in enzyme buffer (25 mM Tris-Cl [pH 8.0], 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol) at a concentration of 1 g mycelium/ml buffer. This suspension was centrifuged at 4° C in a microcentrifuge for 2 min at 16,000 x g. The supernatant was transferred to a new tube and centrifuged under the same conditions for 15 min. The supernatant was again removed and 8.5 μl aliquots were dispensed into two prechilled microcentrifuge tubes. The remaining supernatant was used to confirm that protein concentration was between 0.5 - 1.0 mg protein/ml by the Bradford technique (1976). The standard curve for the Bradford method was performed with BSA dissolved in enzyme buffer (see above) since both Tris-Cl and 2-mercaptoethanol can affect the results of the protein determination.

[6-^{14}C]-OMP was prepared as described by Diez *et al* (1987) utilizing the enzymatic conversion of [6-^{14}C]-orotate (ICN, 50 mCi/mmol) with commercial yeast orotidine monophosphate pyrophosphorylase (orotate phosphoribosyl transferase) and 5-phosphoribosyl-1-pyrophosphate (PRPP). To the first tube containing the crude cell free enzyme extract, 3.5 μl of prepared OMP was added followed by 0.5 μl 0.2 M sodium arsenate. To the second tube, 2.5 μl [6-^{14}C]-orotate

(1.5 mM), 25 mM Tris-Cl [pH 8.0], 8 mM $MgCl_2$), and 1 μl of PRPP (5 mg/ml, made fresh in chilled H_2O) was added. Both tubes were incubated for 2 hrs at 30° C followed by precipitation of proteins by the addition of 6 μl of methanol and storing overnight at -20° C. Tubes were centrifuged 3 min at 16k x g to remove precipitated proteins and 5 μl of the supernatant was spotted onto a 20 cm x 20 cm polyethylene-imine-cellulose thin-layer-chromatography (TLC) plate. Visual (observed under long wave U.V. light) and ^{14}C-labelled standards of orotic acid, OMP, and UMP were used as controls for determining the location and identity of each compound in enzyme reactions. Samples were separated using 0.75 M Tris-Cl [pH 8.0] as the solvent and the dried TLC plates were then analyzed by autoradiography by exposing the TLC plate to Kodak XAR5 film for 48 hrs at -70° C.

Transformation of fungal protoplasts.

Plasmid DNA used for transformation was purified by CsCl density gradient centrifugation (Maniatis *et al*, 1982) and resuspended in TE (10 mM Tris-Cl [pH 8.0], 1 mM EDTA). The polyethylene glycol transformation procedure described by Oakley *et al* (1987) was performed using the following modifications. Instead of using swollen conidia as suggested by Oakley *et al*, conidiospores were grown 12-14 hrs in YES (2% yeast extract, 6% sucrose, pH 5.5) and supplemented with 100

μg/ml uridine for auxotrophic mutants. Harvested mycelium was digested with Novozyme 234 and β-glucuronidase in one half diluted YES osmotically stabilized with 0.6 M KCl as performed by Oakley. However, Driselase was eliminated from the protoplasting solution and enzymatic incubation time was decreased from 5 hrs to 3 hrs. Protoplasts were separated from mycelium by passing the cell suspension (using gravity) through a 60 um nylon mesh filter (Nytex, Tetko Corporation, Switzerland) which allowed only protoplasts and small mycelial fragments to pass through. Protoplasts were then harvested, washed with 0.6 M KCl, 0.5 M $CaCl_2$ and treated with polyethylene glycol and 1-10 μg of DNA as described by Oakley *et al* and spread directly on CZ agar containing 0.6 M KCl. Protoplast preparations were always divided into two equal aliquots, one treated with DNA and the other untreated to serve as a control. Cotransformations were occasionally used to transform *A. parasiticus* CS10 to *pyrG*$^+$. Under these conditions, the *pyrG* selectable marker was included on a separate plasmid (pBP28) from the vector containing the transforming DNA of interest. Plasmid pBP28 was constructed by replacing the 0.28 kb BamH1/SalI fragment from pBR322 with a 2.8 kb BamH1/SalI DNA restriction fragment containing the *pyrG* gene from pPG3J. The vector containing DNA of interest was added in a two fold molar ratio over pBP28 for a total of ≈3 μg DNA in the transformation mixture.

Isolation and analysis of genomic DNA from fungal cells.

Genomic DNA was isolated from fungal mycelium using a phenol/chloroform protocol for mammalian DNA isolation (Ausubel *et al*, 1987) Restriction enzymes were purchased from Bethesda Research Laboratories, Inc (Gaithersburg, Md) and used according to the supplier's instructions. Southern hybridization analysis (Maniatis *et al*, 1982) was performed with ^{32}P labelled DNA probes generated by the random primer procedure (Random Primed DNA Labeling Kit, Boehringer Mannheim Biochemicals). Nitrocellulose filters (Schleicher & Schuell, Inc, Keene, NH) were hybridized to labelled probes overnight at 37° C in 6x SSC (1x SSC is: 0.15 M sodium chloride, 0.015 M sodium citrate)-5x Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA)-40% formamide-0.1% sodium dodecyl sulfate (SDS)-5 mM EDTA-100 μg denatured salmon sperm DNA/ml with probe (10^6 cpm/ml). Following hybridization, filters were washed twice in 2x SSC-0.1% SDS at 37°C for 20 min and then in 0.2x SSC-0.1% SDS at 65° for 20 min. Filters were exposed to Kodak XAR5 film for 20 hrs at -70° C with an intensifying screen (Lightning Plus-Dupont).

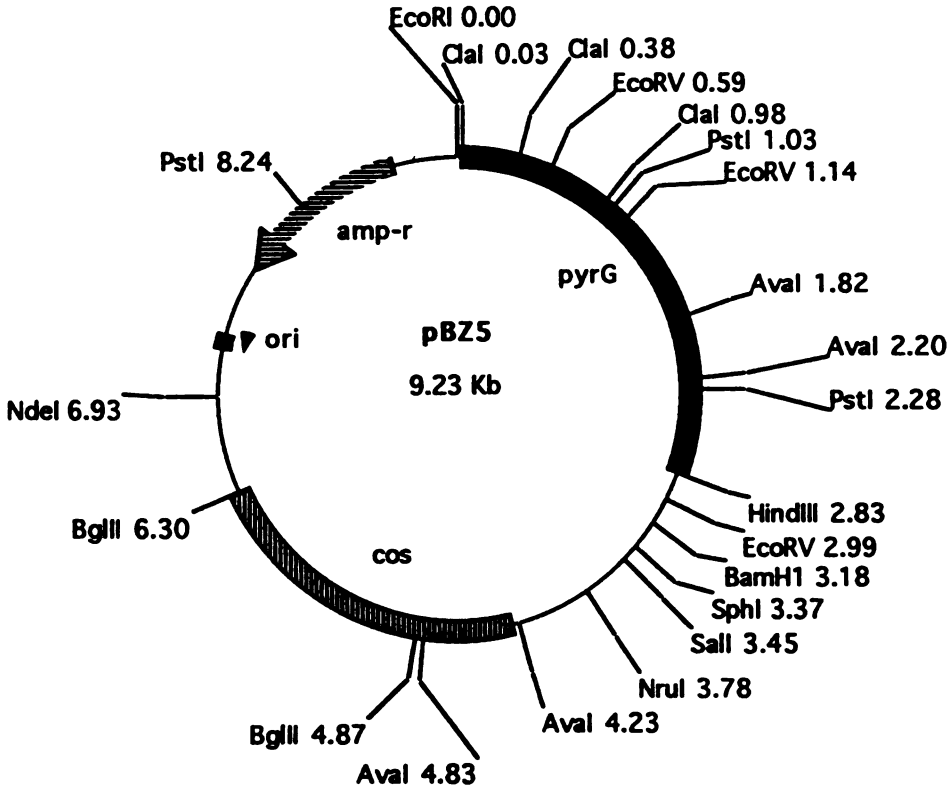
Isolation of the *ver*-1 gene

Preparation of cosmid library.

The cosmid vector pBZ5 (Figure 7) was constructed by ligation of the 2.8 kb BamH1/SalI *pyrG* containing restriction fragment from plasmid pPG3J, into the HindIII restriction endonuclease site of the cosmid vector pHC79 (Stratagene, LaJolla, CA). Ligation of the blunt ended *pyrG* fragment in pBZ5 regenerated the HindIII site since end filling of the SalI and HindIII restriction endonuclease sites completed the recognition sequence for HindIII. The ability to transform *A. parasiticus* CS10 to prototrophy at efficiencies comparable to that obtained with pPG3J confirmed the presence of the *pyrG* gene in pBZ5.

The cosmid genomic DNA library was prepared according to the procedures of Maniatis *et al* (1982). DNA from the wild type Afl^+ strain, *A. parasiticus* NRRL 5862, was used for library preparation. This DNA was partially digested by the restriction endonuclease Sau3A and size fractionated by sucrose density gradient centrifugation. Fractions containing DNA fragments between 30 to 40 Kb in size were ligated into the alkaline phosphatase-treated BamH1 site of pBZ5. Concatemers were then packaged into Lambda phage particles using a commercial packaging extract (Promega Corp., Madison, Wisconsin).

Figure 7. Cosmid vector pBZ5 used in the preparation of an *A. parasiticus* NRRL 5862 cosmid genomic DNA library. This vector was constructed by blunt end ligation of a 2.8 BamH1/SalI *pyrG* containing fragment from plasmid pPG3J (50) into the HindIII restriction endonuclease site of cosmid pHC79 (Stratagene, LaJolla, CA). DNA from a toxigenic *A. parasiticus* strain was digested with Sau3A, size fractionated, and then ligated into the alkaline phosphatase treated BamH1 site of pBZ5.


EcoRI 0.00
ClaI 0.03
ClaI 0.38
EcoRV 0.59
ClaI 0.98
PstI 1.03
EcoRV 1.14
PstI 8.24
amp-r
pyrG
AvaI 1.82
ori
pBZ5
9.23 Kb
AvaI 2.20
PstI 2.28
NdeI 6.93
HindIII 2.83
BglII 6.30
EcoRV 2.99
cos
BamH1 3.18
SphI 3.37
SalI 3.45
NruI 3.78
AvaI 4.23
BglII 4.87
AvaI 4.83

The DNA library was completed by transfecting *E. coli* LE392 with recombinant phage particles and transferring these cells to Luria-Bertani (LB) agar medium containing ampicillin (50 μg/ml). Overnight incubation at 37° C yielded approximately 870 colony forming units per petri plate (11x150 mm). Cells were harvested from ten plates and the suspension was used for cosmid isolation by alkaline lysis and CsCl purification (Maniatis *et al*, 1982).

Preparation of cDNA library.

A. parasiticus NRRL 5862 was inoculated into a glucose mineral salts medium (Adye and Mateles, 1964) and grown for a period of 48 hrs. RNA, isolated from the mycelium using a hot phenol purification protocol (Ausubel *et al*, 1987), was then used to prepare a cDNA library. This library was constructed by Stratagene (LaJolla, CA) using their Uni-Zap XR vector.

Screening of the cosmid genomic DNA library by genetic complementation of *A. parasiticus* CS10.

DNA from the combined cosmid DNA library was used in transformation of *A. parasiticus* CS10. Protoplasts were plated directly onto CAM supplemented with 20 percent sucrose as an osmotic stabilizer. CAM does not contain sufficient

uridine to support the growth of untransformed protoplasts. Only protoplasts receiving the *pyrG* marker, contained on the cosmid, were capable of growth on CAM. Transformant colonies displaying "blue fluorescence" under long wave U.V. light were isolated and reinoculated onto fresh PDA for further analysis and verification of aflatoxin production.

Confirmation of aflatoxin production by transformant clones.

Conidia (1 x 10^7) from transformant colonies were inoculated into 20 ml of YES (2 % yeast extract, 20 % sucrose [pH 5.5]) medium contained in a 50 ml erlenmeyer flask. Each flask also contained four, 4.5 mm glass beads to aid in aeration of the culture and to facilitate dispersed mycelial growth (Heathcote and Hibbert, 1978; Reynolds and Pestka, 1991). Cultures were then grown for 3 days at 28° C in an orbital shaker (150 rpm).

After incubation, cultures were filtered through miracloth (Behring Diagnostics, LaJolla, CA) and the filtrate was used for identification of aflatoxins. Only the filtrate was analyzed for aflatoxins since both aflatoxin B1 and G1 are known to pass efficiently through the mycelial walls into the surrounding medium (Lin *et al*, 1992). Filtrates from cultures of *A. parasiticus* CS10 and *A. parasiticus* NRRL 5862 were included as aflatoxin production negative and positive controls respectively. Filtrates were tested using thin layer

chromatography (TLC) and enzyme linked immunoabsorbent assay (ELISA).

TLC analyses were performed on activated 10 x 10 cm high performance silica TLC plates in an equilibrated chamber using chloroform/methanol (97:3) as a solvent system. Versicolorin A (kindly supplied by L. Lee and D. Bhatnagar, USDA Agriculture Research Service, New Orleans, L.A.) and aflatoxin B1, B2, G1, and G2 were resolved on each plate as reference standards. Direct competitive ELISA analyses were performed according to Pestka (1988) using aflatoxin B1 monoclonal antibodies (kindly provided by J. Pestka, Michigan State University) and aflatoxin B1-horseradish peroxidase conjugate, prepared using the methods of Chu et al (1977).

Production of sclerotia.

Transformant isolates were tested for their ability to produce sclerotia. *A. parasiticus* NRRL 5862 and *A. parasiticus* ATCC 36537 were included for comparisons. Approximately 10^3 previously frozen conidia (in 15% glycerol) of each strain were inoculated on the center of a 100x15 mm petri plate prepared with 25 ml of PDA medium. Cultures were incubated at 30°C and 37°C for a period of 10 days. Sclerotia were harvested according to Cotty (1988), counted, and then dried at 50°C for 48 hrs. Sclerotial diameters were measured using the aid of the Java Video Analysis System (Jandal Corp.,

Corte Madera, CA). Between 32-100 sclerotia were chosen at random from each plate and measured from top to bottom near the estimated center of the vertical plane.

Marker rescue of *amp*^{r} gene from an aflatoxin producing (Afl$^+$) transformant.

Four different restriction endonucleases (SalI, SphI, BglII, and NdeI) were used to digest genomic DNA from one *A. parasiticus* CS10 Afl$^+$ transformant clone. These enzymes were chosen because they cut once within the cosmid vector pBZ5, but do not cut within the *pyr*G or the *amp*^{r} gene. The strategy was designed to allow recovery of fragments that contained the *pyr*G gene, the *amp*^{r} genes, and presumably a piece of the original *A. parasiticus* insert DNA because the second restriction endonuclease site must be situated somewhere within the flanking DNA.

Each restriction enzyme was used separately to digest 50 μg of DNA from the Afl$^+$ transformant. DNA was separated on a 1.2 % agarose gel. Regions of the gel corresponding to the desired fragments containing the *amp*^{r} gene were removed and used for DNA isolation with Prep-A-Gene DNA isolation matrix (Biorad, Richmond, CA). Isolated DNA was diluted to 2 ng DNA/ul prior to T4 DNA ligase treatment to minimize intermolecular ligation. Circularized fragments were transformed into *E. coli* DH5α which was then grown on LB agar

medium containing ampicillin (50 μg/ml).

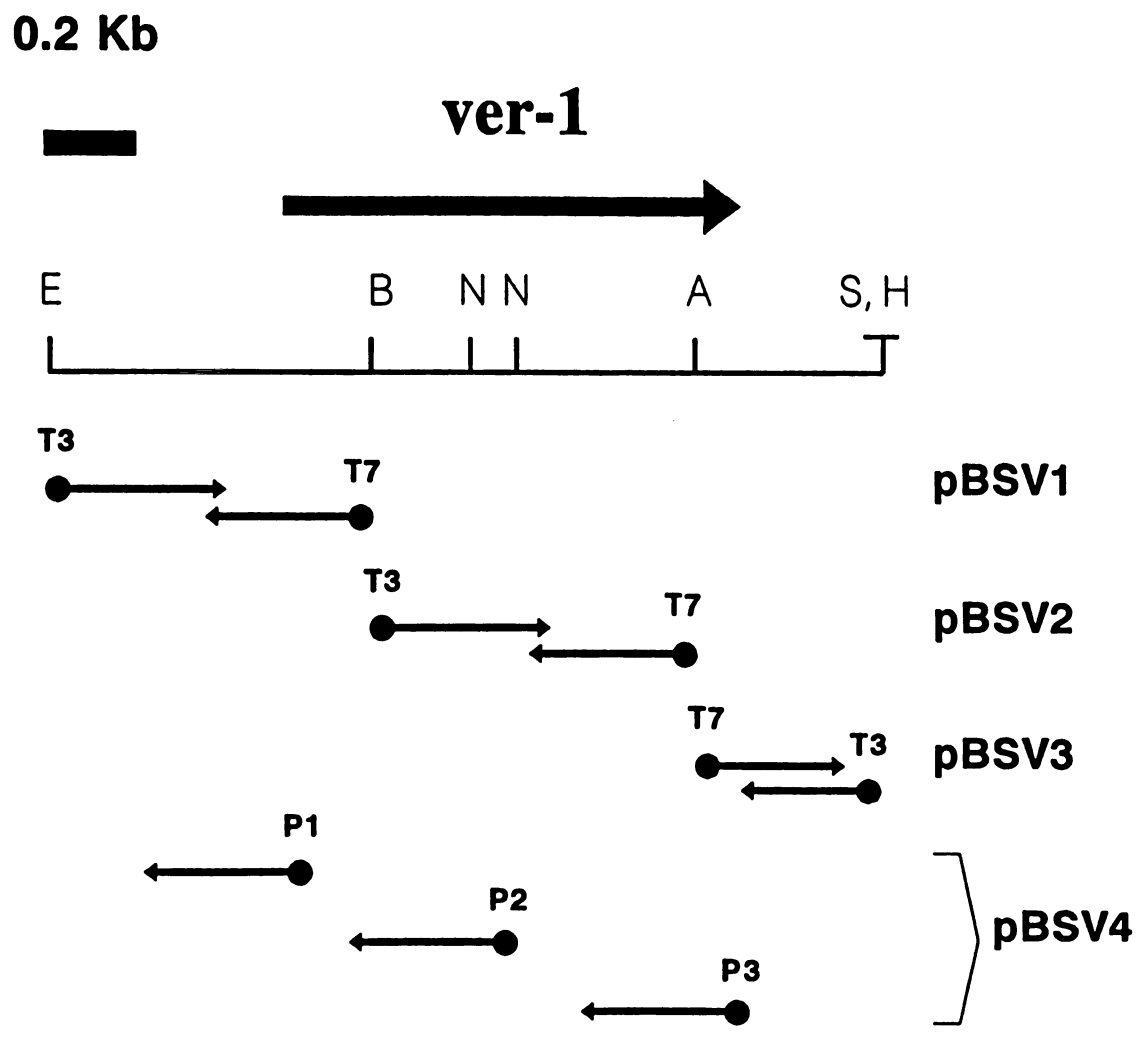
Isolation and analysis of RNA from fungal cells.

Conidia (1 x 10^7) of *A. parasiticus* were inoculated into 100 ml YES broth and grown at 28° C in an orbital shaker (150 rpm) for 3 days. The resulting mycelium was filtered through mira-cloth and then quickly frozen in liquid nitrogen. Ribonucleic acid was then isolated using a hot phenol purification method (Ausebel *et al*, 1987). Polyadenylated RNA was isolated by affinity column chromatography using an oligo-dT matrix (Ausebel *et al*, 1987). Northern analysis of RNA samples was performed as described by Maniatis *et al* (1982).

Analysis of RNA transcripts from the *ver*-1 gene.

The orientation of the *ver*-1 transcript was first determined by Northern hybridization analysis with strand specific riboprobes made from plasmid pBSV1 (Figure 8), in which a 0.7 kb EcoR1/BamH1 *ver*-1 fragment was ligated into the plasmid pBluescriptII KS- (Stratagene, LaJolla, CA). Bluescript plasmids contain T7 and T3 phage promoters on either side of the polylinker region that allow transcription of DNA inserts in both directions. Plasmid pBSV1 was linearized with restriction endonuclease enzymes SacI and SalI

Figure 8. Restriction endonuclease map and sequencing strategy of the 1.8 kb EcoR1/HindIII fragment. The shaded arrow indicates the direction of transcription for the *Aspergillus parasiticus ver*-1 gene. Regions of DNA cloned into pBluescriptII plasmids for the construction of pBSV1, pBSV2, and pBSV3 are illustrated directly beneath the corresponding area of the restriction map. Orientation of T7 and T3 primer regions, contained on pBluescriptII plasmids, are included with arrow designating the direction and extent of sequencing. Each arrow may represent data from more than one set of sequencing reactions. Oligonucleotide primers (P1, P2, and P3) were synthesized and used to sequence plasmid pBSV4 which contains the 1.8 kb EcoR1/SalI fragment cloned into pBluescriptII KS-. E, EcoR1; B, BamH1; N, NdeI; A, AvaI; S, SalI; H, HindIII.


0.2 Kb
ver-1
E
B
N N
A
S, H
T3
T7
pBSV1
T3
T7
pBSV2
T7
T3
pBSV3
P1
P2
P3
pBSV4

(located in the polylinker) to generate transcripts using T3 and T7, respectively. Radiolabelled riboprobes were made according to manufacturer's instructions with α-^{32}P-rCTP.

The 5' and 3' ends of the *ver*-1 transcript were localized using a ribonuclease protection assay (Ausebel *et al*, 1987). Riboprobes for 5' end analysis were transcribed with T7 polymerase using SalI linearized plasmid pBSV1 (Figure 8). Riboprobes for 3' end analysis were prepared using T7 polymerase with *Eco*RI-linearized plasmid pBSV3 (Figure 8) in which a 0.4 kb AvaI/HindIII *ver*-1 fragment was blunt end ligated into the SmaI site of pBluescriptII SK-. Riboprobes were hybridized at 55° C to mRNA from *A. parasiticus* NRRL 5862 or tRNA (control). Samples were treated with RNase T1 and RNase A and separated by electrophoresis on a 5% polyacrylamide gel under denaturing conditions. Acrylamide gels were dried under vacuum and subjected to autoradiography using Kodak XAR5 film.

Sequence analysis.

Nucleotide sequence analysis was performed using the dideoxy chain termination method (Sanger *et al*, 1977) employing Sequenase II (USB, Cleveland, OH) according to the manufacturer's instructions. The DNA inserts in plasmids pBSV1, pBSV2, and pBSV3, were sequenced on both strands using the T7 and T3 primers supplied in the kit (Figure 8). Plasmid

pBSV2 was constructed by ligation of a blunt end 0.6 kb *Bam*H1/*Ava*I internal *ver*-1 fragment into the *Sma*I site of pBluescriptII SK-. From this initial sequence information, oligonucleotide primers (15 nucleotides) were synthesized (MSU, Molecular Structure Facility) and used to reconfirm the sequence and to remove ambiguities. Plasmid pBSV4 (Figure 8), which contains a 1.8 kb EcoR1/SalI fragment, served as a template strand for sequencing with the synthesized oligonucleotide primers.

Sequence analysis of the *ver*-1 cDNA fragment was performed using the TAQuence sequencing kit (USB, Cleveland, OH) which utilized the thermostable ΔTaq DNA polymerase. The cDNA fragment was isolated by *in situ* colony hybridization of the cDNA library using the plasmid pBSV2 which contains an internal portion of the *ver*-1 gene.

Computer analysis of sequence data.

Computer analyses of nucleotide data were performed using the Wisconsin Genetics Computer Group (GCG) package. The location of open reading frames, a translation start codon, and introns were predicted using the GCG software programs *Frames*, *Testcode*, and *codon preference*. Codon usage files were constructed using data from 45 different *A. nidulans* genes reported by LLoyd and Sharp (1991). Amino acid comparisons were made using a window size of 30 and a stringency of 15.

Analysis of RNA expression for the *ver*-1 and *nor*-1 gene

Batch fermentation analysis.

Frozen (-80° C in 15% glycerol) conidia (2 x 10^6) of *A. parasiticus* NRRL 5862 were inoculated into 100 ml of defined Adye and Mateles (1964) minimal medium (AM) contained in a 250 ml silanized erlenmeyer flask. Five glass beads (3-4 mm dia) were included in each flask to aid in aeration of the culture and to facilitate dispersed mycelial growth (Reynolds and Pestka, 1991). Growth was visibly more uniform among duplicate flasks containing glass beads since they prevented the inconsistent aggregation or clumping of the mycelium that is typically observed with *Aspergillus* shake cultures (without beads). All flasks were incubated in the absence of light at 29° C in an orbital shaker (220 rpm). Triplicate flasks were removed at each time point and analyzed individually for mycelial dry weight, pH of medium, and aflatoxin concentration in filtrate. The average of three values and the standard error were then calculated for measurements at each time point. One additional flask for each time point was included for RNA isolation.

Nutritional shift assay.

A modified nutritional shift protocol (Buchanan and Lewis, 1984; Buchanan *et al*, 1987) was employed to determine when the *nor*-1 and *ver*-1 RNA transcripts accumulate relative to a shift from non-aflatoxin supporting conditions to those which support aflatoxin biosynthesis. Three silanized Fernbach flasks containing 500 ml of peptone mineral salts (PMS) medium (Buchanan *et al*, 1987) were each inoculated with 2.5×10^7 *A. parasiticus* NRRL 5862 conidia (from a frozen stock) and grown for 65 hrs at 29° C in an orbital shaker (200 rpm). PMS medium is similar to AM medium (Adye and Mateles, 1964) except that glucose is replaced by peptone which serves as the sole carbon source. This medium does not support or induce aflatoxin biosynthesis (Buchanan and Lewis, 1984). The mycelium from all three flasks was combined, harvested by filtration through cheese cloth and distributed [5 g (wet weight) mycelium/flask] to 250 ml silanized flasks containing 30 mls of either PMS or glucose mineral salts (GMS) medium (Buchanan *et al*, 1987). GMS, which is capable of inducing and supporting aflatoxin biosynthesis is identical to PMS except that peptone (6%) is replaced by glucose (6%). Incubation of cultures after the shift was continued up to 48 hrs under the same conditions. One PMS and one GMS containing flask were removed at each time point so that mycelium could be harvested and used for RNA extraction. Filtrates from each time point were saved for quantitative analysis of aflatoxin B_1.

Analysis of mycelial dry weight, aflatoxin concentration, and RNA for batch fermentation and nutritional shift analysis.

Fungal cultures were removed from the incubator/shaker at specified times and the mycelium harvested by filtration through miracloth (Behring Diagnostics, LaJolla, CA). The collected mycelium from each culture flask was dried to completion at 70° C prior to weighing. A sample of each filtrate was tested for pH and the remainder used for determination of aflatoxin production. Only the filtrate was tested since both aflatoxin B_1 and G_1 are known to efficiently pass through the mycelial wall at 29° C (Lin *et al*, 1980). Aflatoxin B_1 was analyzed using direct competitive enzyme linked immunoabsorbent assay (ELISA) according to Pestka (1988).

Total RNA (nuclear and cytoplasmic) was isolated and purified using a hot phenol protocol (Ausebel *et al*, 1987). Northern analysis of RNA samples was performed as described by Maniatis (1982). Approximately 7 μg of total RNA per sample was separated by electrophoresis in a 1.2% denaturing formaldehyde agarose gel and then transfered by capillary action to a Nytran membrane (Schleicher and Scheull, INC, Keene, NH). A 0.6 kb AvaI/BamHI restriction endonuclease fragment of an internal portion of the *A. parasiticus ver*-1 gene (Skory *et al*, 1992) (Genbank accession # M91369), a 1.5 kb BglII/ClaI fragment containing the *A. parasiticus nor*-1 gene (Chang *et al*, 1992), and a 2.8 kb BamH1/SalI fragment

containing the *A. parasiticus pyrG* gene (Skory *et al*, 1990) were used as DNA probes for Northern analysis.

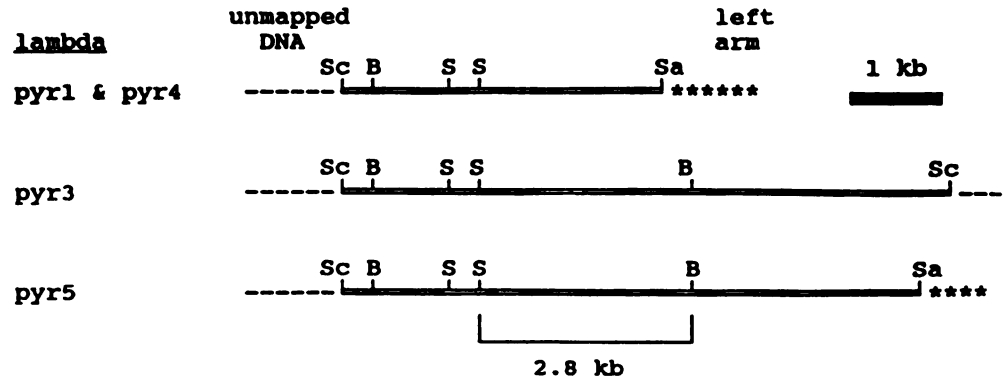
RESULTS/DISCUSSION

Isolation of the *pyrG* gene from *A. parasiticus* ATCC 36537.

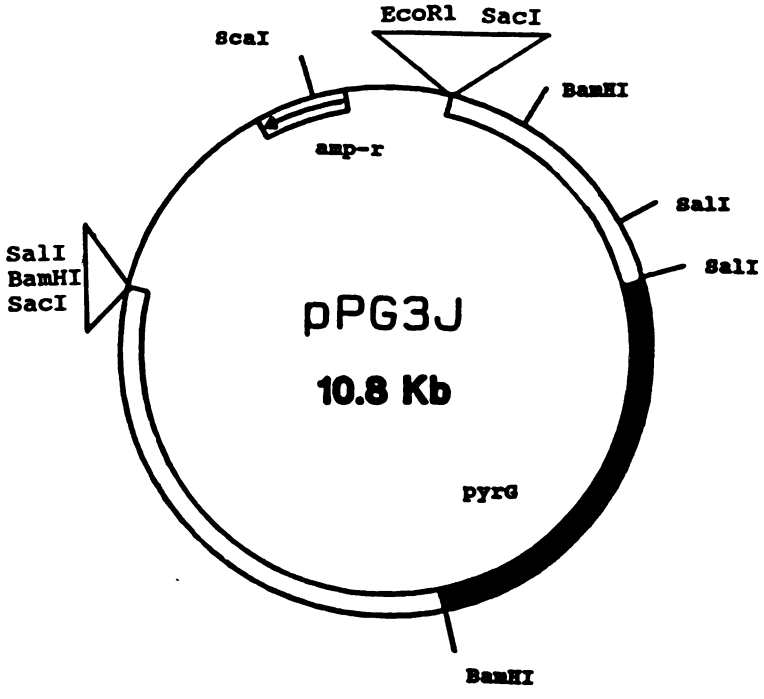
Transformation of *A. parasiticus* ATCC 36537 *pyrG* mutants using the heterologous gene from *A. nidulans* FGSC4 contained on circular pPL6 (Oakley, 1987) was unsuccessful on several attempts and thus prompted the isolation of the homologous *pyrG* gene. Southern analysis of EcoR1 digested genomic DNA from *A. parasiticus* NRRL 5862 exhibited a single band when hybridized to a 1.5 kb HindIII restriction endonuclease fragment containing the *pyrG* gene from *A. nidulans* FGSC4 (Oakley *et al*, 1987). The *pyrG* gene (Skory *et al*, 1990) from *A. parasiticus* NRRL 5862 was then isolated by *in situ* plaque hybridization (Maniatis *et al*, 1987) of a lambda genomic DNA library (Horng *et al*, 1989) using the described *pyrG* gene from *A. nidulans* FGSC4 as a probe. A total of four isolates were obtained, at a frequency of 2 x 10^{-4} clones/Lambda plaque, that contained DNA inserts which hybridized strongly to the *A. nidulans pyrG* probe (Figure 9A). An 8.1 kb SacI fragment which hybridized to the *A. nidulans* probe was purified from one representative phage isolate (lambda pyr3) which appeared

Figure 9. (A) Restriction endonuclease analysis of inserted DNA fragments from four clones of an *A. parasiticus* NRRL 5862 Lambda genomic DNA library which hybridized to the *A. nidulans* FGSC4 *pyrG* probe. The 2.8 kb SalI/BamHI fragment represents the region of probe binding. (B) Restriction endonuclease analysis of plasmid pPG3J (10.8 Kb) which was used for transformation of uridine auxotrophs. The 8.1 kb SacI fragment from Lambda isolate pyr3 was ligated into the SacI (SstI) restriction endonuclease site of the polylinker region of pUC19. Only those enzymes listed in the restriction endonuclease map have tested. Additional sites in the in the polylinker region still remain. The darkened box represents the 2.8 Kb region from *A. parasiticus* which hybridized to the *pyrG* gene from *A. nidulans* FGSC4 while the open box is flanking DNA. Restriction endonuclease sites are as follows: Sc, SacI; B, BamH1; S, Sal1; Sa, Sau3A.

a.


lambda
unmapped DNA
left arm
Sc B S S Sa
1 kb
pyr1 & pyr4
Sc B S S B Sc
pyr3
Sc B S S B Sa
pyr5
2.8 kb

b.


EcoR1 SacI
ScaI
BamHI
amp-r
SalI
SalI
SalI
BamHI
SacI
pPG3J
10.8 Kb
pyrG
BamHI

to contain the hybridizing region within the middle region of the *A. parasiticus* DNA insert. This SacI (SstI) fragment was then subcloned into pUC19 to facilitate restriction endonuclease analysis. The resultant 10.8 kb plasmid was designated pPG3J (Figure 9B). All phage isolates contained overlapping DNA fragments with the region hybridizing to the *pyrG* probe located on a common 2.8 Kb SalI/BamHI fragment (Figure 9A).

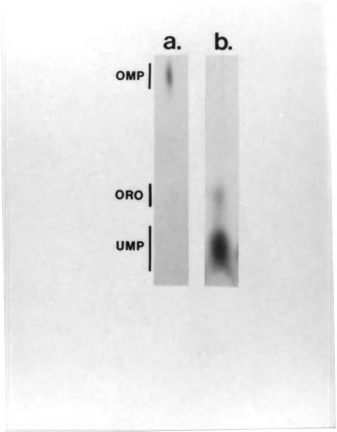
Isolation of uridine auxotrophs from *A. parasiticus* ATCC 36537.

NTG mutagenesis of *A. parasiticus* ATCC 36537 resulted in a 66% survival of conidiospores compared to non-mutagenized conidiospores when inoculated onto PDA. Inoculation of 1.3 x 10^8 viable mutagenized spores onto PDA supplemented with FOA and uridine resulted in FOA resistant (FOA^r) colonies arising at a frequency of 3.5 x 10^{-7} colony forming units/viable conidiospore. Twenty-two percent of these FOA^r isolates were determined to be stable uridine auxotrophs after serial inoculation onto CZ medium supplemented with uridine. All ten strains utilized in further analysis were morphologically indistinguishable from the original parent strain *A. parasiticus* ATCC 36537 and showed no detectable blue fluorescence when grown on coconut agar medium suggesting little or no aflatoxin production.

Enzymatic analysis of *pyr* mutants.

Separation of OMP, orotate, and UMP on TLC plates yielded average Rf values of 0.4, 0.6, and 0.7 respectively. In cell free extracts of three *A. parasiticus* ATCC 36537 *pyr* isolates, CS2, CS3, and CS10, OMP was converted to UMP in trace amounts suggesting that orotidine monophosphate decarboxylase activity was greatly impaired (Figure 10, panel A). Furthermore, [^{14}C]-OMP accumulated when cell free extracts of *A. parasiticus* CS2, CS3, and CS10 were incubated with [^{14}C]-orotate plus PRPP indicating complete conversion of orotate to OMP with subsequent accumulation of OMP indicating active orotate phosphoribosyl transferase activity (data not shown). *A. parasiticus* ATCC 36537 which served as a positive control for functional *pyr*F and *pyr*G enzymatic activity was able to convert both [^{14}C]-OMP and [^{14}C]-orotate to [^{14}C]-UMP (data not shown). *A. nidulans* A722 which lacks orotidine monophosphate decarboxylase activity was used as a negative control. This strain did not accumulate [^{14}C]-UMP when incubated with [^{14}C]-OMP (data not shown). The remaining seven of the ten *pyr* mutants (CS1-CS10) tested, converted [^{14}C]-OMP to [^{14}C]-UMP at levels comparable to that in the *A. parasiticus* ATCC 36537 control suggesting a functional orotidine monophosphate decarboxylase enzyme (data not shown). The results strongly suggested that *A. parasiticus* uridine auxotrophs CS2, CS3, and CS10 were *pyr*G mutants deficient in orotidine monophosphate decarboxylase activity.

Figure 10. Restoration of orotidine-5'-monophosphate activity in a pyrG mutant after transformation. Cell free extracts from *A. parasiticus* were assayed for the conversion of orotidine-5'-monophosphate (OMP) to uridine-5'-monophosphate (UMP). Extracts of *A. parasiticus* CS10 [panel A (uridine auxotrophic mutant, not transformed)] and *A. parasiticus* UT5 [panel B(CS10 transformed with pPG3J)] were incubated with [6-^{14}C]-OMP and separated by thin layer chromatography (TLC). The TLC plate was then exposed 48 hrs to X-ray film. Oro, orotate.


a.
b.
OMP
ORO
UMP

Transformation of *pyrG* mutants with plasmid pPG3J.

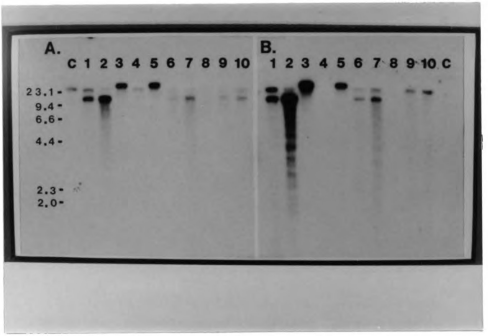
Protoplast preparations of *A. parasiticus* typically yielded 1-3 x 10^6 protoplasts from 10^8 conidia with viability being approximately 25% when cells were grown under nonselective conditions (media supplemented with uridine). Transformation of a total of 10^6 viable protoplasts of *A. parasiticus* ATCC 36537 *pyrG* isolates CS2 or CS10 with 2 μg of pPG3J resulted in 20-50 stable transformants/μg DNA. Control protoplasts not treated with DNA, showed no evidence of germination on CZ (KCl) agar as determined by microscopic examination. Both circular and EcoR1 linearized pPG3J were tested and yielded approximately equal efficiencies. However, linearized plasmids resulted in significantly fewer abortive transformants (colonies which initially grow on selective medium but are unable to do so upon further subculturing). Transformant colonies typically appeared within 2 days but became readily distinguishable from abortive transformants at day 4 by their larger diameter (0.5-1.5 cm compared to abortive colonies which were typically less than 0.2 cm) and abundant conidiation. Transformant colonies transferred to CZ agar medium demonstrated colony morphology similar to *A. parasiticus* ATCC 36537. Efficiencies obtained with the homologous *pyrG* gene were slightly higher than those of Woloshuk et al (1989) in which a heterologous pyrG transformation system in A. flavus was developed.

Southern analysis of prototrophic transformants from plasmid pPG3J.

Southern hybridization analysis of genomic DNA purified from several transformant clones of *A. parasiticus* CS10 suggested that both *pyr*G and pUC19 plasmid sequences integrated into the genome (Figure 11). Transformant isolates 1-5 were transformed with linear pPG3J while isolates 6-10 were transformed with circular pPG3J. A single band was observed in EcoR1 digested genomic DNA from non-transformed *A. parasiticus* CS10 when hybridized with the 2.8 kb Sal1/BamH1 *pyr*G fragment. No hybridizing fragment was seen in the same DNA when probed with pUC19 confirming the lack of sequence similarity with this plasmid. Hybridization patterns for transformant isolates 4 and 8 were the same as the untransformed cells when probed with *pyr*G and did not show any hybridization to pUC19 sequences indicating that a double cross-over recombination event possibly occurred between the *pyr*G gene on the plasmid and the *pyr*G on the chromosome leading to replacement of the homologous DNA. Protoplasts not treated with DNA did not grow on CZ(KCl) medium implying that clones 4 and 8 were not revertants at the pyrG locus.

It was not possible to conclusively determine from the data whether gene replacement also occurred in addition to integration of the plasmid sequences in the remaining transformant clones. A single cross-over and integration event of circular pPG3J into the *pyr*G locus was the most

Figure 11. Southern hybridization analysis of ten *A. parasiticus* *pyrG* transformants. Genomic DNA from untransformed *A. parasiticus* CS10 (lane c), five isolates transformed with linearized EcoR1 pPG3J (lanes 1-5), and five additional isolates transformed with circular pPG3J (lanes 6-10) were digested with EcoR1 and electrophoresed in a 0.8% agarose gel (8 μg DNA per lane). DNA was blotted onto nitrocellulose and probed with ^{32}P labelled (A) 2.8 kb Sal1/BamH1 *pyrG* fragment and (B) pUC19.


A.
C 1 2 3 4 5 6 7 8 9 10
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
B.
1 2 3 4 5 6 7 8 9 10 C

likely event for transformants 9 and 10. Such an event would result in two different EcoR1 fragments of approximately 23 kb and 11 kb each containing *pyrG* sequences, but only the larger of two would contain pUC19 sequences. The Southern data for the remaining transformants suggested integration of all, or part of the plasmid pPG3J into a site other than the *pyrG* locus. Additional hybridizing bands observed in genomic DNA from transformant isolates 1,2,3,5,6, and 7 when probed with the *pyrG* fragment were also noted in the same apparent locations when hybridized to pUC19, indicating that both pUC19 and *pyrG* sequences integrated into the genome. It should be mentioned that transformant isolates 3 and 5 may actually have resulted from integration of pPG3J sequences into the *pyrG* locus resulting in a larger EcoR1 band, although this is difficult to conclude since the intensity and size of this band prevents accurate interpretation. The multiple bands observed with transformant isolate 2 when probed with pUC19 may have resulted from integration and rearrangement of the pUC19 vector occurring independently of that from the *pyrG* DNA. The magnified intensities of the additional *pyrG* hybridizing bands for transformant isolates 1,2,3,5, and 7 might suggest multiple copies of the plasmid have been integrated since equal quantities of genomic DNA were added in each lane.

Enzymatic analysis of *pyrG* transformants.

Enzymatic analysis with [^{14}C]-OMP and [^{14}C]-orotate substrates was performed on cell extracts from five randomly isolated transformant colonies of *A. parasiticus* CS10. All of the transformants tested were able to completely convert OMP to UMP and none accumulated OMP when incubated with orotic acid which demonstrated restored orotidine monophosphate decarboxylase activity (Figure 10, panel B).

In this study, enzymatic analysis was performed on cell free extracts from *A. parasiticus pyr* mutants for two reasons. First, it allowed differentiation between *pyrG* and *pyrF* mutant strains generated through mutagenesis and a positive selection protocol, thus eliminating the need to test all uridine auxotrophs isolated for the ability to be complemented by the cloned *A. parasiticus pyrG* gene. Secondly, it enabled confirmation of ablated orotidine monophosphate decarboxylase enzyme activity in *A. parasiticus* CS10 and restored enzyme activity after complementation with the cloned *pyrG* gene. Although slight conversion of [^{14}C]-OMP to [^{14}C]-UMP seemed to be occurring in all three *pyrG* mutants CS2, CS3, and CS10, suggesting the presence of leaky mutations, none of the mutant strains were able to grow on minimal medium. The reversion frequency in these mutants was shown to be less than 10^{-8}, but it is possible that reversion did occur during cell growth in broth culture thus providing a small amount of functional orotidine monophosphate decarboxylase activity in the cell

free extracts. Results from enzymatic studies of transformants strongly suggest restored orotidine monophosphate decarboxylase activity as indicated by increased UMP formation from [^{14}C] OMP and lack of OMP accumulation when incubated with [^{14}C] orotate as compared to untransformed *A. parasiticus* CS10 controls.

Cosmid library preparation.

DNA purified from *A. parasiticus* NRRL 5862 was confirmed by field inversion gel electrophoresis to be of high molecular weight (100-150 kb). The efficiency of packaging for the ligated concatamers was deduced by estimating the ability of phage particles to transfect *E. coli* LE392 (1.65×10^5 transfectants per μg DNA). Cosmid preparations from twelve randomly isolated *E. coli* transfectant clones revealed the average size of the recombinant cosmid to be approximately 45 kb. Therefore, the average size of *A. parasiticus* DNA inserted into the original cosmid pBZ5 (9.2 kb) was estimated to be 36 kb. Approximately 2,200 clones of this size would provide a 95% probability of obtaining a region of interest if one assumes that the total genome size of *A. parasiticus* is similar to *A. nidulans* (2.6×10^4 kb) (Timberlake, 1990).

Transformation of *A. parasiticus* CS10 with "pooled" DNA library.

A. parasiticus CS10 was transformed with the combined cosmid DNA library at an efficiency of 29 *pyr*G+ transformants/μg DNA. Approximately 400 colony forming units (cfu) were visible after 2 of days incubation at 32° C. No growth was observed on the control plates inoculated with protoplasts untreated with DNA. Almost all of the transformant colonies were observed to have the same morphology as the parent strain, *A. parasiticus* ATCC 36537, grown under similar conditions. One colony fluoresced blue under long wave U.V. light on the fourth day of incubation. Reinoculation of this putative Afl^+ isolate onto CAM demonstrated that it still retained its ability to produce a blue fluorescing compound which was released into the surrounding medium. Additionally, it no longer accumulated significant quantities of the bright yellow fluorescent pigment, as seen in the recipient strain, which contains versicolorin A as a major component (Lee *et al*, 1975).

A green spored transformant was also isolated following transformation with the pooled library. When viewed from the bottom of the petri plate, this isolate appeared visibly identical in both mycelial color and growth patterns to *A. parasiticus* ATTC 36537 after six days of growth (30° C) on PDA medium. When viewed from the top, its morphology was indistinguishable from *A. parasiticus* ATCC 36537, except that

spores appeared green instead of white. Microscopic examination revealed that the majority of conidia were actually yellow to yellow/green. Some areas of entirely green conidia, similar to wild type *A. parasiticus* NRRL 5862, were observed near the center of the colony.

The interest in this particular transformant is the possibility of isolating one or more of the genes responsible for green pigmentation of *A. parasiticus* spores. It has been suggested that spore pigmentation and aflatoxin production are somehow associated, although there is no direct evidence to substantiate this notion. Each of the *A. parasiticus* mutants blocked in aflatoxin biosynthesis possess a spore color very unlike the parent strain. This isolate could prove useful in later research to study such a relationship.

Marker rescue of amp^r gene.

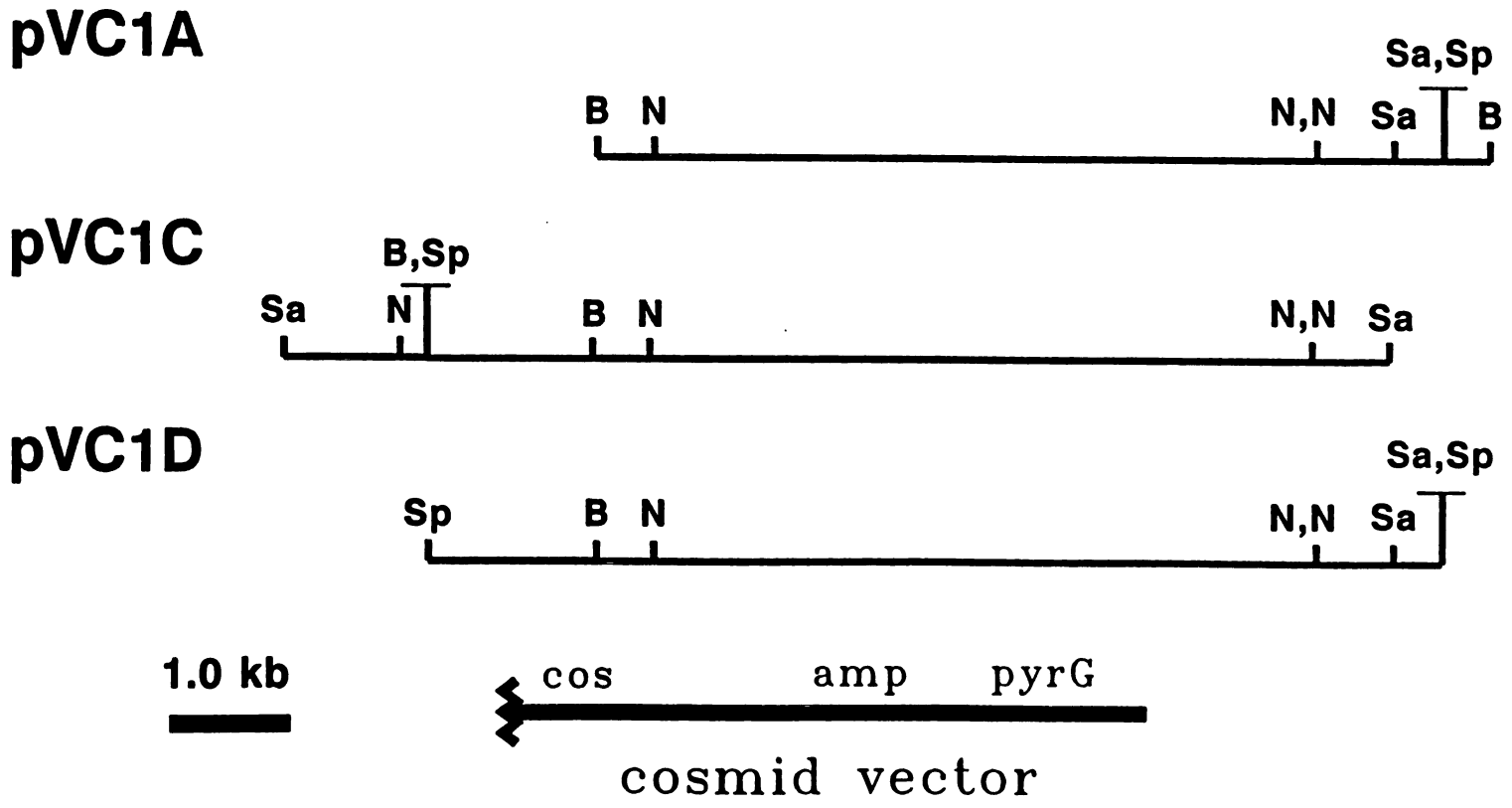
Southern analysis of DNA purified from the putative Afl+ transformant using the cosmid vector pHC79 as a probe suggested that 12.5 kb SalI, 9.8 kb BglII, 11.0 kb SphI, and 7.2 kb NdeI restriction fragments contained the *amp^r* and *pyrG* genes plus a piece of the original *A. parasiticus* DNA insert. Ampicillin resistant clones of *E. coli* DH5α containing plasmids made by circularization of each of these restriction endonuclease fragments were isolated. Restriction endonuclease analysis of each rescued plasmid with the original restriction

enzyme used for its isolation, confirmed that plasmid sizes were consistent with predictions based on Southern analysis of DNA from the Afl+ transformant (Figure 12). Isolated plasmids were designated pVC1A (BglII), pVC1B (NdeI), pVC1C (SalI), and pVC1D (SphI) for the restriction endonuclease enzymes which were used in their recovery. All plasmids appeared to have overlapping restriction endonuclease sites and most of the DNA from cosmid pBZ5 was apparently present except for a deletion of approximately 2 kb in the region of the Lambda cohesive site. It may be possible that only part of the cosmid integrated or that rearrangement of the cosmid occurred during transformation. Deletions are quite common in cosmids of this size (Gibson *et al*, 1987) and may have occurred during replication in *E. coli*.

Transformation of *A. parasiticus* CS10 with pVC1A-D.

Plasmids pVC1A, pVC1B, pVC1C, and pVC1D were used to transform *A. parasiticus* CS10 to test for the presence of the *pyrG* and *ver-1* genes. Pyrimidine prototrophic transformants were obtained using all plasmids except pVC1B. The percentage of Afl^+ isolates in the total number of $pyrG^+$ transformants were 29%, 20%, and, 18% for pVC1A, pVC1C, and pVC1D respectively. The fortuitous complementation of the *ver-1* gene using the rescued fragments eliminated the necessity to conduct *in situ* colony hybridization of the

Figure 12. DNA fragments obtained from an *A. parasiticus* CS10 aflatoxin producing transformant using marker rescue. The region corresponding to the cosmid vector pBZ5 is represented at the bottom of the figure. Plasmid pVC1B is not shown. B, BglII; N, NdeI; Sa, SalI; Sp, SphI; Cos, cohesive site in Lambda.


pVC1A
Sa,Sp
B N
N,N Sa B
pVC1C
B,Sp
Sa N
B N
N,N Sa
pVC1D
Sa,Sp
Sp B N
N,N Sa
1.0 kb
cos amp pyrG
cosmid vector

cosmid genomic library using the rescued fragments as probes.

Analysis of *A. parasiticus* Afl$^+$ transformants.

Five clones transformed with pVC1A (designated P1-P5) which demonstrated blue fluorescence on CAM and five transformants also transformed with pVC1A (designated N1-N5) that lacked blue fluorescence were subcultured onto CAM for further analysis. Upon visual inspection of the cultures, Afl$^+$ transformants (P1-P5) were easily distinguishable from Afl$^-$ strains by the second day of incubation. All Afl$^+$ transformants except P2 accumulated little detectable yellow pigmentation in the mycelium, whereas Afl$^-$ isolates (N1-N5) accumulated abundant quantities of yellow pigment. This yellow pigment has been confirmed by both TLC and ELISA to contain predominantly VA (Reynolds and Pestka, 1991). Under long wave U.V. light, all of the Afl$^+$ transformants produced a blue fluorescent pigment by the third day of incubation at 30° C; No blue fluorescent pigment was detectable in clones N1-N5. One Afl$^+$ transformant, P2, produced a small quantity of a blue fluorescent compound and accumulated less yellow pigment than the Afl$^-$ isolates. By using thin layer chromatography and direct competitive ELISA analysis, it was confirmed that *A. parasiticus* P1, P3, P4, and P5 were producing aflatoxin B_1 in amounts (approximately 20 μg (± 10) aflatoxin/ml filtrate) comparable to *A. parasiticus* NRRL 5862

grown under identical conditions. *A. parasiticus* P2 produced aflatoxin B_1 at an approximately 10 fold lower level. No aflatoxins were detected (1 ng/ml, minimum detectable level for monoclonal antibody employed) in filtrates from *A. parasiticus* N1, N2, N3, N4, N5, or *A. parasiticus* ATCC 36537. TLC analysis demonstrated VA was present in small amounts in the filtrates from all of the non-aflatoxin producing strains, *A. parasiticus* ATCC 36537, and also from *A. parasiticus* P2. VA is not normally found in the filtrate since it does not traverse the cell wall efficiently (Lee *et al*, 1975). However, it is likely that some of this pigment is released into the broth medium when the integrity of the cell wall is lost during cell death or as cells are physically disrupted by the glass beads.

Each of the Afl^+ and Afl^- transformants were reinoculated onto PDA and grown for 10 days at 30°C and 37°C. Sclerotia, which are composed of a highly pigmented dense mass of hyphae, were abundant at 30° C in the wild type *A. parasiticus* NRRL 5862 and in all of the Afl^+ transformants except *A. parasiticus* P2 which was not producing any of these dormant structures (Table II). No sclerotia were observed in any of the Afl^- transformants or in the mutant strain *A. parasiticus* ATCC 36537 when grown on PDA under identical conditions. However, *A. parasiticus* ATCC 36537 did produce sclerotia at 37°C which is inhibitory to aflatoxin biosynthesis (and VA biosynthesis) (Niehaus, 1989); no yellow pigmentation of the mycelium was evident at this temperature. Transformants could

Table II. Sclerotial production of various *A. parasiticus* strains grown on PDA medium for a period of 10 days.

	30° C		37° C	
strain	# sclerotia/ 10 cm^2	avg. dia (μm)[a]	# sclerotia/ 10 cm^2	avg. dia. (μm)
P1	130	317±53	--[b]	--
P2	0	---	--	--
P3	180	360±56	--	--
P4	7	332±51	--	--
P5	120	329±50	--	--
N1-N5	0	---	--	--
NRRL 5862	69	338±55	32	341±55
ATCC 36537	0	---	33	372±54

[a]Between 32-100 sclerotia were choosen at random for each strain.

[b]Growth was negligible (colony diameter <0.5 cm) at 37°C for all pyrimidine transformants tested.

not be analyzed at 37°C because of lack of growth presumably because the *pyrG* gene used for transformation was not effectively expressed at this temperature.

These intriguing observations strongly support previous hypotheses (Bennett *et al*, 1986; Cotty, 1988, 1989) that sclerotia development and aflatoxin biosynthesis in *A. parasiticus* are associated. However, it is not clear whether regulation of these two processes are somehow interrelated or if there may also be a direct functional relationship (i.e. aflatoxins or pathway intermediates affect sclerotia development). The appearance of sclerotia in *A. parasiticus* ATCC 36537 grown at 37°C (an inhibitory temperature for aflatoxin biosynthesis) might suggest that the restored sclerotia production is a result of the disappearance of versicolorin A. If VA is inhibitory to sclerotia production, then preventing VA accumulation by either increasing its conversion to aflatoxin B_1 or by suppressing its synthesis should restore sclerotia. Secondary metabolism in *Streptomyces* is also believed to be linked with morphological development (Horinouchi *et al*, 1986). This hypothesis was supported by the isolation of a pleiotropic gene (*afs*B) which positively regulates the biosynthesis of A-factor, the polyketide derived antibiotic actinorhodin, and undecylprodigiosin. However, our data suggest that the *ver*-1 gene is a structural gene encoding a ketoreductase. It seems improbable that a gene product with enzymatic activity would also have direct regulatory functions. However, aflatoxin B_1

or pathway intermediates which are produced in *pyrG*$^+$ Afl$^+$ *A. parasiticus* CS10 transformants could have a direct or indirect effect on regulatory proteins involved in aflatoxin biosynthesis and/or sclerotia development.

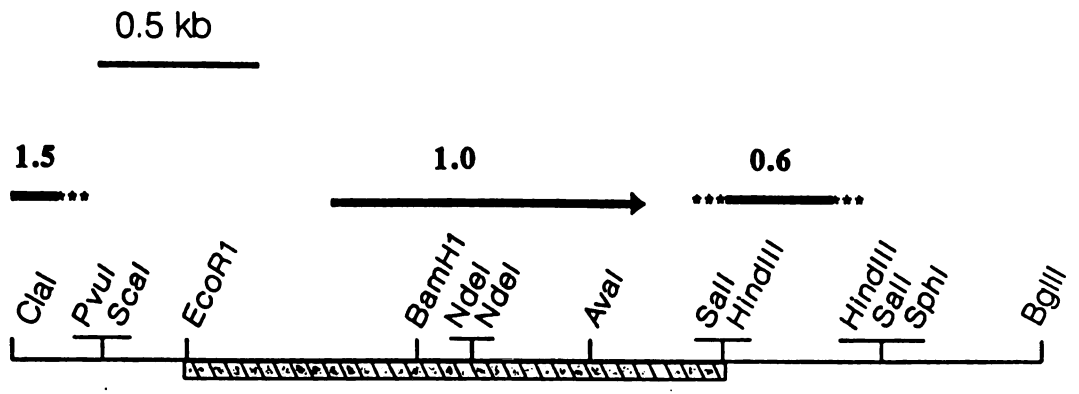
Southern analysis was performed on BamH1 digested genomic DNA from *A. parasiticus* P1-P5, and CS10 using the 2.6 kb PvuI/SphI *ver*-1 containing restriction endonuclease fragment as probe (data not shown). One additional hybridization band that did not appear in the *A. parasiticus* CS10 control was observed in approximately the same location for *A. parasiticus* P1-P5. The relative intensities of these bands suggested that only one or at most two additional copies of transforming *ver*-1 DNA had integrated into the genome. Additional studies will be performed to determine if integration of *ver*-1 at a specific locus is required for full complementation activity. Differences in the percentage of Afl$^+$ isolates among the total number of transformants may be a function of the various *ver*-1 containing fragments having *preferred* sites of integration which may or may not complement the *ver*-1 mutation. Since selection of transformants was conducted based on a functional *pyrG* gene, it is probable that integration of the transforming plasmid into the chromosome may have resulted in nonfunctional copies of the *ver*-1 gene and therefore less than 100 % restoration of aflatoxin biosynthesis among the total number of transformants.

Localization of mRNA transcripts encoded on the Afl$^+$ complementing DNA fragment.

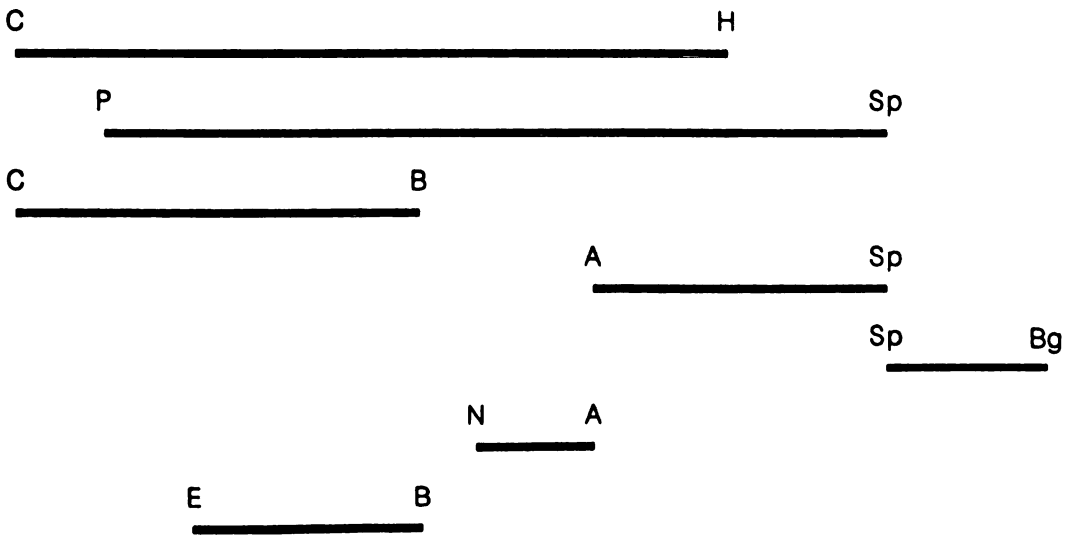
We were able to predict the approximate location of the region responsible for the *ver*-1 complementation activity by identifying the smallest common restriction endonuclease fragment present in the *ver*-1 complementing plasmids pVC1A, pVC1C, and pVC1D. Since we knew from control transformations that Afl$^+$ complementing activity was not contained on the cosmid vector pBZ5, we could determine that it must lie between the *pyrG* gene in the vector and the first SalI restriction endonuclease site within the insert (Figure 12).

Northern analysis was used to determine the size and location of transcription units contained on the Afl$^+$ complementing DNA fragments (Figure 13). Overlapping restriction fragments were used as DNA probes for hybridization to RNA purified from *A. parasiticus* NRRL 5862 or *A. parasiticus* ATTC 36537. No significant differences in hybridization patterns were noted between the two strains. Three different RNAs (0.6, 1.0, and 1.5 kb) were detected by Northern analyses using radiolabeled probes made from the 3.4 kb ClaI/BglII restriction fragment. Based on hybridization patterns observed with the DNA probes, it was predicted that the 0.6 kb RNA was encoded primarily on the 0.5 HindIII restriction fragment with a small portion of the transcription coding region extending into the 0.4 kb Ava/HindIII fragment. The region encoding the 1.0 kb RNA was localized to the 1.8 kb

Figure 13. Summary of Northern analyses using probes made from DNA fragments subcloned from the 3.4 kb ClaI/BglII region of pVC1A. Poly-adenylated RNA was used for hybridization to the labelled fragments. The approximate location of transcripts is represented by solid bars at the top of the diagram. The arrow designates direction of transcription. Asterisks on the ends of the bars reflect approximate locations of transcript ends. Shaded area beneath the 1.8 kb EcoR1/HindIII shows the smallest fragment to functionally complement *ver*-1. ND, none detected.



Probes used:



Size of detected RNA transcript

0.6, 1.0, 1.5 kb

0.6,1.0

1.0,1.5

0.6,1.0

nd

1.0

1.0

EcoR1/SalI restriction fragment. Finally, it was estimated that only part of the 1.5 kb RNA transcript was encoded on the 0.3 kb ClaI/PvuI restriction fragment.

Location of the *ver*-1 transcript.

It was initially postulated that either the 1.0 kb or the 0.6 kb transcript was responsible for complementation of the *ver*-1 mutation in *A. parasiticus* CS10. We did not believe that the region encoding the 1.5 kb transcript would be able to complement such a high percentage of transformants to Afl^+ when only a small part of the RNA coding region (less than 0.3 kb) was present. In addition, the region encoding the entire 0.6 kb transcript (1.9 kb BamH1/BglII cloned into pBZ5) was unable to restore *A. parasiticus* CS10 to Afl^+. By a process of elimination, it was predicted that the 1.0 kb transcript was responsible for complementing the *ver*-1 mutation. This hypothesis was confirmed when the 1.8 kb EcoI/SalI fragment from pVC1A cloned into pBluescriptII KS- (pBSV4) restored aflatoxin production to five of a total of 46 (11%) $pyrG^+$ *A. parasiticus* CS10 transformants following cotransformation with the *pyr*G containing plasmid, pBP28. Although pBSV4 does contain some of the DNA encoding the 0.6 kb transcript, a DNA fragment encoding the entire 0.6 kb RNA was unable to complement the *ver*-1 mutation.

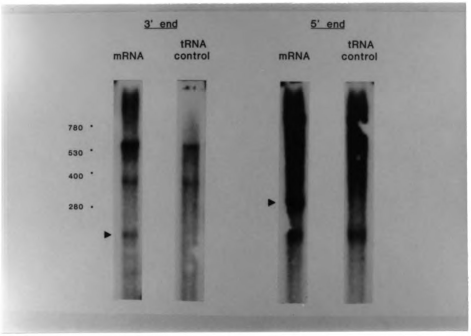
Analysis of transcripts from the *ver*-1 gene.

Strand specific riboprobes were used in Northern analysis of *A. parasiticus* NRRL 5862 total RNA to determine that the direction of transcription of the *ver*-1 gene proceeds from the BamHI to the AvaI restriction endonuclease sites. The approximate location of the transcription initiation site and polyadenylation site on the *ver*-1 transcript were determined by RNase protection assay. Several protected fragments of similar size were present in both mRNA and tRNA control lanes (Figure 14). However, a unique 290 bp fragment was protected in the mRNA 5' end assay and not the tRNA control, suggesting that transcription of the *ver*-1 RNA initiates approximately 290 bp upstream from the BamH1 restriction endonuclease site in the *ver*-1 gene. Similarly, the 3' end of the *ver*-1 transcript (poly-A site) was localized to a region approximately 230 bp downstream from the AvaI restriction endonuclease site. These data correlate well with a total transcript size of 1.0 kb observed in Northern analysis and the location of ends of the transcript predicted by cDNA sequence analysis.

Nucleotide Sequence analysis of the *ver*-1 gene.

The nucleotide sequence of the entire *ver*-1 region was determined. A short sequence characteristic of a Hogness box

Figure 14. RNase protection assay of 5' and 3' ends of the *ver*-1 transcript. Radiolabelled riboprobes were transcribed from the 0.4 kb AvaI/HindIII or 0.7 kb EcoR1/BamHI restriction fragments for 3' and 5' end analysis respectively and hybridized to poly-A RNA isolated from *A. parasiticus* or tRNA (control). Samples were treated with RNase and protected fragments separated by polyacrylamide gel electrophoresis under denaturing conditions. Unique 230 and 290 bp fragments (shown by arrows) were protected in 3' and 5' reactions respectively. The location and size (nt) of RNA reference standards are included to the left of the diagram.


3' end
5' end
mRNA
tRNA control
mRNA
tRNA control
780
530
400
280

(TATATAT) was observed in the same general region that a transcription initiation site was localized by RNase protection (Figure 15). The two closest ATG initiation codons downstream from the TATA box were 8 bp and 90 bp downstream. The latter, which was followed by a long open reading frame, was chosen as the most likely translation initiation codon. Protein coding regions were predicted by using the Wisconsin GCG software to test the randomness of codons and codon preference within the 1.8 kb EcoR1/HindIII restriction fragment. The location of intervening sequences could then be predicted using this information with open reading frame analyses. Splice junctions for introns were tentatively identified by comparing consensus sequences of intron boundaries and internal regions associated with lariat formation *Aspergillus* spp (Gurr *et al*, 1988). Two introns were predicted for the *A. parasiticus ver*-1 sequence which would allow translation consistent with the predicted open reading frames. Intron location and length (50 bp and 61 bp) were confirmed by analysis of a cDNA clone. The codon TAA in the final open reading frame (132 bp in length) was believed to be the translation termination codon because it was located approximately 130 bp upstream from the location of the polyadenylation site that was predicted by RNase protection and confirmed by cDNA sequence analysis. No other reasonable open reading frames could be found beyond this point. The putative spliced *ver*-1 sequence encodes a predicted polypeptide sequence of 262 amino acids.

Figure 15. Nucleotide sequence of the 1.8 kb EcoR1/HindIII restriction fragment containing the *ver*-1 gene. The predicted amino acid sequence is shown below the corresponding codon. Probable consensus sequences are underlined as follow: ____ Hogness box; _____ polyadenylation motif. """", region of transcription initiation (predicted by RNase protection); ^, polyadenylation site (predicted by RNase protection and confirmed by cDNA sequence); ~, nucleotides detected only in cDNA sequence.

```
   1  GAATTCACTTCTAAATGATACAAGCGCGAATATCTCCGATTAAGCCCACGTTAAGAGTATTTTCCAAGACATGCAGGGACAGATACAGACTTCCCTCAAG   100
 101  GTTAGAATCAACGAAAGGTTCCCTAGGCGACCAGTGAGAGATTGGCTTTGGATAGAGGCAGAGGCAGAGCAACATCCCAGGTACACGAAGCCAACCGTTT   200
 201  TCGTTCATTATTTTGTTTTTGGTGTGATTGGTCCAGAGCCTGCTCCTATTCTCAGCTTCCTATGCTTTCAGCCTGCCATAAACAAGATGTATTACTGCAT   300
 301  AGAAGTTTTAGGCTCGCCGCCCGATGAGCTACTGGTCTTCAGATATTCGGTCTCCGAGGAAAGATTTGTTTGGTGGCCAACCATCCATAGCTGCGTATAT   400

 401  ATGTACTACATGCCCGTTCCCCTGGGTCACCGTTTTCACAGAACTACACATCATTTTGCCTCCACAAAATCTCTACCATACACGATCCCGTCAGCATGTC   500
   1                 ****                                                                                N  S    2

 501  GGATAATCACCGTTTAGATGGCAAAGTGGCCTTGGTGACAGGCGCCGGCCGCGGCATCGGTGCTGCCATCGCCGTCGCCCTTGGTGAGCGCGGAGCCAAA   600
   3   D  N  N  R  L  D  G  K  V  A  L  V  T  G  A  G  R  G  I  G  A  A  I  A  V  A  L  G  E  R  G  A  K    35

 601  GTCGTGGTTAACTATGCCCACTGCCGCAGGCCGCGGAGAAAGTGGTTGAACAGATCAAGGCCAATGGTACCGATGCTGTCGCAATCCCAGGCCGATGTCG   700
  36  V  V  V  N  Y  A  H  C  R  R  P  R  R  K  W  L  N  R  S  R  P  N  V  P  N  L  S  Q  S  Q  A  D  V  G    69

 701  GGGATCCTGAGGCGACAGCGAAATTAATGGCGGAGACGGTGCGCCATTTTGGCTACCTGGACATCGTGTCATCGAACGCTGGAATTGTATCGTTCGGTCA   800
  70   D  P  E  A  T  A  K  L  N  A  E  T  V  R  N  F  G  Y  L  D  I  V  S  S  N  A  G  I  V  S  F  G  N    102

 801  CCTGAAAGACGTGACCCCAGAAgtatgaaccacagataacgcattcaaggcatatgctaaagaaaacactagGAGTTTGACAGGGTCTTCCGGGTCAACA   900
 103   L  K  D  V  T  P  E                                                     E  F  D  R  V  F  R  V  N  T    119

 901  CTCGTGGCCAGTTCTTCGTGGCGCGGGAGGCCTATCGCCATATGCGGGAAGGAGGCCGGATTATCCTGACCAGCTCTAACACCGCTTGCGTCAAGGGGGT   1000
 120   R  G  Q  F  F  V  A  R  E  A  Y  R  N  M  R  E  G  G  R  I  I  L  T  S  S  N  T  A  C  V  K  G  V    152

1001  ACCCAAACATGCTGTATACTCCGGTTCCAAGGGGGCTATTGACACCTTTGTTCGCTGCATGGCCATTGACTGCGGAGACAAGAAAATCACCGTGAATGCG   1100
 153   P  K  N  A  V  Y  S  G  S  K  G  A  I  D  T  F  V  R  C  M  A  I  D  C  G  D  K  K  I  T  V  N  A    185

1101  GTGGCTCCTGGAGCCATCAAGACTGATATGTTTTTGGCTGTGTCGCGGGAGTATATCCCCAATGGTGAGACTTTCACCGATGAGCAGGTAGACGAGgtca   1200
 186  V  A  P  G  A  I  K  T  D  M  F  L  A  V  S  R  E  Y  I  P  N  G  E  T  F  T  D  E  Q  V  D  E         217

1201  gctttcccccccataaactgcgtcttgttgggttcccgcttaacgaagtcttatctagTGTGCCGCTTGGCTCTCTCCTTTGAACCGCGTGGGCCTCCCTG   1300
 218                                                          C  A  A  W  L  S  P  L  N  R  V  G  L  P  V    232

1301  TGGATGTCGCCCGGGTAGTGAGCTTCCTGGCATCTGACACAGCCGAATGGGTAAGTGGAAAGATCATTGGGGTGGATGGTGGCGCTTTTCGATAAACCTT   1400
 233   D  V  A  R  V  V  S  F  L  A  S  D  T  A  E  W  V  S  G  K  I  I  G  V  D  G  G  A  F  R  *          262

1401  TACCGCTATATACTCGTGGGTGAAGTGTATTCTCTCGTATTATAAAGAGCTAGACGTCGTATTTGATAGGATTTGCTAGTTAAACTACAACGTAATATAA   1500

1501  GCTCTACTGCTCCCAGGTAGCGGGGAAAAAGACCTTGTATATATGCTTGAAAACCTTTCACATTACACTAATCACGGTAACTTCATATATCCAATGCGGC   1600

1601  CGTTGTGAGGTGGACAATTCGCAGTTCATTGCGTCGTTTTTCTCACTTCACCAAGCACCACCGCTCTCATTTTGGACCGATCTGTGAATCTATCCTCGTC   1700
1701  CTCCGCCACCTCCGTAGTCGACATAACAGGACAAATTGTTGAAATGCGCGTTCGCTCTCAAAGCT   1765
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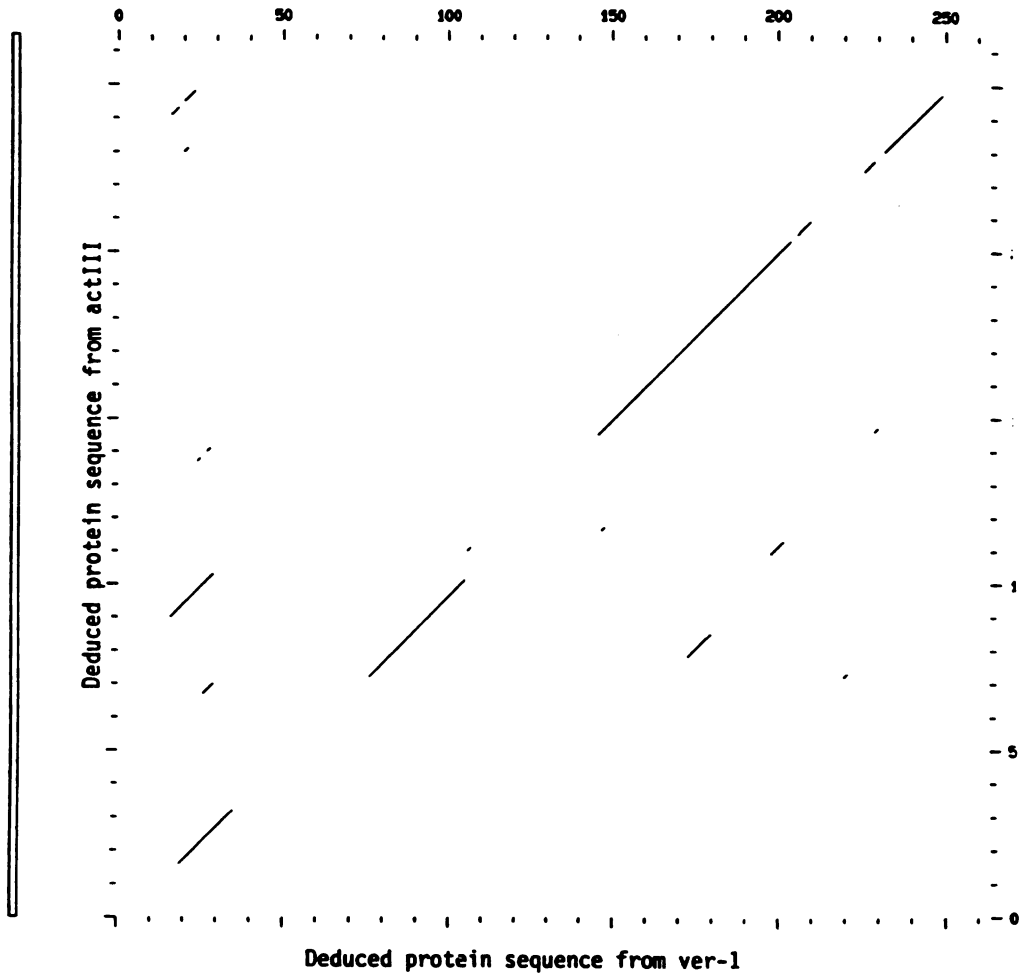
A search of the EMBL and GenBank database libraries using the Wisconsin Genetics Computer Group *FastA* protocol revealed that the predicted amino acid sequence of the *ver*-1 protein shared abundant identity with numerous reductases and dehydrogenases. A dotplot comparison (Figure 16) of the predicted *ver*-1 amino acid sequence with the known polypeptide sequence from the *Streptomyces coelicolor act*III gene (Hallam *et al*, 1988), encoding a ketoreductase associated with biosynthesis of the polyketide actinorhodin, demonstrates the extent of identity between these proteins. Similar dotplots were observed when peptide sequences from the *Azospirillum brasilense nod*G gene (Delledonne *et al*, 1990), encoding a ketoreductase associated with plant nodulation, and the *Bacillus megaterium gdh*A gene (Heilmann *et al*, 1988), encoding a glucose dehydrogenase, were compared to the predicted *ver*-1 amino acid sequence. All three proteins used in these comparisons were nearly identical in size (within 1-2 amino acids) to the predicted *ver*-1 protein. Best fit analysis using the amino acid sequence from *A. parasiticus ver*-1 determined an average 52 percent similarity (includes conservative substitutions) and 33 percent identity with all three of the proteins over their entire length. A predicted adenine nucleotide binding motif, Gly-X-Gly-X-X-Ala-12X-Lys consistent with NAD(P) or ATP binding proteins (Hopwood and Sherman, 1990; Kamps *et al*, 1984), can be found starting at Gly-18.

Figure 16. Dotplot analysis comparing the deduced peptide sequence from the *A. parasiticus ver*-1 gene and the deduced peptide sequence from the *Streptomyces act*III gene (ketoreductase). Window=30, stringency=15.


0
50
100
150
200
250
1
5
0
Deduced protein sequence from actIII
Deduced protein sequence from ver-1

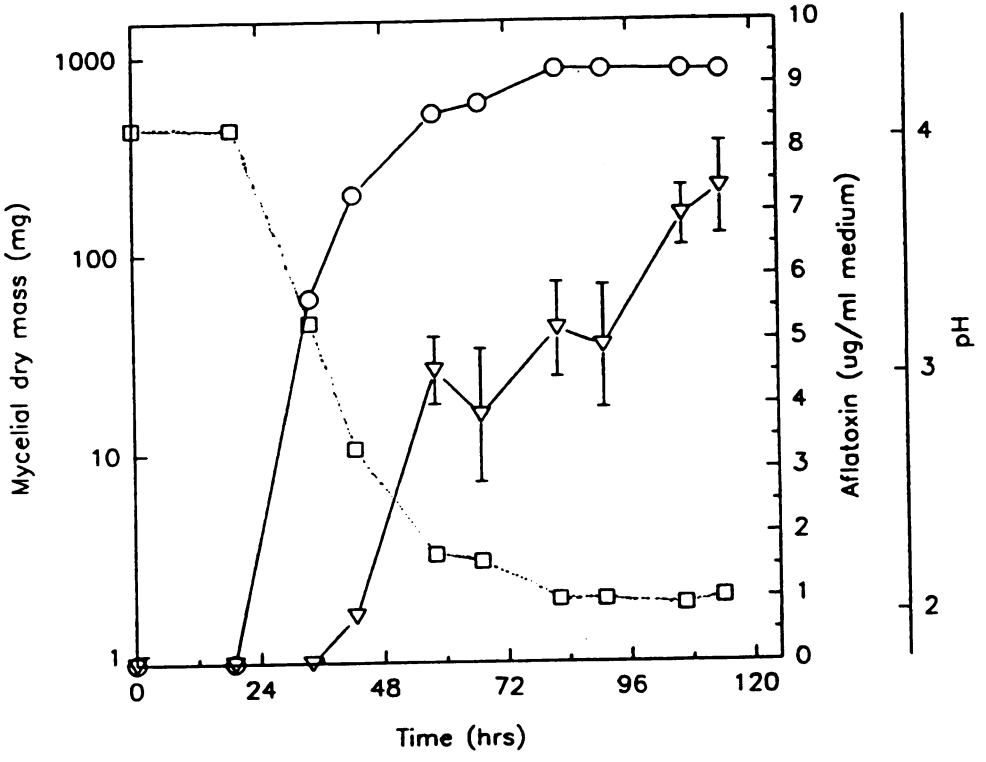
Linkage of *nor*-1 and *ver*-1 genes.

Parasexual analysis with Afl$^-$ mutants in *A. parasiticus* has generated conflicting results regarding the genetic linkage of *nor*-1 and *ver*-1 (Bennett *et al*, 1980; Bradshaw *et al*, 1983). We have found a cosmid using *in situ* colony hybridization (Ausubel) that hybridizes to both the *nor*-1 and *ver*-1 genes. It is possible that random association of separate restriction fragments carrying *ver*-1 and *nor*-1 occurred during construction of the cosmid library. However, the probability of such a rare event supports a physical linkage of these genes in the *A. parasiticus* genome. While structural genes are infrequently linked in *Aspergillus*, there are numerous exceptions to this pattern (Gurr *et al*, 1987). The close proximity of these two genes might suggest that many of the aflatoxin genes are tightly clustered as are the polyketide derived pigments in *Streptomyces* (Caballero *et al*, 1991; Davis and Chater, 1990; Hallam *et al*, 1988; Sherman *et al*, 1989). In support of this hypothesis, Papa (1984) concluded that nine out of eleven aflatoxin variants of *A. flavus* were in linkage group VII, which contains the *nor*-1 locus. The remaining two were localized to linkage groups II and VIII.

Expression of aflatoxin associated genes during batch fermentation.

Wild type *A. parasiticus* NRRL 5862 was grown in AM medium under conditions which support aflatoxin biosynthesis. The pattern of aflatoxin accumulation and mycelial growth (Figure 17) correlated with previous growth studies involving aflatoxigenic *A. parasiticus* strains (Sharma *et al*, 1980). Aflatoxin B_1 was first detected approximately 42 hrs after inoculation or near the end of trophophase (growth phase). Onset of trophophase was accompanied by a rapid decrease in the pH of the medium. The concentration of accumulated aflatoxin B_1 in the growth medium increased rapidly well into stationary phase with the greatest rate of accumulation occurring between 42 and 58 hrs after inoculation. The onset of stationary phase (approximately 60 hrs) was accompanied by a brief plateau of aflatoxin accumulation before the concentration again increased at approximately 96 hrs. The levels of aflatoxin did decrease slightly between 120-150 hrs (data not shown in figure). The error between duplicate samples was negligible for pH and mycelial mass in the continuous growth study (note: error bars for pH and mycelial mass are too small to be detected in figure 17). However, aflatoxin accumulation in the filtrate was somewhat variable. This inconsistency may be explained by variations in efficiency of aflatoxin B_1 transport out of the cell and probably could have been partly eliminated by analyzing total

Figure 17. Production of aflatoxin during batch fermentation. *A. parasiticus* NRRL 5862 was inoculated into defined medium (time 0) and grown at 29° C under shake conditions. Triplicate samples were removed for each time point and used for analysis of: o, mycelial dry mass; □, pH; and v, aflatoxin B_1 (μg/ml medium). Standard error is shown as vertical bars for all three measurements.


Mycelial dry mass (mg)
1000
100
10
1
0
24
48
72
96
120
Time (hrs)
10
9
8
7
6
5
4
3
2
1
0
Aflatoxin (ug/ml medium)
4
3
2
pH

aflatoxin B_1 (mycelium and filtrate). A period of decreased aflatoxin accumulation noted between 58 and 91 hrs is believed to be a result of the inoculum size. This type of biphasic pattern of aflatoxin accumulation often occurs when the initial spore concentration is greater than 10^3 conidia/ml medium (Sharma *et al*, 1980).

Northern hybridization analysis using *ver*-1 and *nor*-1 DNA probes was conducted on total RNA purified from mycelial cultures harvested at various time points during batch fermentation. The size of transcripts detected during this analysis were determined to coincide with previously published results (Skory *et al*, 1992; Chang *et al*, 1992) of approximately 1.0 kb and 1.3 kb for the *ver*-1 and *nor*-1 transcripts respectively. RNA transcripts from each gene were not detected until 36 hrs after inoculation (Figures 18 & 19). This was approximately 8 hrs previous to the first observation of aflatoxin B_1 in the filtrate. Maximum accumulation of these transcripts occurred between 60-72 hrs after inoculation. Evidence for transcript degradation during sample preparation or as a result of *in vivo* digestion, indicated by smearing below the band, first appeared at 48 hrs and increased after this time. Degradation products from the *ver*-1 and *nor*-1 transcripts could still be detected as late as 146 hrs after inoculation.

The accumulation of RNA transcribed from the *pyrG* gene, which encodes an orotidine monophosphate decarboxylase involved in uridine biosynthesis (Figure 6) was also examined.

Figure 18. Accumulation of *ver*-1 transcript during batch fermentation. Approximately 7 μg (per sample) of total RNA isolated from various time points during the growth study was separated by denaturing gel electrophoresis. RNA was transferred to Nytran membranes and then hybridized to ^{32}P labelled probes containing the *ver*-1.

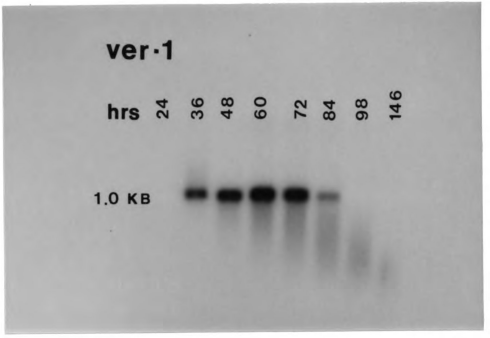
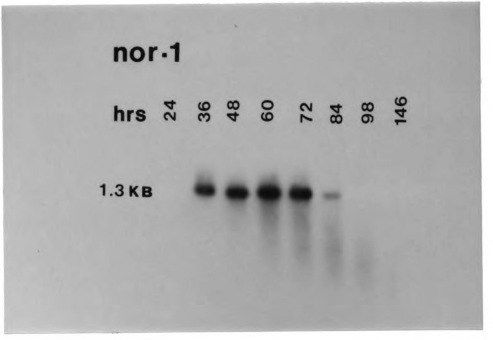

ver·1
hrs 24 36 48 60 72 84 98 146
1.0 KB

Figure 19. Accumulation of *nor*-1 transcript during batch fermentation. Approximately 7 μg (per sample) of total RNA isolated from various time points during the growth study was separated by denaturing gel electrophoresis. RNA was transferred to Nytran membranes and then hybridized to ^{32}P labelled probes containing the *nor*-1.


nor-1
hrs 24 36 48 60 72 84 98 146
1.3 KB

This analysis was used to compare the accumulation of RNA transcribed from genes associated with aflatoxin biosynthesis to RNA transcribed from a gene involved in primary metabolism. The *pyrG* transcript was first detected at 24 hrs (Figure 20). Maximum accumulation of the transcript is believed to be between 24 and 36 hrs after inoculation and corresponds to the period of greatest rate of mycelial mass production. Accumulated levels of transcript then decreased after 36 hrs and could not be detected beyond the onset of stationary phase (approximately 84 hrs after inoculation). The *pyrG* transcript revealed little degradation, especially at the early time points confirming that the absence of the *ver*-1 and *nor*-1 transcript at 24 hrs was not a result of the quality of the RNA preparation.

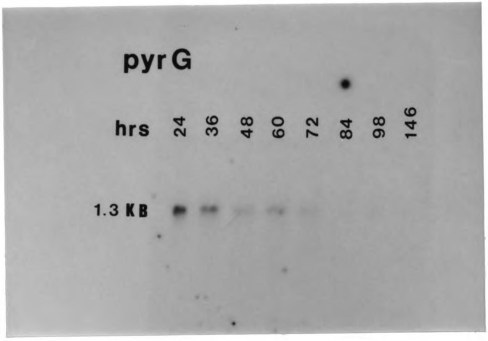
During batch fermentation of *A. parasiticus*, the *ver*-1 and *nor*-1 RNA transcripts were observed to accumulate in a manner similar to what might be expected of genes involved in secondary metabolism (Drew and Demain, 1977). RNA transcribed from these genes was not detected until near the end of trophophase and this accumulation was followed approximately 8 hours later by idiophase when aflatoxins are produced. The pattern of accumulation of the *pyrG* transcript which is involved in primary metabolism differed significantly from the accumulation of the *ver*-1 and *nor*-1 transcripts. Maximum accumulation of the *pyrG* transcript occurred during trophophase (approximately 24 hrs after inoculation), a period when the *ver*-1 and *nor*-1 transcripts were not detectable,

Figure 20. Accumulation of *pyrG* transcript during batch fermentation. Northern analysis was performed as described in figure 2, except a *pyrG* containing DNA fragment was used to prepare the ^{32}P labelled probe.

batch

bed in

sed to



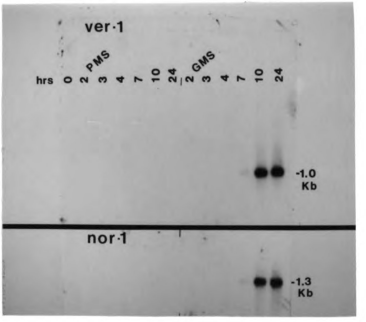
whereas maximum accumulation of the *ver*-1 and *nor*-1 transcripts was observed just prior to the onset of stationary phase (approximately 60-72 hrs after inoculation). Additionally, the presence of the *ver*-1 and *nor*-1 transcripts continued well beyond that which was observed with the *pyr*G gene.

The exact mechanisms involved in the regulation of induction for aflatoxin biosynthesis are not fully understood. However, synthesis of this secondary metabolite generally occurs during a period of repressed tricarboxylic acid (TCA) cycle activity (Buchanan *et al*, 1985). We might then anticipate a reduction in pyrimidine biosynthetic activity during aflatoxin production since the precursors for uridine biosynthesis are derived from metabolites of the TCA cycle. Such a relationship is reflected in the maximal accumulation of *pyr*G RNA transcripts early in the growth of the fungus, while *nor*-1 and *ver*-1 RNA appear to reach maximum accumulation near stationary phase of growth; a period where TCA activity would be expected to be reduced.

Expression of aflatoxin associated genes following nutritional shift.

The *ver*-1 and the *nor*-1 transcripts were not detected in RNA isolated from the mycelium initially grown in PMS medium which does not support aflatoxin biosynthesis (Figure 21).

Figure 21. Accumulation of *ver*-1 and *nor*-1 transcripts following nutrition shift. *A. parasiticus* NRRL 5862 grown for 65 hrs in PMS medium was equally distributed to fresh PMS and GMS medium and growth was continued under the same incubation conditions. RNA was isolated previous to the transfer (time 0) and at various times following transfer from the spent PMS to fresh GMS and PMS media. Approximately 7 μg of total RNA (per sample) was analyzed as described in figure 2. Top panel: *ver*-1 probe. Bottom panel: *nor*-1 probe.


ver·1
hrs
0
2 PMS
3
4
7
10
24
2 GMS
3
4
7
10
24
-1.0
Kb
nor·1
-1.3
Kb

Likewise, *ver*-1 and *nor*-1 transcripts could not be detected in RNA isolated from mycelium after transfer to fresh PMS medium. However, these transcripts were observed 7 hrs after transfer to GMS medium which has been shown to induce aflatoxin biosynthesis. The *pyr*G transcript was barely detectable in all of the RNA samples tested suggesting that the absence of detectable *ver*-1 and *nor*-1 transcripts was not due to a lack of RNA quality. The low level of *pyr*G transcript is presumably a result of limited growth following transfer of mycelium that was harvested during stationary phase of growth. Aflatoxin B_1 was found in the medium 24 hrs after transfer from PMS to fresh GMS medium (Table III). No aflatoxins could be detected even 48 hrs after transfer from the initial PMS medium to the fresh PMS medium.

It was previously shown by Buchanan *et al* (1987) that peptone, used in the nutritional shift study, is not inhibitory to aflatoxin biosynthesis. Instead, glucose is most likely involved in a carbon catabolite repression of NADPH generating enzymes and TCA cycle enzymes (Buchanan and Lewis, 1984). This repression would contribute to lowering the cellular NADPH/NADP ratio which has been implicated in being involved in induction of aflatoxin biosynthesis (Neihaus and Jiang, 1989; Bhatnagar *et al*, 1986). Our finding that accumulation of the *ver*-1 and *nor*-1 RNA transcripts occurs between 4-7 hrs after transfer to the carbohydrate containing medium corresponds with predicted values obtained in a similar study using transcription inhibitors to study induction of

Table III. Aflatoxin accumulation following nutritional shift.

	Aflatoxin concentration (μg/ml filtrate)[a]	
Hrs[b]	PMS	GMS
10	nd	nd
24	nd	0.6
48	nd	3.7

[a]Determined by direct competitive ELISA.
[b]Hours after transfer from initial PMS medium to either fresh PMS or GMS.
PMS, peptone mineral salt medium; GMS, glucose mineral salts medium; nd, none detected.

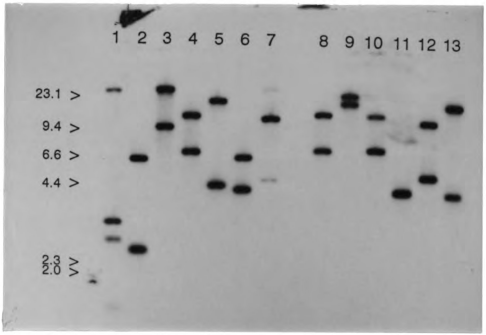
aflatoxin biosynthesis (Buchanan *et al*, 1987).

The timing of appearance of the *ver*-1 and *nor*-1 transcripts relative to aflatoxin detection in batch fermentation and nutritional shift analysis strongly suggest that aflatoxin biosynthesis is partly regulated by the accumulation of the *ver*-1 and *nor*-1 RNA transcripts. It is generally believed that genes of the filamentous fungi are usually regulated at the transcriptional level (Gurr *et al*, 1987). We speculate the observed RNA accumulation parallels the rate of RNA transcription for the aflatoxin genes examined. However, it will not be possible to conclusively determine if this accumulation is controlled primarily through the rate of RNA transcription or by mechanisms affecting RNA stability until these processes are measured directly.

Southern analysis of *A. parasiticus* NRRL 5862

Genomic DNA from *A. parasiticus* NRRL 5862 was digested with restriction endonucleases, separated by agarose gel electrophoresis, and examined by Southern hybridization analysis using the *ver*-1 containing 1.8 Kb EcoR1/SalI fragment as probe (Figure 22). The data from Southern analysis were compared to known restriction endonuclease sites determined by restriction endonuclease mapping and nucleotide sequence analysis. Two hybridizing bands of almost equal intensities were noted for DNA digested with restriction endonucleases

Figure 22. Southern hybridization analysis of genomic DNA from *A. parasiticus* NRRL 5862 digested with assorted restriction endonucleases. Digested DNA was electrophoresed in a 0.8% agarose gel (0.8 μg per lane), transferred to Nytran membranes (Schleicher and Schuell, INC, Keene, NH) and probed with a *ver*-1 containing 1.8 Kb EcoR1/SalI DNA fragment. 1) BamH1, 2) EcoR1, 3) XbaI, 4) SalI, 5) SphI, 6) PvuII, 7) SmaI, 8) SalI, 9)HindIII 10) BglII, 11) ClaI, 12) PstI, 13) PstI and 14) XhoI.


1 2 3 4 5 6 7 8 9 10 11 12 13
23.1 >
9.4 >
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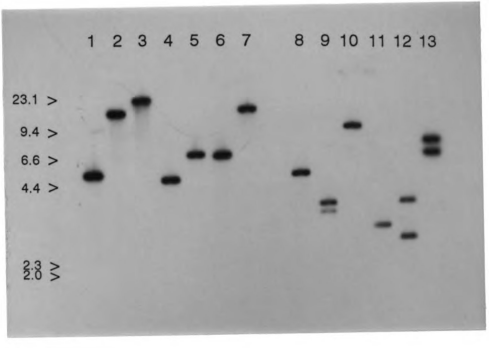
(EcoR1, XbaI, SalI, SphI, PvuII, HindIII, BglII, PstI, and XhoI) which do not digest within the 1.8 kb region and four hybridizing bands were observed with BamHI which cuts once within the 1.8 kb region. These data strongly suggest that an additional region of the genome shares significant sequence identity with the *ver*-1 gene and may represent a gene duplication or a pseudogene. The *ver*-1 gene and this *ver*-1 like sequence are believed to be in close proximity since only one 4.4 kb ClaI hybridizing fragment was detected when using the *ver*-1 as probe. However, it is possible that both *ver*-1 like sequences reside on different, but similar size ClaI restriction fragments.

The same membrane was stripped and reprobed with the *nor*-1 containing 1.5 kb EcoR1/ClaI restriction endonuclease fragment (Figure 23). Only one hybridizing band was observed for all restriction enzymes which were previously determined not to digest within the *nor*-1 sequence. Two hybridizing bands were noted for the restriction endonucleases HindIII, PstI, and XhoI. However, this result was expected since these enzymes are known to digest once within the *nor*-1 sequence used as probe. These data confirm that only one copy of *nor*-1 gene exists in the *A. parasiticus* genome.

There appears to be a strong degree of sequence identity, as suggested by similar intensities in Southern analysis, between the *ver*-1 gene and the *ver*-1 like region. Additionally, both the *ver*-1 gene and the *ver*-1 like region contain a BamH1 restriction endonuclease site, yet lack

Figure 23. Southern hybridization analysis of genomic DNA from A. parasiticus NRRL 5862 digested with assorted restriction endonucleases. Digested DNA was electrophoresed in a 0.8% agarose gel (0.8 μg per lane), transferred to Nytran membranes (Schleicher and Schuell, INC, Keene, NH) and probed with a *nor*-1 containing 1.5 Kb EcoR1/ClaI DNA fragment. 1) BamH1, 2) EcoR1, 3) XbaI, 4) SalI, 5) SphI, 6) PvuII, 7) SmaI, 8) SalI, 9)HindIII 10) BglII, 11) ClaI, 12) PstI, 13) PstI and 14) XhoI.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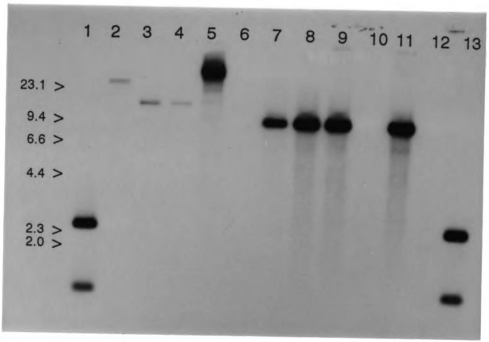
restriction sites for the nine other enzymes tested. We hypothesize that the additional copy of the *ver*-1 like sequence most likely arose by gene duplication. We do not currently know the extent of identity between these two regions or if this *ver*-1 like region represents a functional gene. Pseudogenes are commonly found in gene clusters but are usually defective in transcription, intron excision, and/or translation (Freifelder, 1987). This *ver*-1 like region is being isolated for further study. Sequence analysis should characterize the extent of this identity and help determine the functionality, if any, of this region.

Southern analysis of related *Aspergillus* species

Genomic DNA from related *Aspergillus* spp. was digested with the restriction endonuclease BamH1, separated by agarose gel electrophoresis, and examined by Southern hybridization analysis using a 0.7 kb EcoR1/BamH1 DNA fragment that contains the 5' region of the *ver*-1 gene (Figure 24). DNA fragments hybridizing to this probe were detected for all species tested except for *A. tamarii* and *A. versicolor*. Band intensities for *A. nidulans* were significantly lighter than for the remaining fungi. Only one hybridization band was detected for all species except for *A. parasiticus* NRRL 5862 which contained two bands of sizes 2.6 kb and 1.1 kb. Insufficient digestion of *A. flavus* SRRC 1273 DNA prevented size

Figure 24. Southern hybridization analysis of genomic DNA from various *Aspergillus* spp. digested with BamH1. Digested DNA was electrophoresed in a 0.8% agarose gel (0.8 μg per lane), transferred to Nytran membranes (Schleicher and Schuell, INC, Keene, NH) and probed with a *ver*-1 containing 1.8 Kb EcoR1/SalI DNA fragment. 1) *A. parasiticus* NRRL 5862, 2) *A. nidulans* ATCC 26451, 3) *A. nidulans* SRRC 1076, 4) *A. nidulans* ATCC 32610, 5) *A. flavus* SRRC 1273, 6) *A. tamarii* SRRC 1244, 7) *A. sojae* SRRC 1120, 8) *A. sojae* ATCC 42251, 9) *A. sojae* ATCC 9362, 10) *A. versicolor* ATCC 26939, 11) *A. oryzae* ATCC 14895, 12) *A. tamarii* (var *flavus*) SRRC 2045, 13) *A. parasiticus* NRRL 5862.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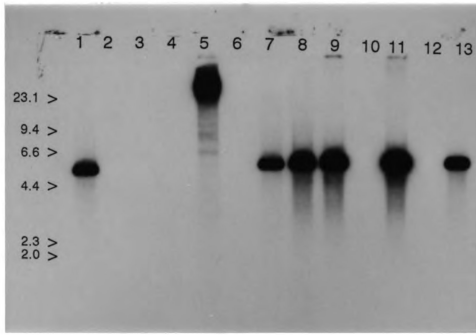
determination of the hybridizing fragment. However, preliminary data in further studies suggest that the *ver*-1 gene hybridizes to a 2.6 kb fragment in other *A. flavus* species. The presence of only one hybridizing fragment for *A. sojae* (8.9 kb), *A. oryzae* (8.9 kb), *A. nidulans* ATCC 26451 (>23 kb), *A. nidulans* SRRC 1079 (11.2 kb), and *A. nidulans* ATCC 32610 (11.2 kb) might suggest that only *A. parasiticus* contains two copies of *ver*-1 like sequences. However, Southern hybridization analysis with additional restriction endonucleases would be required before this could be concluded since restrictions endonuclease maps most likely differ between these species.

The same membrane was stripped and reprobed with the *nor*-1 containing 1.5 EcoR1/ClaI fragment (Figure 25). A 5.8 kb DNA fragment hybridizing to the *nor*-1 was observed for all species except for *A. nidulans*, *A. tamarii*, and *A. versicolor* which did not have any detectable hybridization signals. Intensities were of approximately equal values for all samples that hybridized to the *nor*-1 gene.

These data suggest that nucleotide sequences similar to the *ver*-1 and *nor*-1 aflatoxin biosynthetic genes are not unique to *A. parasiticus*. Both genes were found to hybridize very strongly to *A. sojae* sp. which shares a high degree of overall DNA relatedness with *A. parasiticus*. Likewise, *A. oryzae* sp. which shares a great degree of DNA relatedness with *A. flavus* also hybridized to these genes (Kurtzman *et al*, 1987; Klich and Pitt, 1988). Aflatoxin production in *A.*

Figure 25. Southern hybridization analysis of genomic DNA from various Aspergillus spp. digested with BamH1. Digested DNA was electrophoresed in a 0.8% agarose gel (0.8 μg per lane), transferred to Nytran membranes (Schleicher and Schuell, INC, Keene, NH) and probed with a *nor*-1 containing 1.5 Kb EcoR1/ClaI DNA fragment. 1) *A. parasiticus* NRRL 5862, 2) *A. nidulans* ATCC 26451, 3) *A. nidulans* SRRC 1076, 4) *A. nidulans* ATCC 32610, 5) *A. flavus* SRRC 1273, 6) *A. tamarii* SRRC 1244, 7) *A. sojae* SRRC 1120, 8) *A. sojae* ATCC 42251, 9) *A. sojae* ATCC 9362, 10) *A. versicolor* ATCC 26939, 11) *A. oryzae* ATCC 14895, 12) *A. tamarii* (var *flavus*) SRRC 2045, 13) *A. parasiticus* NRRL 5862.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oryzae and *A. sojae* has always been a concern because these strains are commonly used in food fermentations. However, extensive research with these fungi have failed to demonstrate their ability to produce aflatoxins (Barbesgaard *et al*, 1992).

A. nidulans sp. and *A. versicolor* sp. produce sterigmatocystin (Chung *et al*, 1989; Hajjar *et al*, 1990; Hamasaki *et al*, 1967) and thus might be expected to contain similar aflatoxin biosynthetic genes. However, we found that the *ver*-1 only weakly hybridized to the *A. nidulans* DNA (Figure 24) and that no hybridization could be detected with the *nor*-1 gene. Likewise, no hybridization signal was detected for *A. versicolor* when probed with the *ver*-1 or the *nor*-1 genes (Figures 24 and 25). The lack of homology between these genes could suggest that the aflatoxin biosynthetic pathway has been present for a very long period of time. It is possible that the ability to produce sterigmatocystin was present in a common ancestor and evolved to eventually produce aflatoxin B_1 in the related species *A. parasiticus*, *A. flavus*, and *A. nomius*. Many of these evolved fungi retained their ability to produce sterigmatocystin while some such as *A. oryzae*, and *A. sojae* lost that ability. We would expect genetic changes in these aflatoxin biosynthetic genes since secondary metabolites are not essential for growth and therefore are not under immense selective pressure to retain the same genetic code. Organisms such as *A. parasiticus* and *A. sojae* have a high degree of DNA relatedness, suggesting that they have only recently evolved from a common ancestor,

might be expected to share much more homology between aflatoxin biosynthetic genes than for *A. parasiticus* and *A. versicolor* which are believed to have diverged much earlier.

While these ideas are purely speculative, they do help to explain the commonness of sterigmatocystin production and the uniqueness of aflatoxin B_1 production. Further analysis using additional aflatoxin biosynthetic genes should help identify the diversity of these genes and allow the proposal of divergent or possibly convergent evolutionary models. It will also be interesting to compare the RNA expression of aflatoxin biosynthetic genes found in both aflatoxin B_1 producers and nonproducers. Studies comparing the DNA relatedness and rRNA will also help define the genetic variation between related fungi. This will eventually allow the proposal of a workable model which helps to explain the genetic evolution of aflatoxin biosynthesis.

CONCLUDING REMARKS

The *A. parasiticus* transformation system based on restoration of uridine prototrophy (*pyrG*) was used successfully in isolating a gene (*ver*-1) associated with aflatoxin biosynthesis. The data strongly suggest that this *ver*-1 gene is associated with the conversion of VA → ST because it complements Afl^- mutants which are unable to carry

out this biotransformation step. Since this complex conversion of an anthraquinone moiety to a xanthone structure is believed to occur through a number of enzymatic steps, it is probable that the *ver*-1 polypeptide must cooperate with the products of other genes. Nucleotide sequence analysis proved to be extremely useful in predicting that the *ver*-1 gene encodes a protein which has enzymatic activity associated with this bioconversion. The predicted *ver*-1 protein displays significant conservation of size and amino acid sequence with reductases and dehydrogenases involved in modification of ring structures. Further support of this predicted enzymatic function is the hypothesis of a NADPH dependent reductase involved in the conversion of VA to ST (Bhatnagar *et al*, 1992; Dutton, 1988). Following complementation with the *ver*-1 gene in Afl$^-$ mutants, biosynthesis of aflatoxins B_2 and G_2 were also restored in addition to aflatoxins B_1 and G_1 (Bhatnagar, personal communication). This suggests that the product of *ver*-1 gene is also associated with the conversion of versicolorin B to dihydrosterigmatocystin (Lin and Anderson, 1992; Yabe *et al*, 1991). This proposal is consistent with theories that speculate that one set of enzymes carries out parallel functions on alternative aflatoxin intermediates, at a branch in the pathway, to allow for the synthesis of aflatoxins B_1 then G_1 at one branch of the pathway and B_2 then G_2 at the other branch (Figure 5) (Bhatnagar *et al*, 1992).

Although we cannot conclusively rule out the possibility that *ver*-1 encodes a suppressor activity, the data strongly

suggest that the restoration of aflatoxin biosynthesis in *ver*-1 transformants is due to a direct complementation of a missing enzyme activity. 1) Nucleotide sequence analysis predicts a reductase function for the product of the *ver*-1 gene. 2) No Afl^+ transformants have ever been observed when *A. parasiticus* CS10 is transformed with only the *pyrG* gene, suggesting that the *ver*-1 mutation is quite stable (i.e. it is improbable that we are seeing reversion of a mutation). 3) Versicolorin A is converted to aflatoxin in Afl^+ transformants at an efficiency comparable to the wild type strain. This efficiency would not be anticipated in transformants with only one additional copy of a gene encoding an enzyme which fortuitously converts VA to St. 4) RNA transcripts from the *ver*-1 gene accumulate only during idiophase suggesting that the gene functions in secondary metabolism.

The results of this study also imply an unknown but useful purpose for aflatoxin biosynthesis. Possible functions or benefits of aflatoxin production have been studied with ambiguous results (Heathcote, 1978). To put such a tremendous amount of cellular energy into biosynthesis of a secondary metabolite without any function might be considered wasteful for the organism. However, it has been proposed that the lack of discernable biological activity for a secondary metabolite simply reflects that the correct screening assays have not been employed (Maplestone *et al*, 1992; Vinning, 1992). Finding sequences, in a number of related *Aspergillus* species, that hybridize very strongly to the aflatoxin biosynthetic

genes *ver*-1 and *nor*-1 might suggest that aflatoxin (or sterigmatocystin) biosynthesis has been around for quite sometime. The linkage of aflatoxin biosynthetic genes also provides evidence that this secondary metabolite provides a beneficial function. Genes for secondary metabolism are often clustered together presumably because of selective pressure. If production of a secondary metabolite is useful to the organism, then it might be favorable if the genes were closer together to facilitate signaling between genes and to increase the probability that they would be passed on as a unit to further generations (Maplestone *et al*, 1992). It would make little sense that such a pathway would evolve and remain as a clustered unit if there were not selective pressure to keep it together.

The information and tools obtained from this research project should allow for the continued success in the study of molecular control of aflatoxin biosynthesis. Preliminary studies suggest that the aflatoxin gene cluster is able to complement at least two additional mutants blocked at bioconversions steps. Additionally, it appears that disruption of aflatoxin biosynthetic genes is feasible and could possibly be used in field studies for the prevention of aflatoxin contamination by methods of competitive exclusion. Methods of aflatoxin regulation should soon begin to be understood as studies with gene expression continue. It is hoped that this research will eventually benefit efforts to facilitate the elimination of aflatoxin contamination.

LITERATURE CITED

Adye, J. and R.I. Mateleles. 1964. Incorporation of labelled compounds into aflatoxin. Bichim. Biophys. Acta. 86:418-420.

Anderson, J.A. and C.H. Chung. 1990. Conversion of versiconal acetate to versiconal and versicolorin C in extracts from *Aspergillus parasiticus*. Mycopathologia 110:31-35.

Anderson, J.A., C.H. Chung, S.U. Cho. 1990. Versicolorin A hemiacetal, hydroxydihydrosterigmatocystin, and aflatoxin G_{2a} reductase activity in extracts from *Aspergillus parasiticus*. Mycopathologia 111:39-45.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (eds). 1987. Current protocols in molecular biology, vol I. John Wiley & Sons. New York.

Barbesgaard, P., H.P. Heldt-Hansen, and B. Diderichsen. 1992. On the safety of *Aspergillus oryzae*: a review. Appl. Microbiol. Biotechnol. 36:569-572.

Beck, J., S.Ripka, A. Siegner, E. Schiltz, and E. Schweizer. 1990. The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum*. Eur. J. Biochem. 192:487-498.

Bennett, J.W., and L.A. Goldblatt. 1973. The isolation of mutants of *Aspergillus flavus* and *Aspergillus parasiticus* with altered aflatoxin producing ability. Sabouraudia 11:235-241.

Bennett, J.W., L.S. Lee, and A.F. Cucullu. 1976. Effect of dichlorvos on aflatoxin and versicolorin A production in *Aspergillus parasiticus*. Bot. Gaz. 137(4):318-324.

Bennett, J.W., L.S. Lee, S.M. Shoss, and G.H. Boudreaux. 1980. Identification of averantin as an aflatoxin B_1 precursor: placement in the biosynthetic pathway. Appl. Environ. Microbiol. 39:835-839.

Bennett, J.W., C.H. Vinnett, and W.R. Goynes,JR. 1980. Aspects of parasexual analysis in *Aspergillus parasiticus*. Can. J. Microbiol. 26:706-713.

Bennett, J.W., P.M. Leong, S. Kruger, and D. Keyes. 1986. Sclerotial and low aflatoxigenic morphological variants from haploid and diploid *Aspergillus parasiticus*. Experientia 42:848-851.

Bennett, J.W., and K.E. Papa. 1987. The aflatoxigenic *Aspergillus* spp. Adv. Plant Pathol. 6:263-280.

Berry, Colin. 1988. The pathology of mycotoxins. J. Path. 154:301-311.

Bhatnagar, D., A.H.J. Ullah, and T. E. Cleveland. 1988. Purification and characterization of a methyltransferase from *Aspergillus parasiticus* SRRC 163 involved in aflatoxin biosynthetic pathway. Prep. Biochem. 18(3):321-349.

Bhatnagar, D., T.E. Cleveland, and E.B. Lillehoj. 1989. Enzymes in aflatoxin B_1 biosynthesis: Strategies for identifying pertinent genes. Mycopathologia 107:75-83.

Bhatnagar, D., and T.E. Cleveland. 1990. Purification and characterization of a reductase from *Aspergillus parasiticus* SRRC 2043 involved in aflatoxin biosynthesis. FASEB J. 4:A2164.

Bhatnagar, D., T.E. Cleveland, and D.G. Kingston. 1991. Enzymological Evidence for Separate Pathways for Aflatoxin B_1 and B_2 Biosynthesis. Biochem. 30(17);4343-4350.

Bhatnagar, D., K.C. Erlich, and T.E. Cleveland. 1992. Oxidation-reduction reactions in biosynthesis of secondary metabolites and their control by environmental factors, p. 255-286. *In* D. Bhatnagar, E.B. Lillehoj, and D.K. Arora (ed.) Handbook of applied mycology, vol V. Marcel Dekker, Inc, New York.

Bhatnagar, R.K., S. Ahmad, K.G. Mukerji, and T.A. Venkitasubramanian. 1986. Nitrogen metabolism in *Aspergillus parasiticus* NRRL 3240 and *A. flavus* NRRL 3537 in relation to aflatoxin production. J. Appl. Bact. 60:203-211.

Biollazz, M., G. Buchi, and G. Milne. 1970. The biosynthesis of aflatoxins. J. Am. Chem. Soc. 92:1035-1043.

Boeke, J.D., F. LaCroute, and G.R. Fink. 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. 197: 345-346.

Bradford, M.M.. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72:248-254.

Bradshaw, R.E., J.W. Bennett, and J.F. Peberdy. 1983. Parasexual analysis of *Aspergillus parasiticus*. J. Gen. Microbiol. 129:2117-2123.

Buchanan, R.L., and D.F. Lewis. 1984. Regulation of aflatoxin biosynthesis: Effect of glucose on activities of various glycolytic enzymes. Appl. Environ. Micro. 48(2):306-310.

Buchanan, R.L., D. Federowicz, and H.G. Stahl. 1985. Activities of tricarboxylic acid cycle enzymes in an aflatoxigenic strain of *Aspergillus parasiticus* after peptone to glucose carbon source shift. Trans. Br. Mycol. Soc. 84(2):267-275.

Buchanan, R.L., S.B. Jones, W.V. Gerasimowicz, L.L. Zaika, H.G. Stahl, and L.A. Ocker. 1987. Regulation of aflatoxin biosynthesis: Assesment of the role of cellular energy status as a regulator of the induction of aflatoxin production. Appl. Environ. Micro. 53(6):1224-1231.

Caballero, J.L., E. Martinez, F. Malpartida, and D.A. Hopwood. 1991. Organisation and function of the *act*VA region of the actinorhodin biosynthetic gene cluster of *Streptomyces coelicolor*. Mol. Gen. Genet. 230:401-412.

Campbell, T.C., J. Chen, C. Liu, J. Li, and B. Parpia. 1990. Nonassociation of aflatoxin with primary liver cancer in a cross-sectional ecological survey in the People's Republic of China. Cancer Res. 50:6882-6893.

Campbell, T.C., J. Chen, T.A. Brun, C. Liu, and C.A. Geissler. 1991. Aflatoxin and liver cancer. The Lancet. 335:1165.

CAST (Council for Agicultural Science and Technology). 1989. Mycotoxins: Economic and health risks. Library of Congress Task force report. 29-31.

Chang, P.K., C.S. Skory, and J.E. Linz. 1992. Cloning of a gene associated with aflatoxin B_1 biosynthesis in *Aspergillus parasiticus*. Curr. Gen. 21(3):

Chu, F.S., M.S. Hsia, and P.S. Sun. 1977. Preparation and characterization of aflatoxin B_1-1-(O-carboxymethyl) oxime. J. Assoc. Off. Anal. Chem. 60(4):791-794.

Chu, F.S. 1991. Mycotoxins:food contamination, mechanisms, carcinogenic potential, and preventative measures. Mutation Res. 259:291-306.

Chung, D.H., M.M. Abouzied, and J.J. Pestka. 1989. Immunochemical assay applied to mycotoxin biosynthesis:ELISA comparisons of sterigmatocystin production by *Aspergillus versicolor* and *Aspergillus nidulans*. Mycopath. 107:93-100.

Chuturgoon, A.A., M.F. Dutton, and R.K. Berry. 1990. The preparation of an enzyme associated with aflatoxin biosynthesis by affinity chromatography. Biochem. Biophys. Res. Commun. 166(1):38-42.

Chuturgoon, A.A. and M.F. Dutton. 1991. The affinity purification and characterization of a dehydrogenase from

Aspergillus parasiticus involved in aflatoxin B_1 biosynthesis. Prep. Biochm. 21(2&3):125-140.

Cleveland, T.E. 1987. Appearance of enzyme activities catalyzing conversion of sterigmatocystin to aflatoxin B_1, in late-growth phase *Aspergillus parasiticus* cultures. Appl. Environ. Microbiol. 53:1711-1713.

Cleveland, T.E. 1989. Conversion of dihydro-O-methyl-sterigmatocystin to aflatoxin B_2 by *Aspergillus parasiticus*. Arch. Environ. Contam. Toxicol. 18:429-433.

Cleveland, T.E. and D. Bhatnagar. 1990. Evidence for *de novo* synthesis of an aflatoxin pathway methyltransferase near the cessation of active growth and the onset of aflatoxin biosynthesis in *Aspergillus parasiticus* mycelia. Can. J. Micro. 36:1-5.

Cole, R.J., J.W. Dorner, P.D. Blankenship, and B. Horn. 1991. The use of biocompetitive agents for preharvest aflatoxin control in peanuts. 1991 Aflatoxin elimination workshop. Atlanta, Georgia.

Cotty, P.J. 1988. Aflatoxin and sclerotial production by *Aspergillus flavus*: Influence of pH. Phytopath. 78(9). 1250-1253.

Cotty, P.J. 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79(7):808-814.

Cotty, P.J. 1990. Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. Plant Disease 74(3):233-235.

Cotty, P.J. and D.J. Daigle. 1991. Update on *Aspergillus flavus* populations and prevention of aflatoxin through intraspecific competition. 1991 Aflatoxin elimination workshop. Atlanta, Georgia.

Coupland, K. and W.G. Niehaus. 1987. Effect of nitrogen supply, Zn^{+2}, and salt concentration on Kojic acid and versicolorin biosyntheis by *Aspergillus parasiticus*. Exp. Mycol. 11:206-213.

Cusumano, V., G.B. Costa, and S. Seminara. 1990. Effect of aflatoxins on rat peritoneal macrophages. Appl. Environ. Micro. 56(11):3482-3484.

Davis, N.D., S.K. Iyer, and U.L. Diener. 1987. Improved method of screening for aflatoxin with a coconut agar medium. Appl. Environ. Microbiol. 53:1593-1595.

Davis, N.K. and K.F. Chater. 1990. Spore colour in *Streptomyces coelicolor* A3(2) involves the developmentally regulated synthesis of a compound biosynthetically related to polyketide antibiotics. Molec. Micro. 4(10):1679-1691.

Delledonne, M., R. Porcari, and C. Fogher. 1990. Nucleotide sequence of the *nod*G gene of *Azospirillum brasilense*. Nucleic Acids Res. 18(21):6435.

Diez, B., E. Alvarez, J.M. Cantoral, J.L. Barredo, and J.F. Martin. 1987. Selection and characterization of *pyr*G mutants of *Penicillium chrysogenum* lacking orotidine-5'-phosphate decarboxylase and complementation by the *pyr*4 gene of *Neurospora crassa*. Curr. Genet. 12:277-282.

Donadio, S., M.J. Staver, J.B. McAlpine, S.J. Swanson, and L. Katz. 1991. Modular organization of genes required for complex polyketide biosynthesis. Biochem. Biophy. Res. Comm. 47:1051-1055.

Donkersloot, J.A., R. I. Mateles, and S.S. Yang. 1972. Isolation of averufin from a mutant of *Aspergillus parasiticus* impaired in aflatoxin biosynthesis. Biochem. Biophys. Res. Commun. 47:1051-1055.

Dorner, J.W., R.J. Cole, and U.L. Diener. 1984. The relationship of *Aspergillus flavus* and *Aspergillus parasiticus* with reference to production of aflatoxins and cyclopiazonic acid. Mycopathologia 87:13-15.

Drew, S.W., and A.L. Demain. 1977. Effects of primary metabolites on secondary metabolism. Ann. Rev. Microbiol. 31:343-56.

Dutton, M.F. 1988. Enzymes and aflatoxin biosynthesis. Microbiol. Rev. 53:274-95.

Ehrlich, K., 1987. Effect on aflatoxin production of competetion between wild-type and mutant strains of *Aspergillus parasiticus*. Mycopathologia 97:93-96.

Ellis, W.O., J.P. Smith, and B.K. Simpson. 1991. Aflatoxins in food: Occurence, biosynthesis, effects on organisms, detection, and methods of control. Crit. Rev. Food Sci. Nutr. 30(3):403-439.

Essigmann, J.M., R.G. Croy, R.A. Bennett, and G.N. Wogan. 1982. Metabolic activation of aflatoxin B_1: patterns of DNA adduct formation, removal, and excretion in relation to carcinogenesis. Drug. Metab. Rev. 13:581-602.

Feng, G.H., F.S. Chu, and T.J. Leonard. 1992. Molecular cloning of genes related to aflatoxin biosynthesis by

differential screening. Appl. Environ. Micro. 58(2):455-460.

Fincham, J.R. 1989. Transformation in fungi. Microbiol. Rev. 53:148-170.

Foreman, J.E. and W.G. Niehaus. 1985. Zn^{+2} induced cooperativity of mannitol-1-phosphate dehydrogenase from *Aspergillus parasiticus*. J. Biol. Chem. 260:10019-10022.

Freifelder, D. 1987. Molecular Biology, 2nd ed. Jones and Bartlett Publishers, Inc. Boston:510.

Fujimoto, Y., L.L. Hampton, L.D. Luo, P.J. Wirth, and S.S. Thorgeirsson. 1992. Low frequency of p53 gene mutation in tumors induced by aflatoxin B_1 in nonhuman primates. Canc. Res. 52:1044-1046.

Gibson, T.J., A.R. Coulson, J.E. Sulston, and P.R. Little. 1987. Lorist 2, a cosmid with transcriptional terminators insulating vector genes from interference by promoter within the insert: effect on DNA yeild and cloned insert frequency. Gene. 53:275-281.

Gurr, S.J., S.E. Unkles, and J.R. Kinghorn. 1987. The structure and organization of nuclear genes of filamentous fungi. In:Kinghorn JR (ed) Gene structure in eukaryotic microbes. IRL Press, Oxford, 95-138.

Hallam, S., F. Malpartida, and D. Hopwood. 1988. Nucleotide sequence, transcription and deduced function of a gene involved in polyketide antibiotic synthesis in *Streptomyces coelicolor*. Gene 74:305-320.

Hamasaki, T., Y. Hatsuda, N. Terashima, and M. Renbutsu. 1967. Studies on the metabolites of *Aspergillus versicolor*. Agr. Biol. Chem. 31(1):11-17.

Hayes, A.W. 1980. Mycotoxins: A review of biological effects and their role in human diseases. Clin. Toxicol. 17:45-63.

Heathcote, J.G. and J.R. Hibbert, *Aflatoxins: Chemical and biological aspects*, (New York: Elsevier Scientific, 1978), pg. 1-201.

Heilmann, J.J., H.J. Maegert, and H.G. Gassen. 1988. Identification and isolation of glucose dehydrogenase genes of *Bacillus megarterium* M1286 and their expression in *Escherichia coli*. Eur. J. Biochem. 174:485-490.

Hendricks, J.D., R.O. Sinnhuber, J.H. Wales, M.E. Stack, and D.P.H. Hsieh. 1980. Hepatocarcinogenicity of sterigmatocystin and versicolorin A to rainbow trout (*Salmo gairdneri*) embryos. JNCI 64(6):1503-1506.

Hopwood, D.A. and D.H. Sherman. 1990. Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. Ann. Rev. Gen. 24:37-66.

Horng, J.S., J.E. Linz, and J.J. Pestka. 1989. Cloning and characterization of the *trp*C gene from an aflatoxigenic strain of *Aspergillus parasiticus*. App. Environ. Micro. 55:2561-2568.

Horinouchi, S., H. Suzuki, and T. Beppu. 1986. Nucleotide sequence of *afs*B, a pleiotropic gene involved in secondary metabolism in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. J. Bact. 168(1):257-269.

Hsieh, D.P.H., C.C. Wan, and J.A. Billington. 1989. A versiconal hemiacetal acetate converting enzyme in aflatoxin biosynthesis. Mycopathologia 107:121-126.

Jelinek, C.F., A.E. Pohland, and G.E. Wood. 1989. Worldwide occurrence of mycotoxins in foods and feeds- an update. J. Assoc. Anal. Chem. 72:223-230.

Kamps, M.P., S.S. Taylor, and B.M. Sefton. 1984. Direct evidence that oncogenic tyrosine kinases and cyclic AMP-dependent protein kinase have homologous ATP-binding sites. Nature 310(16):589-591.

Keller, N., H.C. Dischinger, D. Bhatnagar, T.E. Cleveland, and A.H.J. Ullah. 1991. Purification of a second methyltransferase active in the aflatoxin biosynthetic pathway. FASEB J. A2461.

Kitada, M., M. Taneda, K. Ohta, K. Nagashima, K. Itahashi, and T. Kamataki. 1990. Metabolic activation of aflatoxin B_1 and 2-amino-3-methylimidazo[4,5,-f]-quinoline by human adult and fetal livers. Cancer Res. 50:2641-2645.

Klich, M.A. and J.I. Pitt. 1988. Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. Trans. Br. Mycol. Soc. 91(1):99-108.

Kurtzman, C.P., B.W. Horn, and C.W. Hesseltine. 1987. *Aspergillus nomius*, a new aflatoxin-producing species related to *Aspergillus flavus* and *A. tamarii*. Antonie Van Leeuwenhook 53:147-158.

Lee, L.S., J. W. Bennett, L.A. Goldblatt, and R.E. Lundin. 1970. Norsolorinic acid from a mutant strain of *Aspergillus parasiticus*. J. Am. Oil Chem. Soc. 48:93-94.

Lee, L.S., J.W. Bennett, A.F. Cucullu, and J.B. Stanley. 1975. Synthesis of versicolorin A by a mutant strain of *Aspergillus parasiticus* deficient in aflatoxin production. J. Agr. Food Chem. 23:1132-1134.

Lin, B.K. and J.A. Anderson. 1992. Purification and properties of versiconal cyclase from *Aspergillus parasiticus*. Arch. Biochm. Biophys. 293(1):67-70.

Lin, Y.C., J.C. Ayres, and P.E. Koehler. 1980. Influence of temperature cycling on the production of aflatoxins B1 and G1 by *Aspergillus parasiticus*. App. Environ. Micro. 40(2):333-336.

Lloyd, A.T. and P.M. Sharp. 1991. Codon usage in *Aspergillus nidulans*. Mol. Gen. Genet. 230:288-294.

Maniatis, T., E.F. Fritsch, and J.Sambrook (eds). 1982. Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Maplestone, R.A., M.J. Stone, and D.H. Williams. 1992. The evolutionary role of secondary metabolites - a review. Gene. 115:151-157.

McCormick, S.P., D. Bhatnagar, and L.S. Lee. 1987. Averufanin is an aflatoxin B_1 precursor between averantin and averufin in the biosynthetic pathway. Appl. Environ. Micro. 53(1):14-16.

Nesbitt, B.F., J. O'Kelly, K. Sargeant, and A. Sheridan. 1962. Toxic metabolites of *Aspergillus flavus*. Nature 195:1060-1062.

Niehaus, W.G. and L.J. Failla. 1984. Effect of zinc on versicolorin production by a mutant strain of *Aspergillus parasiticus*. Exp. Mycol. 8:80-84.

Niehaus, W.G. and W.P. Jiang. 1989. Nitrate induces enzymes of the mannitol cycle and suppresses versicolorin synthesis in *Aspergillus parasiticus*. Mycopath. 107:131-137.

Niehaus, W.G. 1989. Versicolorin synthesis by *Aspergillus parasiticus*:regulation by temperature and zinc. Exp. Mycol. 13:20-26.

Novak, L.A. and L.M. Kohn. 1990. Developmental proteins in *Aspergillus*. Exp. Mycol. 14:339-350.

Oakley, B. R., J. E. Rinehart, B. L. Mitchell, C. E. Oakley, C. Carmona, G. L. Gray, and G. S. May. 1987. Cloning, mapping and molecular analysis of the pyrG (orotidine-5'-phosphate decarboxylase) gene of Aspergillus nidulans. Gene. 61:385-399.

Papa, K.E. 1984. Genetics of *Aspergillus flavus*:linkage of aflatoxin mutants. Can. J. Microbiol. 30:68-73.

Park, D.L., L.S. Lee, R.L. Price, and A.E. Pohland. 1988. Review of the decontamination of aflatoxins by ammoniation: Current status and regulation. J. Assoc. Off. Anal. Chem.

71(4):685-703.

Parkin, D.M., E. Laara, and C.S. Muir. 1988. Estimates of the worldwide frequency of sixteen major cancers in 1980. Int. J. Cancer 41:184-197.

Payne, G.A., and C.P. Woloshuk. 1989. Transformation of *Aspergillus flavus* to study aflatoxin biosynthesis. Mycopathologia 107:139-144.

Payne, G.A. 1991. Report to the aflatoxin technical advisory group. 1991 Aflatoxin elimination workshop. Atlanta, Georgia.

Pestka, J.J. 1988. Enhanced surveillance of foodborne mycotoxins by immunochemical assay. J. Assoc. Off. Anal. Chem. 71(6):1075-1081.

Pestka, J.J. and G.S. Bondy. 1990. Alteration of immune function following dietary mycotoxin exposure. Can. J. Physiol. 68:1009-1016.

Pestka, J.J. and W.L. Casale. 1990. Naturally occuring fungal toxins. In J.O. Ariam and M.S. Simmons (ed). Food contamination from environmental sources. John Wiley & Sons.

Reddy, T.V., L. Viswanathan, and T.A. Venkitasubramanian. 1971. High aflatoxin production on a chemically defined medium. Appl. Micro. 22(3):393-396.

Reddy, T.V., L. Viswanathan, and T.A. Venkitasubramanian. 1979. Factors affecting aflatoxin production by *Aspergillus parasiticus* in a chemically defined medium. J. Gen. Micro. 114:409-413.

Reynolds, G.N. and J.J. Pestka. 1991. Enzyme-linked immunosorbent assay of versicolorin A and related aflatoxin biosynthetic precursors. J. Food Protect. 54(2):105-108.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. 74:5463-5467.

Schroeder, H. W., and W.H. Kelton. 1975. Production of sterigmatocystin by some species of the genus *Aspergillus* and its toxicity to chicken embryos. Appl. Micro. 30:589-591.

Sharma, A., A.G. Behere, S.R. Padwal-Desai, and G.B. Nadkarni. 1980. Influence of inoculum size of *Aspergillus parasiticus* spores on aflatoxin production. Appl. Environ. Micro. 40(6):989-993.

Sherman, D.H., F. Malpartida, M.J. Bibb, H.M. Kieser, M.J. Bibb, and D.A. Hopwood. 1989. Structure and deduced function

of the granaticin-producing polyketide synthase gene cluster of *Streptomyces violaceoruber* Tu22. EMBO J. 8(9):2717-2725.

Shimada, T. and F.P. Guengerich. 1989. Evidence for cytochrom P-450, the nifedipine oxidase, being the principle enzyme involved in the bioactivation of aflatoxins in the human liver. Proc. Natl. Acad. Sci. U.S.A. 86:462-465.

Singh, R., and D.P. Hsieh. 1976. Enzymatic conversion of sterigmatocystin into aflatoxin B_1 by cell-free extracts of *Aspergillus parasiticus*. Appl. Environ. Micro. 31(5):743-745.

Skory, C.D., J.S. Horng, J.J. Pestka, and J.E. Linz. 1990. Transformation of *Aspergillus parasiticus* with the homologous gene (*pyrG*) involved in pyrimidine biosynthesis. App. Environ. Micro. 56(11):3315-3320.

Skory, C.D., P.K. Chang, and J.E. Linz. 1991. Isolation of a gene involved in the aflatoxin biosynthetic pathway of *Aspergillus parasiticus*. ASM Annual Meeting. Dallas, Texas.

Skory, C.D., P.K. Chang, and J.E. Linz. 1992. Isolation of a gene from *Aspergillus parasiticus* associated with the conversion of versicolorin to sterigmatocystin in aflatoxin biosynthesis. Appl. Environ. Micro. In press.

Stark, A.A. 1980. Mutagenicity and carcinogenicity of mycotoxins: DNA binding as a possible mode of action. Ann. Rev. Microbiol. 34:235-62.

Stoloff, L. 1976. Occurrence of mycotoxins in food and feed. In Rodricks, J.V. (ed.) Mycotoxins and other fungal related food problems. American Chemical Society, Washington, DC, pp. 23-50.

Stoloff, L. 1989. Aflatoxin is not a probable human carcinogen: The published evidence is sufficient. Regulatory Tox. and Pharm. 10:272-283.

Timberlake, W.E. 1990. Molecular genetics of *Aspergillus* development. Annu. Rev. Genet. 24:5-36.

Townsend, C.A., K.A. Plavcan, K. Pal, and S.W. Brobst. 1988. Hydroxyversicolorone: isolation and characterization of a potential intermediate in aflatoxin biosythesis. J. Am. Chem. Soc. 53:2472-2477.

Tyagi, J.S. and T.A. Venkitasubramanian. 1981. The role of glycolosis in aflatoxin biosynthesis. Can. J. Micro. 27:1276-1282.

Vining, L.C. 1992. Secondary metabolism, inventive evolution and biochemical diversity - a review. Gene 115:135-140.

Wild, C.P., Y.Z. Jiang, S.J. Allen, L.A.M. Jansen, A.J. Hall, and R. Montesano. 1990. Aflatoxin-albumin adducts in human sera from different regions of the world. 11(12):2271-2274.

Wild, C.P., R. Montesano, J. Van Benthem, E. Scherer, and L. DenEngelse. 1990. Intercellular variation in levels of adducts of aflatoxin B_1 and G_1 in DNA from rat tissues: a quantitative immunocytochemical study. J. Cancer Res. Clin. Oncol. 116:134-140.

Woloshuk, C.P., E.R. Seip, G.A. Payne, and C.R. Adkins. 1989. Genetic transformation system for the aflatoxin-producing fungus Aspergillus flavus. Appl. Environ. Micro. 55(1):86-90.

Wong, J.J., R. Singh, and D.P. Hsieh. 1977. Mutagenicity of fungal metabolites related to aflatoxin biosynthesis. Mutat. Res. 44:447-450.

Yabe, K., Y. Ando, and T. Hamasaki. 1988. Biosynthetic relationship among aflatoxins B_1, B_2, G_1, and G_2. Appl. Environ. Microbiol. 54:2101-2106.

Yabe, K., Y. Ando, J. Hashimoto, and T. Hamasaki. 1989. Two distinct O-methyltransferases in aflatoxin biosynthesis. Appl. Environ. Micro. 55(9):2172-2177.

Yabe, K., Y. Nakamura, H. Nakajima, Y. Ando, and T. Hamasaki. 1991. Enzymatic conversion of norsolorinic acid to averufin in aflatoxin biosynthesis. Appl. Environ. Micro 57(5):1340-1345.

Yabe, K., Y. Ando, and T. Hamasaki. 1991. A metabolic grid among versiconal hemiacetal acetate, versiconol acetate, versiconol and versiconal during aflatoxin biosynthesis. J. Gen. Micro. 137:22469-2475.

Yabe, K., Y. Ando, and T. Hamasaki. 1991. Desaturase activity in the branching step between aflatoxins B_1 and G_1 and aflatoxins B_2 and G_2. Agric. Biol. Chem. 55(7):1907-1911.

Yao, R.C., and D.P. Hsieh. 1974. Step of dichlorvos inhibition in the pathway of aflatoxin biosynthesis. Appl. Micro. 28(1):52-57.

Yu, F.L., W. Bender, and I.H. Geronimo. 1990. Base and sequence specificities of aflatoxin B_1 binding to single and double-stranded DNAs. Carcinogenesis 11(3):475-478.

Zaika, L.L. and R.L. Buchanan. 1987. Review of compound affecting the biosynthesis or bioregulation of aflatoxins. J. Food Protect. 50(8):691-708.