THE FUNCTION, ACCUMULATION AND LOCALIZATION OF THE NOR-1 PROTEIN INVOLVED IN AFLATOXIN BIOSYNTHESIS; THE FUNCTION OF THE *FLUP* GENE ASSOCIATED WITH SPORULATION IN *ASPERGILLUS PARASITICUS*

BY

RENQING ZHOU

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition
and
Multidisciplinary Graduate Program in Environmental Toxicology

1997

ABSTRACT

THE FUNCTION, ACCUMULATION AND LOCALIZATION OF THE NOR-1 PROTEIN INVOLVED IN AFLATOXIN BIOSYNTHESIS; THE FUNCTION OF THE *FLUP* GENE ASSOCIATED WITH SPORULATION IN *ASPERGILLUS PARASITICUS*

BY

Renqing Zhou

Aflatoxin contamination can cause public health and economic problems in many areas of the world. A study of the mechanisms regulating aflatoxin biosynthesis and the biology of the aflatoxigenic fungus *Aspergillus parasiticus* may play a role in controlling aflatoxin contamination.

To study the function of the Nor-1 protein, which is directly involved in aflatoxin B_1 biosynthesis in *A. parasiticus*, a *nor*-1 cDNA was expressed in *Escherichia coli.* The resulting Nor-1c protein was evaluated in an enzyme activity assay.

To study the accumulation of the Nor-1 protein in *A. parasiticus*, polyclonal antibodies were raised against the Nor-1c protein and were used to monitor the accumulation of the Nor-1 protein. A *nor*-1/GUS reporter construct was also used to monitor the activity of the *nor*-1 promoter.

Cell fractionation/Western blot analysis and in situ immunolocalization experiments were used to study the localization of the Nor-1 protein. A *nor*-1/GUS reporter construct was used to localize the activity of the *nor*-1 promoter.

Gene disruption was used to study the function of the *flu*P gene which encodes a putative polyketide synthase in *A. parasiticus.*

The data presented in this dissertation suggest that: The Nor-1 protein is a ketoreductase which converts the aflatoxin B_1 pathway intermediate norsolorinic acid to averantin. The accumulation of the Nor-1 protein is regulated temporally in liquid media and temporally/spatially on solid media. The Nor-1 protein is mainly localized in the cytosol of the vegetative cell. The Nor-1 protein is localized in developing conidial head, conidial stalks, vegetative hyphal cells, and mature conidia in the order from the highest to the nondetectable level, respectively. The localization of the Nor-1 protein and the localization of *nor*-1 promoter activity follow a temporal and spatial pattern and are correlated to the process of sporulation in *A. parasiticus* grown on solid media. Disruption of *flu*P resulted in slow growth with cotton-like hyphae, no sporulation, and reduced aflatoxin accumulation suggesting that *flu*P is associated with the vegetative cell growth, sporulation, and in turn indirectly influences the accumulation of aflatoxin in *A. parasiticus.*

This work is dedicated to my aunt (Zhou Chunlan), my uncle (Ma Zhichun), my grand mother (Cong Guicang) and my sister (Ma Lijun) for their emotional and financial support during the most difficult time in my life; and to my daughter (Zhou Yingchiao) for her understanding and self sacrifice during my seven-year absence, working and studying in the United States.

ACKNOWLEDGMENTS

I would like to deeply acknowledge the academic and technical guidance and advice of Dr. John E. Linz throughout my graduate study at Michigan State University. It was his continuous encouragement and inspiration that helped me succeed in these studies.

I am very grateful to receive the advice and help from Dr. James J. Pestka, Dr. William G. Helferich, and Dr. L. Patrick Hart who have graciously served on my guidance committee.

Special thanks are also expressed to Matt Rarick for his assistance in sequence analysis, and last but not least to Dr. Tzong-Shoon Wu, Dr. Ludmila Rose, Dr. Ninee Mahanti and all of my colleagues in the Food Microbiology Laboratory, the Department of Food Science and Human Nutrition (Michigan State University) for their technical expertise.

TABLE OF CONTENTS

CHAPTER 5

APPENDICES

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Aflatoxins are extremely toxic mycotoxins produced by certain strains of *Aspergillus parasiticus*, *A. flavus*, and *A. nomius*. These ubiquitous fungi grow on a variety of food and feed crops and may cause serious aflatoxin contamination. Aflatoxin contamination poses a threat to public health and in turn causes economic problems in many areas of the world. Therefore, reducing or eliminating aflatoxin contamination is an urgent need.

My research is one part of a long term research project aimed at the elimination of preharvest contamination of crops by aflatoxin B1 (AFB1). The work is designed to provide an understanding of the mechanisms which regulate aflatoxin biosynthesis at the molecular level and an understanding of the general biology of the toxigenic fungi (e.g. regulatory mechanisms involved in hyphal growth, hyphal differentiation, and sporulation). This information may lead to the development of new fungicides, the improvement of agronomic practices, the development of resistant crops (by genetic engineering or classical breeding), and the development of biocontrol agents (such as nontoxigenic competitive fungi) for control of aflatoxin contamination.

In the currently accepted general pathway of aflatoxin biosynthesis (Figure 1-22) (Bhatnagar *et al.*, 1992; Dutton, 1988; Yabe *et al.*, 1993), the decaketide-derived norsolorinic acid (NA) is the earliest stable pathway intermediate isolated (Dutton, 1988). NA is converted to averufin (AVF) via one or more alternative pathways by converting NA to averantin (AVN) first or to averufanin (AVNN) first (Figure 2-1) (Bennett and Christensen, 1983; Button, 1988; McCormick *et al.*, 1987; Yabe *et al.*, 1991b). Disruption of the *nor*-1 gene resulted in NA accumulation and a significant reduction of aflatoxin in an aflatoxigenic strain of *A. parasiticus* (Trail *et al.*, 1994). Therefore the *nor*-1 gene may function in one of these alternative pathways. Analysis of the predicted amino acid sequence of *nor*-1 provided evidence that *nor*-1 encodes a ketoreductase (Trail *et al.*, 1994). Comparison of the chemical structure of NA and AVN (Figure 2-1) demonstrated that the 5'-keto group of NA is converted to the 5'-hydroxyl group of AVN suggesting that this reaction is catalyzed by a ketoreductase. The accumulation of *nor*-1 transcript is subjected to temporal regulation in liquid culture (Skory *et al.*, 1993; Trail *et al.*, 1994). Aflatoxins have been found to be concentrated in spores and sclerotia (Wicklow and Cole, 1982; Cotty, 1988). These data suggest that the accumulation of the Nor-1 protein is also subjected to temporal regulation in liquid culture and the localization of the Nor-1 protein is correlated to the process of sporulation.

Based on the background data, three hypotheses related to the Nor-1 protein are proposed. (1) The Nor-1 protein encoded by *nor*-1 is a ketoreductase which converts NA to AVN in the AFB_1 pathway. (2) The accumulation of the Nor-1 protein is subjected to temporal regulation in liquid media and to temporal and spatial regulation on solid media. (3) The Nor-1 protein resides either in the cytosol or in organelles. The localization of the Nor-1 protein is correlated to the process of sporulation in *A. parasiticus* grown on solid media.

Previous data showed that a 680-bp genomic DNA fragment from *A. parasiticus* was able to hybridize to a cDNA fragment of a polyketide synthase (PKS) gene which encodes the β-ketoacyl synthase functional domain of the 6-methylsalicylic acid synthase (MSAS) complex in

Penicillium patulum (Beck *et al.*, 1990). As expected, the deduced amino acid sequence from the 680-bp DNA fragment was similar (72% identity) to that of the β-ketoacyl synthase functional domain of MSAS. A search of the EMBL and GenBank data base libraries showed that the 680-bp DNA fragment is also very similar to a variety of other PKS genes (35.3% to 70.8% identity in 216 amino acid overlap compared with 6 PKS genes). Because aflatoxins are derived from a polyketide pathway (Kurtzman *et al.*, 1987), the producing fungi are predicted to have a polyketide synthase (PKS) related to aflatoxin biosynthesis.

It was initially hypothesized that *fluP* could be involved in aflatoxin biosynthesis because there had been no published report on identifying patulin and/or its precursor 6-methylsalicylic acid produced by *A. parasiticus*.

Polykides are secondary metabolites and are often involved in regulation of development or differentiation (Summers *et al.*, 1995; Revill *et al.*, 1995; Keller and Adams, 1995). Therefore an alternative hypothesis was that this PKS-like gene (*flu*P) containing the 680-bp DNA fragment might be related to some kind of secondary metabolism (such as pigment synthesis, hyphal growth and/or sporulation).

In order to test these hypotheses, four goals were proposed: (1) to determine the enzymatic function of the Nor-1 protein; (2) to determine the accumulation pattern of the Nor-1 protein; (3) to localize the accumulation of the Nor-1 protein; and (4) to test the function of the *flu*P gene.

Four experiments were designed to fulfill the four goals and in turn to address the four hypotheses. (1) The Nor-1c protein was produced in a host bacterium transformed with a *nor-1* cDNA expression vector. The Nor-1c protein was used to test the enzymatic function of the native Nor-1 protein in the AFB1 pathway. (2) Polyclonal antibodies (PAb) were raised against the Nor-1c protein and used to monitor the accumulation of the native Nor-1 protein in *A. parasiticus* grown in liquid media and on solid media. A *nor*-1/GUS reporter construct was used to monitor the activity of the *nor*-1 promoter (which could indirectly indicate the accumulation of the Nor-1 protein during aflatoxin biosynthesis). (3) Cell fractionation plus Western blot analysis and in situ immunolocalization were used to localize the Nor-1 protein at the cellular level and the hyphal level. A *nor*-1/GUS reporter construct was used to localize the activity of the *nor*-1 promoter (which could indirectly indicate the localization of the Nor-1 protein during aflatoxin biosynthesis) at the hyphal level and at the colony level. (4) Southern blot and Northern blot analyses were used to clone the *flu*P gene. Gene disruption was used to analyze the function of the *fluP* gene.

CHAPTER 1

LITERATURE REVIEW

My dissertation focuses attention on two genes. The first is the *nor*-1 gene which is involved in aflatoxin B_1 (AFB_1) biosynthesis. The second is a suspected polyketide synthase (PKS) gene, the *flu*P gene, which is associated with growth and sporulation and indirectly influences AFB_1 biosynthesis in the filamentous fungus *Aspergillus parasiticus*. AFB_1 is just one of the numerous mycotoxins produced by *Aspergillus*. The first part of this review is a general introduction to mycotoxin biosynthesis in *Aspergillus*, which includes a brief discussion of secondary metabolism and its possible functions and several biosynthetic pathways for representative mycotoxins produced by species of *Aspergillus*. This dissertation is a part of a long term research project designed to provide an understanding of the regulation of aflatoxin biosynthesis which may lead to the development of biocontrol agents for control of aflatoxin contamination. The second part of this review includes a discussion of aflatoxin toxicity and biosynthesis, and biocontrol of aflatoxin contamination.

PART 1. MYCOTOXIN BIOSYNTHESIS IN *ASPERGILLUS*

SECONDARY METABOLISM

Mycotoxins are a large and highly varied group of natural products referred to as secondary metabolites. Chemical compounds are synthesized and degraded by means of a series of chemical reactions mediated by enzymes in the living organism. These processes are known as metabolism which is comprised of catabolism (degradation) and anabolism (synthesis). All organisms possess very similar primary metabolic pathways by which they synthesize and degrade certain biochemicals such as sugars, amino acids, common fatty acids and nucleotides to generate energy and to build the polymers derived from them (polysaccharides, proteins, lipids, RNA, and DNA). Compounds derived from primary metabolic pathways are essential for the survival and well-being of the organism and are called primary metabolites. Most organisms also utilize other metabolic pathways to produce compounds which appear to have no apparent utility. These compounds are called secondary metabolites. The pathways of synthesis and degradation of secondary metabolites constitute secondary metabolism. These pathways are perhaps only activated during particular stages of growth and development or during periods of stress caused by factors such as nutritional limitation or microbial attack. Most secondary metabolites are produced only by a specific species or a small number of closely related species. For example, aflatoxins have been found to be produced only by certain strains of three *Aspergillus* species.

Primary metabolites have the same or similar structures such as the 20 common amino acids, RNA and DNA, while secondary metabolites have a wide diversity of structures. Their unusual chemical structures include β-lactam rings, cyclic peptides containing 'unnatural' and non-protein amino acids, unusual sugars and nucleosides, unsaturated bonds of polyacetylenes and polyenes and large macrolide rings. Examples of some of the well known secondary metabolites with unusual structures include ephedrine (for respiratory ailments), ricinine (a purgative), and salicin (aspirin is a synthetic analogue) which are medicines; strychnine, coniline, and rotenone which are poisons; morphine (from opium), tetrahydrocanabinol (from marijuana), and cocaine which are narcotics and hallucinogens; caffeine which is a stimulant; geraniol (from rose oil), linalol (from lavender), cinamaldehyde (from cinamon), eugenol (from cloves) and diallyl disuphide (from garlic) which are perfumes and spices.

The organisms which can produce secondary metabolites are usually considered to be limited to bacteria, fungi, and plants. But some scientists believe that all organisms including humans can produce secondary metabolites. For example, defensins, which are endogenous antibiotic peptides produced by human leukocytes may be considered as secondary metabolites (Ganz *et al.*, 1985).

There is no unanimous definition of secondary metabolism. However, one proposed definition may provide some hints about the nature of secondary metabolism. Davies (1992) proposed that primary metabolism enables an organism to produce the next generation. Secondary metabolism is responsible for interactions between the organism and its environment and is concerned with what is going on outside the producing organism, rather than events going on inside.

FUNCTIONS OF SECONDARY METABOLITES

The role of secondary metabolites for the producing organisms is still an area of vigorous debate, but there is a growing consensus that secondary metabolism benefits the organism and this benefit usually reflects the nature of the metabolites themselves (Davies, 1992). Davies has proposed ten potential biological functions of secondary metabolites: (1) competitive weapons against other organisms including bacteria, fungi, plants, amoebae, and insects; (2) metal-transporting agents; (3) plant-microbe symbiosis; (4) nematode-microbe symbiosis; (5) insect-microbe symbiosis; (6) sexual hormones (pheromones); (7) differentiation effectors between and within cells; (8) excretion of unwanted products; (9) products of 'selfish' DNA; (10) reserve pool of new biochemical pathways.

BIOSYNTHESIS OF MYCOTOXINS BY *ASPERGILLUS*

Fungal secondary metabolites are relatively small molecules characterized not only by their structural diversity (Turner and Aldridge, 1983), but also by their diversity of biological activity which includes antibiotic activity, phytotoxicity, animal toxicity and a very large array of physiological and pharmacological activities in a mammalian system.

The toxic secondary metabolites produced by fungi are called mycotoxins. The diversity of mycotoxins produced by *Aspergillus* was reviewed by Moss (1977, 1994). The precursors of mycotoxins produced by *Aspergillus* species are certain amino acids, acetyl CoA, and isopentenyl pyrophosphate. The polymers of the three basic building units are polypeptides (from amino acids), polyketides (from acetyl CoA), and isoprenoids (from isopentenyl pyrophosphate). Acetyl CoA is also the precursors of some amino acids and isopentenyl pyrophosphate. Not only can the mycotoxins from *Aspergillus* be derived from the three kinds of precursors respectively, but also from all the possible combinations of the three kinds of precursors. The broad range of mycotoxins produced by *Aspergillus* are contained in seven groups: (1) isoprenoids; (2) amino acid-isoprenoids; (3) polyketides; (4) amino acid-polyketides; (5) polypeptides; (6) isoprenoid-polyketides; (7) amino acid-isoprenoid-polyketides.

Some of the well known mycotoxins produced by *Aspergillus* are listed in Figure 1-1 along with their likely producers (may not be the major producers). In addition to the mycotoxins listed in Figure 1-1, many other *Aspergillus* mycotoxins (each with one producer) have been identified such as sulochrin *(A. terreus)*, dicoumarol (*A. fumigatus*), aflatrem (*A. flavus*), fumitremorgin (*A. fumigatus*), emodin (*A. alutaceus*), kojic acid (*A. alutaceus*), neoaspergillic acid (*A. alutaceus*), penicillic acid (*A. alutaceus*), secalonic acid A (*A. alutaceus*), gandidulin (*A. candidus*), terphenyllin (*A. candidus*), xanthoascin (*A. candidus*), ascladiol (*A. clavatus*), clavatol (*A. clavatus*), kotanine (*A. clavatus*), aflavinin (*A. flavus*), aspergillic acids (*A. flavus*), paspalinin (*A. flavus*), fumigaclavines (*A. fumigatus*), fumigatin (*A. fumigatus*), fumitoxins (*A. fumigatus*), spinulosin (*A. fumigatus*), verruculogen (*A. fumigatus*), naphthoquinones (*A. oryzae*), nigragillin (*A. niger*), aspergillomarasmin (*A. oryzae*), 3-nitropropionic acid (*A. oryzae*), oryzacidin (*A. oryzae*), nidulatoxin (*A. sydowii*), griseofulvin (*A. sydowii*), terrein (*A. terreus*), terreic acid (*A. terreus*), terretonin (*A. terreus*), terredionol (*A. terreus*), austamid (*A.ustus*), wentilacton (*A. wentii*), and physicon *(A. wentii)* (Frisvad, 1988).

With some exceptions such as 3-nitropropionic acid from *A. oryzae*, the biosynthetic pathways of most mycotoxins (as for many other secondary metabolites) in *Aspergillus*

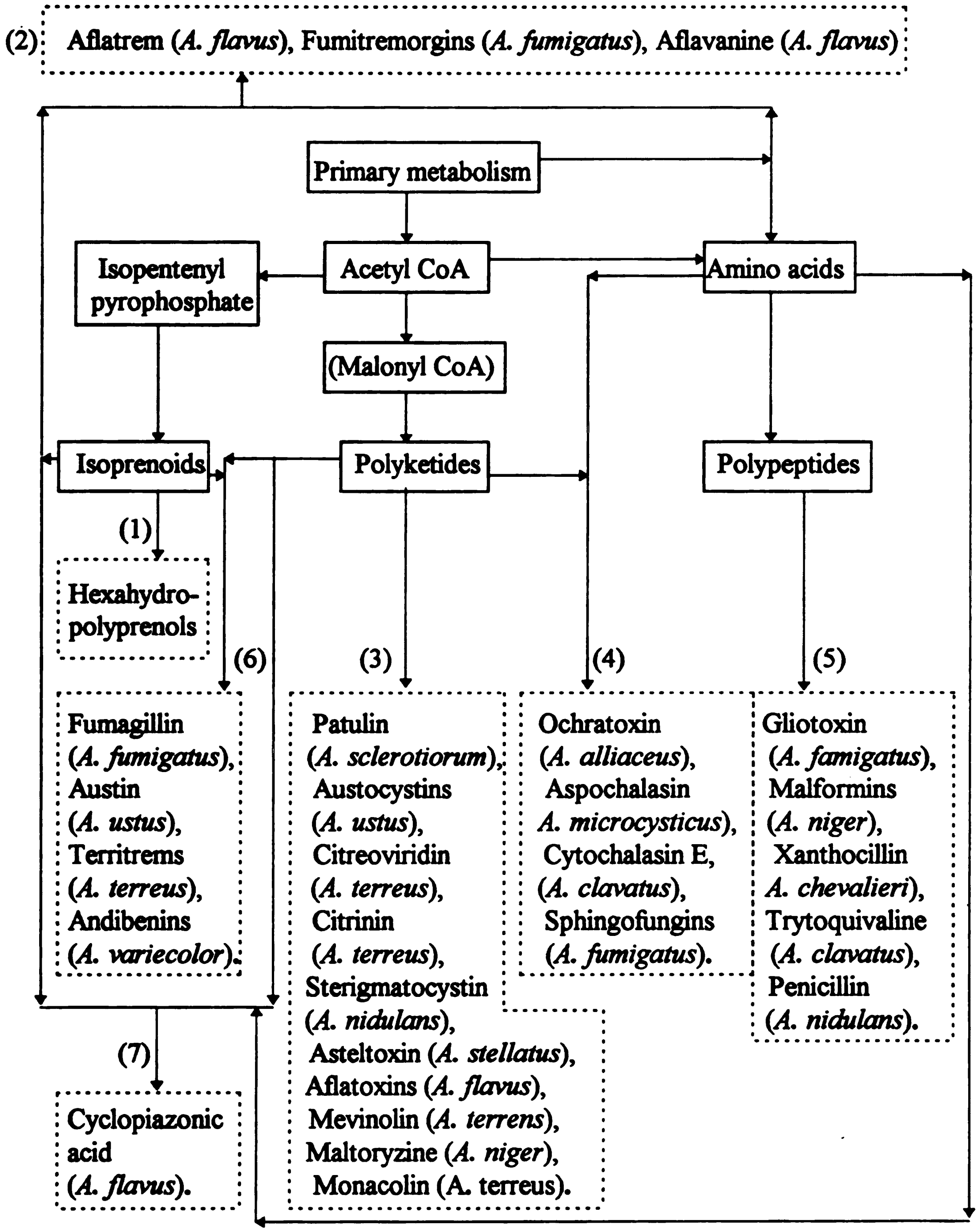


Figure 1-1. Representatives of mycotoxins produced by species of *Aspergillus*.

involve two distinct phases. The first phase is the polymerization phase during which the precursor molecules are linked together by enzyme complexes such as polyketide synthases. The second phase is the modification phase during which the products of the polymerization phase are modified by enzymes which carry out hydroxylations, oxidative cleavages and rearrangements leading to the remarkable diversity of mycotoxins produced by *Aspergillus*.

Although hexahydropolyprenol and penicillin may not be considered as mycotoxins, their biosynthetic pathways have been well studied (Manitto, 1981; Baldwin and Abraham, 1988), and these two secondary metabolites are typically used as examples to discuss isoprenoid mycotoxin biosynthesis and polypeptide mycotoxin biosynthesis. Biosynthetic pathways of each of the seven groups of *Aspergillus* mycotoxins are described with one or more examples. The best studied biosynthetic pathway is that utilized for AFB_1 biosynthesis which is discussed separately.

Biosynthesis Of Isoprenoid Mycotoxins

Secondary metabolites containing a carbon backbone comprised of five-carbon isoprene [$CH_2C(CH_3)CHCH_2$), 2-methylbutadiene] units are called terpenes. If the carbon backbone of a terpene is altered by addition or loss of carbon atoms, or by other modifications, such a modified compound is called an isoprenoid.

Isopentenyl pyrophosphate. All isoprenoids originate from isopentenyl pyrophosphate which is formed from acetyl CoA through an important intermediate mevalonic acid (Figure 1-2).

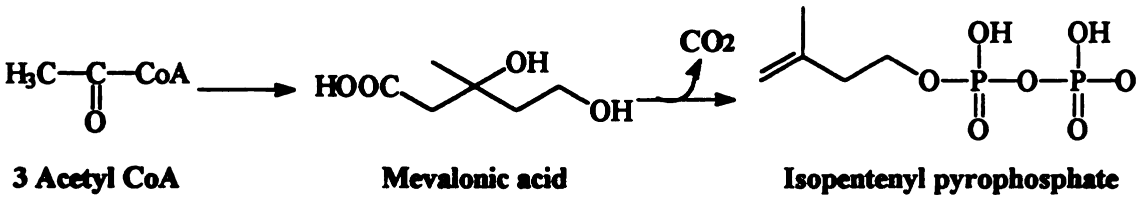


Figure 1-2. Biosynthesis of isopentenyl pyrophosphate from acetyl CoA.

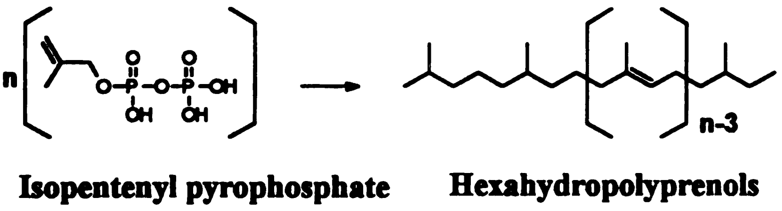


Figure 1-3. Biosynthesis of hexahydropolyprenols in *Aspergillus fumigatus*.

Hexahydropolyprenols. A large group of isoprenoids are the polyisoprenes which are made up of more than 10 isoprene units, bound head to tail. Polyisoprenes which exist in the alcohol form are called polyprenols. Among the polyprenols, hexahydropolyprenols were isolated from *Aspergillus fumigatus* and their synthetic pathway (Figure 1-3) was described by Manitto (1981).

Biosynthesis Of Amino Acid-Isoprenoid Mycotoxins

Most isoprenoids produced by *Aspergillus* species are not mycotoxins, but many amino acid-isoprenoids are mycotoxins. A large group of tremorgenic mycotoxins, such as aflatrem and fumitremorgin, are isoprenoids with an indole nucleus provided by tryptophan.

Aflatrem. Aflatrem was the first mycotoxin with tremorgenic activity isolated from the mycelium and the sclerotium of a strain of *A. flavus* by Wilson and Wilson (1964) and its structure was then elucidated by Gallagher and Wilson (1978). Later, other aflatrem-related tremorgenic compounds from *Aspergillus* were isolated, such as paspalinine (Gallagher *et al.*, 1980), aflavarin, β-aflatrem (TePaske *et al.*, 1992), sulpinine, secopenitrem B, and aflatrem B (Laakso *et al.*, 1992). Indeed, paspalinine, which may be a precursor of aflatrem, was isolated from an aflatrem-producing isolate of *A. flavus* along with the very unusual indole-isopentenyl pyrophosphate metabolites aflavinine and dihydroxyaflavinine (Cole *et al.*, 1981). The biosynthetic pathway of aflatrem in *A. flavus* was proposed by Steyn and Vleggaar (1985). Aflatrem is formed from tryptophan,

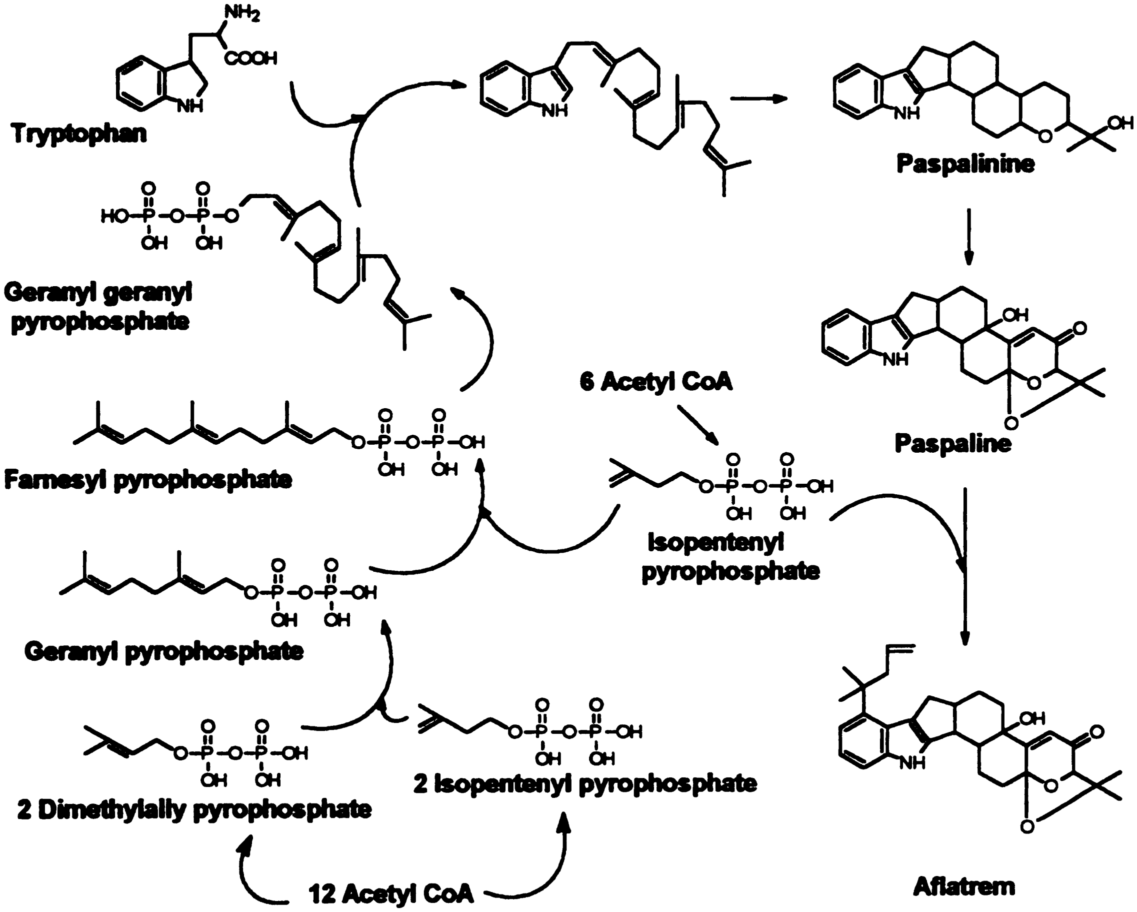


Figure. 1-4. Biosynthesis of aflatrem in *Aspergillus flavus.*

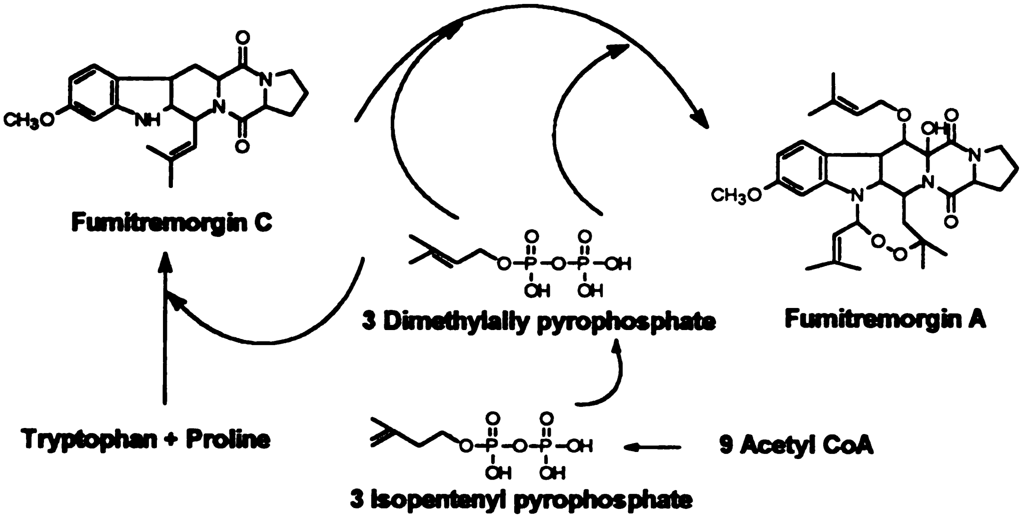


Figure 1-5. Biosynthesis of fumitremorgin A in *Aspergillus fumigatus.*

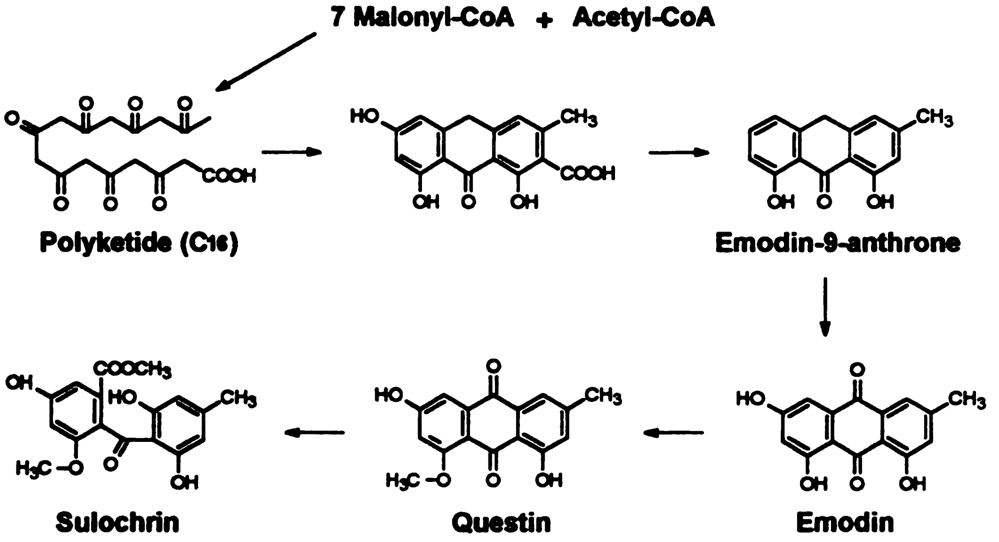


Figure 1-6. Biosynthesis of sulochrin in *Aspergillus terreus.*

geranylgeranyl pyrophosphate, and isopentenyl pyrophosphate with the loss of a methyl group during the formation of the polycyclic structure (Figure 1-4).

Fumitremorgin. During a survey of toxigenic food-borne fungi in Japan, Yamazaki *et al.* (1971) found metabolites from certain strains of *Aspergillus fumigatus* which caused vigorous tremor and convulsions in experimental animals. Two indole-containing metabolites, designated fumitremorgin A and B, were isolated from extracts of this fungus. These tremorgenic metabolites are a family of compounds derived from diketopiperazine. Based on feeding experiments in *A. fumigatus* with [^{14}C]-labeled tryptophan, proline and mevalonic acid, Yamazaki *et al.* (1980) proposed that fumitremorgin C is synthesized from two amino acids, tryptophan and proline, to which a C_5 unit from dimethylally pyrophosphate is added. Fumitremorgin A is synthesized by adding two more C_5 units from dimethylallyl pyrophosphate (Figure 1-5). Their discovery and structural characterization have been concisely reviewed by Yamazaki (1980). A derivative of fumitremorgin C was also found in *A. fumigatus* (Abraham and Arfmann, 1990).

Biosynthesis Of Polyketide Mycotoxins

Polyketides may represent the largest group of secondary metabolites (Weiss and Edward, 1980). These structurally diverse compounds typically contain a carbon backbone with oxygen atoms at alternate positions. The name 'polyketide' was coined more than 100 years ago by Collie and Myers (1893). They imagined that poly-β-keto compounds could be produced by treatment of polyacetyl compounds with weak alkali. Biochemical support for this idea was provided by Birch and Donovan (1953). According to the isotopic labeling pattern of several fungal metabolites, they proposed that polyketides must be formed from acetic and malonic acids by a process similar to the biosynthesis of fatty acids. Polyketide chain growth, however, is different from fatty acid biosynthesis, because it lacks the faithful removal of each β-keto group by a process necessary in fatty acid biosynthesis. This hypothesis to explain the mechanism of polyketide synthesis was further supported by the application of the isotopic labeling method, and later by the development of sophisticated nuclear magnetic resonance spectroscopic techniques (Simpson, 1987; Vederas, 1987).

Knowledge of the enzymology of the polyketide synthase (PKS) enzymes was obtained very slowly prior to 1985 because of the difficulty encountered in enzyme assay and purification. Only three PKS's were purified by 1985: 6-methylsalicylic acid synthase (6-MSAS) from the fungus *Penicillium patulum* (Dimroth *et al.*, 1970), naringenin chalcone synthase (NCS) from the parsley plant *Petroselinum hortense* (Kreuzaler *et al.*, 1979), and resveratrol (stilbene) synthase from the peanut plant *Arachis hypohaea* (Schoppner and Kindl, 1984). Studies on these three enzymes established the basic characteristics of PKS, even though they displayed distinctly different properties. The synthase of 6-MSA is a large (800 kDa), tetrameric multifunctional protein to which all the substrates are covalently attached, whereas NCS is a homodimer composed of 42 kDa subunits that acts on the CoA esters of the substrates and lacks a functional equivalent to an acyl carrier protein (ACP). From 1986 until late 1996, several other PKS's or their components have been identified and/or purified. The multienzyme of the erythromycin-producing polyketide synthase, DEBS1, DEBS2 and DEBS3 (6-deoxyerythronolide B synthase) were identified and purified from *Saccharopolyspora erythraea* (Caffrey *et al.*, 1992). A proposed component of the dimeric polyketide synthase (actinorhodin acyl carrier protein-dependent malonyltransferase) for the antibiotic actinorhodin was purified from *Streptomyces coelicolor* A3(2) (Revill *et al.*, 1995). With advanced molecular biology techniques, other PKS's or their components were expressed from cDNAs, and then purified. Acyl carrier proteins (ACPs) of the polyketide synthases for the aromatic antibiotics actinorhodin, granaticin, frenolicin and oxytetracycline were expressed in and purified from *Escherichia coli* (Crosby *et al.*, 1995).

The biosyntheses of sulchrin, patulin, citrinin, sterigmatocystin, sterigmatin, and autocystin in some species of *Aspergillus* have been well studied and are taken as examples to discuss the charateristics of polyketide biosynthesis.

Sulochrin. Sulochrin is a derivative of a group of mycotoxins called ergochromes which were first isolated from ergot, a name for the sclerotium of *Claviceps purpurea*. Ergochrome BB was isolated from *Aspergillus ochraceus*, and ergochrome AC and ergochrome BC from *Aspergillus aculeatus*. All of the known ergochromes are dimers of the monoxanthones which are synthesized from polyketide precursors. The structure and the possible biosynthetic pathway of sulochrin (Figure 1-6) were described by Franck (1980).

Patulin. Patulin and penicillic acid are synthesized primarily by *Penicillium* and *Aspergillus* species. The biosyntheses of these two secondary metabolites are very similar. Only the biosynthesis of patulin is discussed in this section. Patulin was first discovered by Birkinshaw *et al.* (1943) and its chemical structure was identified by Woodward and Singh

(1949). The toxicity of patulin has led to an increased awareness of its occurrence in fresh apple juice which originates from brown rot of apples contaminated with *Penicillium expansum* Link. The production of patulin by *Aspergillus clavatus* growing on spent malted barley was implicated in the poisoning of cattle when this material was used as a feed additive. Birch *et al.* (1955) demonstrated the biosynthetic pathway of 6-methylsalicylic acid (6-MSA) in experiments with [1-^{14}C]-labeled acetate fed to *Penicillium patulum*. 6-MSA is converted to patulin by a complex sequence of oxidation, cleavage and rearrangements which form the second stage in the biosynthesis of patulin. Zamir (1980) confirmed that 6-MSA is converted to gentisaldehyde, then phyllostine which is the substrate for the oxidative cleavage reaction and the rearrangement which results in the formation of patulin. The major steps are outlined in Figure 1-7. The biosynthesis of patulin is a clear example of the two stages in the production of a secondary metabolite. The polymerization of three molecules of malonyl CoA with a molecule of acetyl CoA, during which a reduction and dehydration occur, leads to a tetraketide derivative which readily cyclizes to form 6-methylsalicylic acid (6-MSA). Although a relatively small molecule, 6-MSA has a special place in the history of our understanding of polyketide biosynthesis.

Citrinin. Citrinin, first isolated from *Penicillium citrinum* by Hetherington and Raistrick (1931), is produced by numerous *Penicillium* and *Aspergillus* species (Rodig *et al.*, 1966). Citrinin exhibited marked antibiotic activity *in vitro*, but its nephrotoxigenic properties prevented therapeutic application (Krogh *et al.*, 1973). The biosynthesis of citrinin was determined initially by Birch *et al.* (1958). Addition of sodium [1-^{14}C]-labeled

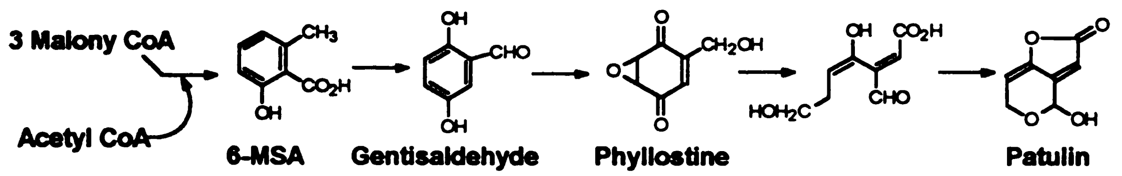


Figure 1-7. Biosynthesis of patulin in *Penicillium patulum.*

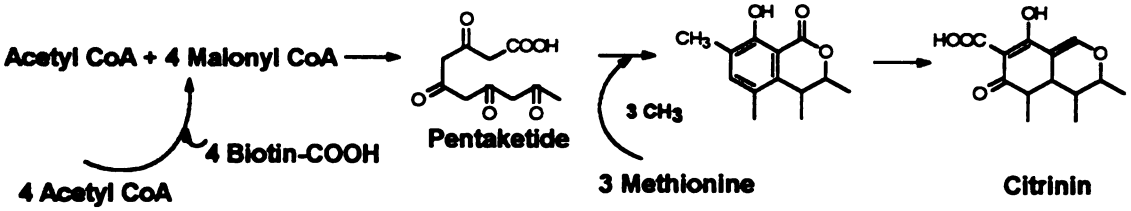


Figure 1-8. Biosynthesis of citrinin in *Aspergillus candidus.*

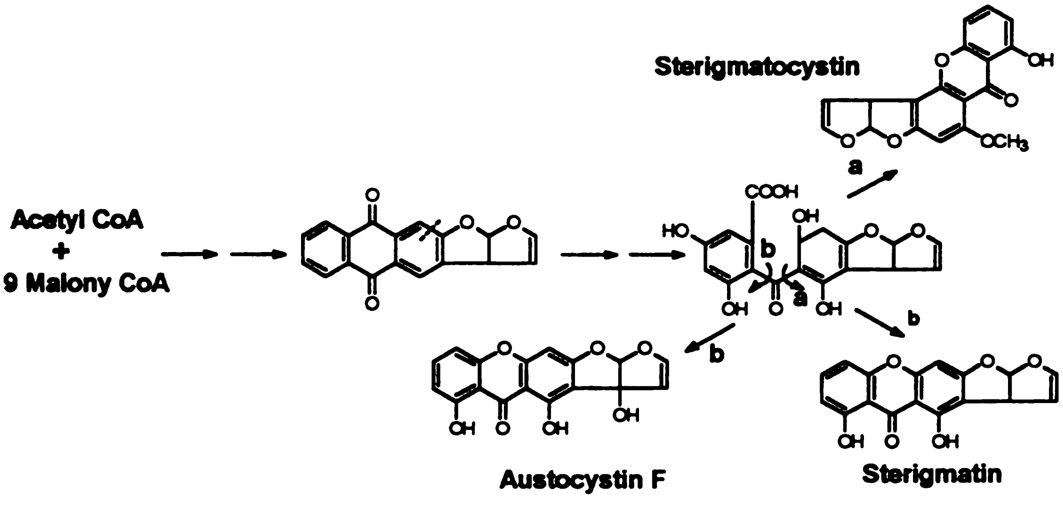


Figure 1-9. Biosynthesis of sterigmatocystin, sterigmatin, and austocystin in *Aspergillus versicolor.*

acetate to cultures of *Aspergillus candidus* gave [^{14}C]-citrinin. Gererally, during the building of a polyketide chain, a number of additional groups can be added, especially to the reactive mythylene groups, providing another source of diversity in structure. In the biosynthesis of citrinin, three additional C_1 groups are added to the pentaketide chain from the biochemically active one carbon pool involving methionine (Figure 1-8). The common occurrence of C-methyl groups in polyketide-derived metabolites produced by fungi is often provided by malonyl CoA. These additional C_1 groups are frequently further oxidized. Thus, one of the three additional branched carbons in citrinin is oxidized to a carboxylic acid group.

Sterigmatocystin, sterigmatin, and autocystin. Sterigmatocystin (Figure 1-9), a xanthone which contains an angularly fused bisdihydrofuran system, was originally isolated from the mycelium of *Aspergillus versicolor* Tiraboschi and proved to be hepatocarcinogenic in rats (Hatsuda and Kuyama, 1954). Sterigmatocystin is an intermediate in the biosynthetic pathway leading to the aflatoxins. Sterigmatocystin is synthesized from a decaketide, which is probably derived from hexanoyl CoA with the subsequent addition of seven more malonyl CoA units (Townsend *et al.*, 1984). With the development of an understanding of the molecular biology of aflatoxin biosynthesis, several genes and enzymes involved in sterigmatosystin synthesis have been identified (Skory *et al.*, 1992; Keller *et al.*, 1994; Yu and Leonard, 1995; Keller *et al.*, 1995).

Sterigmatin (Figure 1-9), a metabolite isolated from *A. versicolor*, was the first metabolite found with a linearly fused xanthone and bisdihydrofuran moiety (Hamasaki *et al.*, 1973). Steyn and Vleggaar (1975) obtained other linear xanthones from corn cultures of *A. ustus.* The linear xanthones, called austocystins, frequently contain a chlorine atom, or an isopentyl side chain. They are essentially substituted derivatives of sterigmatin produced (along with sterigmatocystin) by *Aspergillus versicolor.* They are believed to share the early pathway of aflatoxin biosynthesis (Hamasaki *et al.*, 1973). A key intermediate in this pathway is the postulated benzophyenone carboxylic acid (BCA) (Figure 1-9). Rotation about bond (**a**) would lead to the sterigmatocystins, whereas no rotation or rotation about bond (**b**) would lead to sterigmatin and the austocystins.

Biosynthesis Of Amino Acid-Polyketide Mycotoxins

This group of mycotoxins is synthesized first by forming a polyketide backbone to which amino acids are added. Ochratoxin and cytochalasin E are two examples of amino acid-polyketide mycotoxins produced by *Aspergillus.*

Ochratoxin. Ochratoxin is a nephrotoxic metabolite of some species of *Aspergillus.* Ochratoxin A is also a weak antibiotic (Heller *et al.*, 1975). The discovery of toxigenic strains of *Aspergillus ochraceus* (Scott, 1965) led to the isolation and structural elucidation of ochratoxin A and B. The biosynthesis of ochratoxin A in *Aspergillus ochraceus* was studied in feeding experiments by Seary *et al.* (1969) with [^{14}C]-labeled phenylalanine, by Steyn *et al.* (1970) with [^{14}C]-labeled acetate and methionine, by Wei *et al.* (1971) with [^{36}Cl]-labeled sodium chloride, and by Maebayashi *et al.* (1972) with [^{13}C]-labeled formate. These studies made it clear that the biosynthesis of ochratoxin A (Figure 1-10) consists of the generation of a pentaketide which is modified by the addition

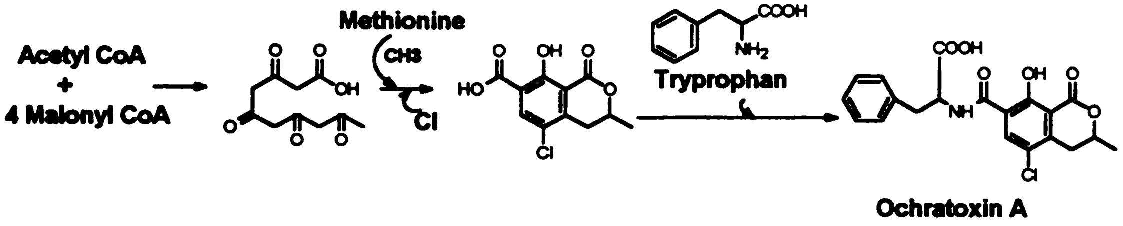


Figure 1-10. Biosynthesis of ochratoxin in *Aspergillus ochraceus*.

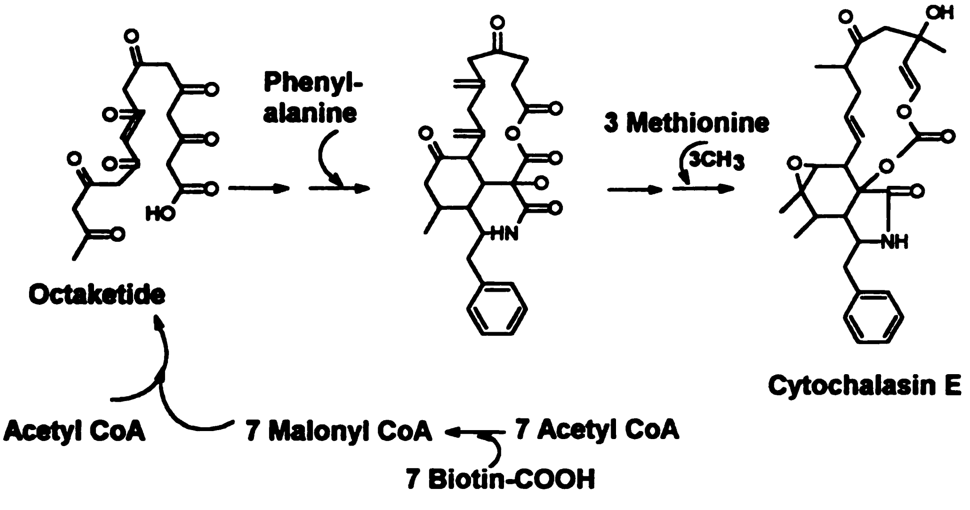


Figure 1-11. Biosynthesis of cytochalasin E in *Aspergillus clavatus*.

of a single methyl group from the C_1 pool. This additional methyl group is oxidized to a carboxyl group through which L-phenylalanine is linked by an amide bond.

Cytochalasin E. The discovery of cytochalasans was the result of the observation that the culture filtrate of certain microorganisms produced morphological changes in hyphal cells of test fungi and unusual effects in mammalian cells in tissue culture (Rothweiler and Tamm, 1966). Cytochalasin E, one of more than 20 cytochalasans, was found in certain species of *Asperillus* (Buchi *et al.*, 1973). Cytochalasans are characterized by a highly substituted perhydroisoindolone group, to which is fused a macrocyclic ring (which is either a carbocyclic, a lactone, or a cyclic carbonate). The structure of cytochalasin E was determined by Buchi *et al.* (1973). A detailed account of the biosynthetic pathway leading to cytochalasans was provided by Tamm (1980). Here, cytochalasin E produced by *A. clavatus* (Buchi *et al.*, 1973) is utilized as an example to describe a possible biosynthetic pathway for cytochalasans. The proposed pathway of cytochalasin E synthesis starts with the formation of an octaketide which is produced from one molecule of acetyl CoA and 7 molecules of malonyl CoA, followed by addition of a phenylalanine to form an amino acid-polyketide complex to which three C_1 group from methionine are added. The synthesis is terminated by formation of an epoxide (Figure 1-11).

Biosynthesis Of Polypeptide Mycotoxins

Amino acids are the precursors of several distinct families of secondary metabolites including polypeptides, cyclic polypeptides, amino acid-polyketides, and amino acid-isoprenoids. The last two groups have already been described. Some mycotoxins produced by *Aspergillus* are synthesized using one or two amino acids as building blocks. Coumarin and related compounds are the derivatives of one moleclue of L-phenylalanine or one molecule of L-tyrosine in several species of *Aspergillus*. Aspergillic acid is synthesized from one molecule of leucine and one molecule of isoleucine in *Aspergillus flavus*. Many amino acid-derived mycotoxins or antibiotics are synthesized from more than two amino acids such as dicoumarol, gliotoxin, tryptoquivaline M, and penicillin.

Dicoumarol. During fermentation of hay, *Aspergillus fumigatus* transforms coumarin into dicoumarol which is a powerful blood anticoagulant and can cause fatal hemorrhage in cattle eating the spoiled hay. The immediate precursor of dicoumarol is 4-hydroxycoumarin which is derived from either L-phenylalanine or L-tyrosine. The methylene group which connects the two 4-hydroxycoumarins is derived from formaldehyde which is also produced during fermentation. The possible biosynthetic pathway of dicoumarol in *Aspergillus fumigatus* (Luckner, 1990) is shown in Figure 1-12.

Gliotoxin. Gliotoxin was originally described as a metabolite with antifungal activity. The producing organism was subsequently identified as *Gliocladium fimbriatum* Gilman & Abbott (Webster and Lomas, 1964). Gliotoxin is also produced by several species of *Penicillium* and *Aspergillus* such as *A. chevalieri* (Wilkinson and Spilsbury, 1965). Gliotoxin belongs to a large group of toxins called epipolythiodioxopeperazines with phenylalanine, tryptophan, or tyrosine and sometimes alanine as their precursors, and with methionine or cysteine as their sulfur suppliers. Gliotoxin causes genomic DNA degradation preferentially in certain blood cell types including T lymphocytes and

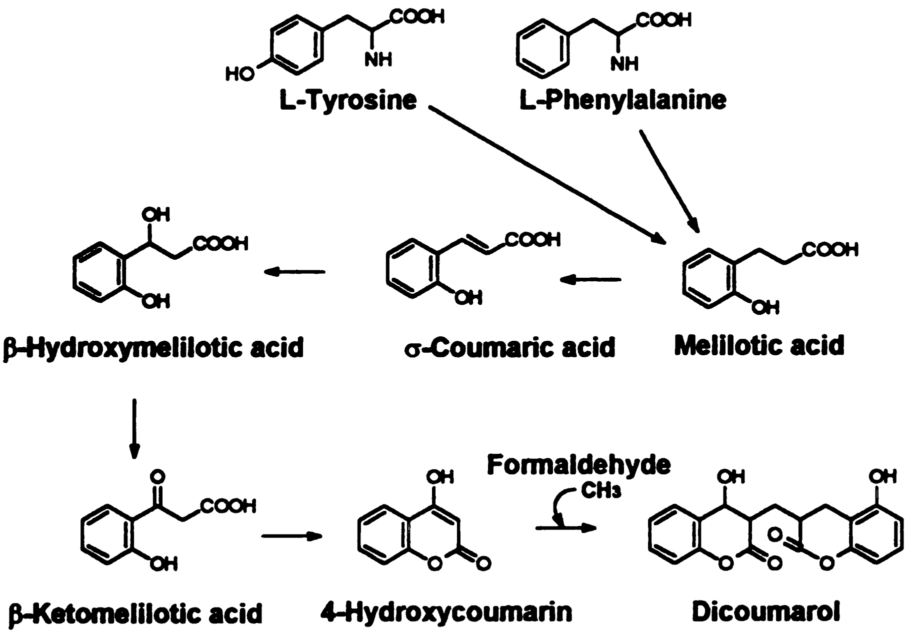


Figure 1-12. Biosynthesis of dicoumarol in *Aspergillus fumigatus.*

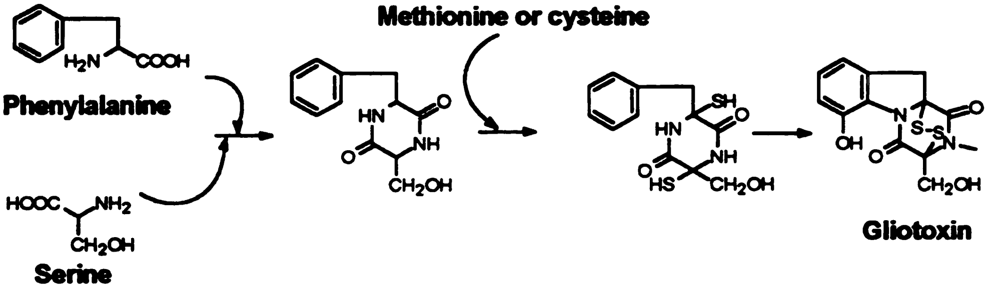


Figure 1-13. Biosynthesis of gliotoxin in *Trichoderma viridi.*

macrophages. Gliotoxin has previously been used to treat murine allogeneic bone marrow prior to transplantation into irradiated recipients, and in this situation the drug prevents development of graft-versus-host disease. This group of toxins includes hyalodendrin, aranotin, epicorazine, sporidesmin, chaetocin, verticillin, chetomin, sirodesmin, and a large number of their derivatives.

An understanding of the gliotoxin biosynthetic pathway has been partially achieved (Figure 1-13). Suhadolnik and Chenoweth (1958) were the first to demonstrate that phenylalanine provides the reduced indole nucleus of gliotoxin in *Trichoderma viridi*. Almost 20 years later, Kirby *et al.* (1978) confirmed that cyclo-(L-phenylalanyl-L-seryl) is an intermediate in gliotoxin biosynthesis in *Trichoderma viridi*. For the biosynthetic mechanism for introduction of sulfur into gliotoxin, no firm conclusions have been reached. Cysteine (possibly in combination with pyridoxal) is suggested for the sulfur donor, and a dehydrodioxopiperazine is suggested for the sulfur acceptor. Consecutive introduction of two thiol groups would be sufficient for oxidative cyclization to an epidisulfide compound (Kirby and Robins, 1980). The biosynthesis of gliotoxin is dealt with in some detail by Kirby and Robins (1980). Gliotoxin was the first mold metabolite to be recognized as belonging to the diketopiperazines with polysulfur bridges.

Tryptoquivaline M. Two tremor-causing metabolites, tryptoquivaline and tryptoquivalone, were isolated from a sample of rice implicated in the death of a child in Thailand, and then demonstrated to be produced in a toxigenic strain of *Aspergillus clavatus* by Clardy *et al.* (1975). Later other tryptoquivaline-related metabolites were isolated from *Aspergillus fumigatus* (Buchi *et al.*, 1977; Yamazaki *et al.*, 1979).

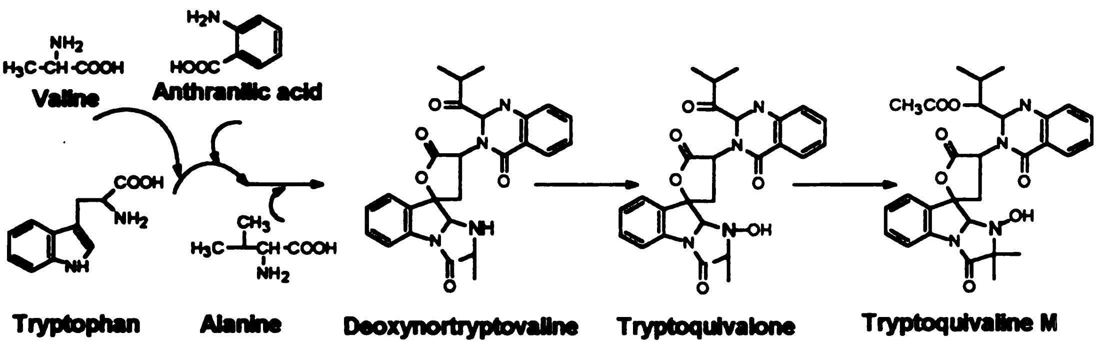


Figure 1-14. Biosynthesis of tryptoquivaline M in *Aspergillus fumigatus.*

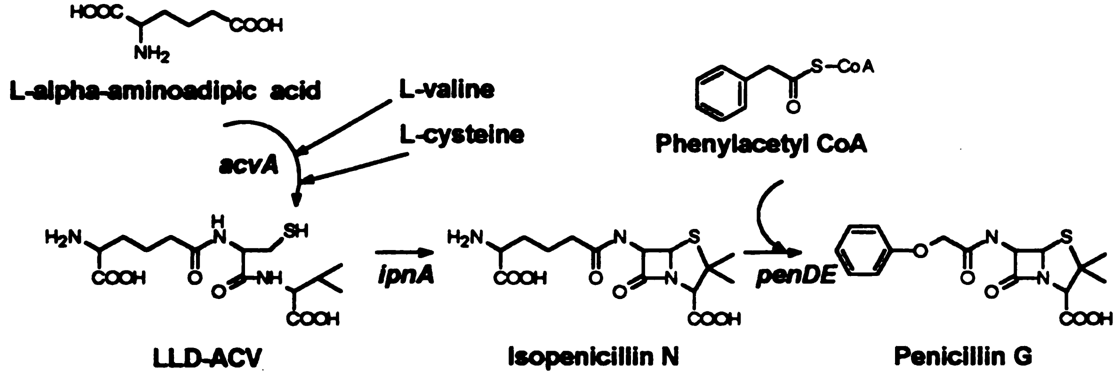


Figure 1-15. Biosynthesis of penicillin in *Aspergillus nidulans.*

The basic precursors involved in biosynthesis of these complex molecules are probably the amino acids tryptophan, valine, alanine and anthranilic acid, the latter being also an intermediate in the shikimate pathway to tryptophan. In this section tryptoquivaline M is utilized as an example to describe the possible pathway of tryptoquivaline biosynthesis in *Aspergillus fumigatus* (Yamazaki *et al.*, 1979). Synthesis appears to be initiated to form a tripeptide composed of anthranilic acid, tryptophan, and valine. Deoxynortryptoquivalone may be the first metabolite formed in the pathway of tryptoquivaline by addition of another amino acid alanine. The oxidation of dexoynortryptoquivalone results in the formation of tryptoquivalone which is converted to nortryptoquivaline. Nortryptoquivaline is then converted to tryptoquivaline M by simple epimerization (Figure 1-14).

Penicillin. The discovery of penicillin has a special place in the history of human medicine. In September 1928, Alexander Fleming, a Scottish bacteriologist working in Almroth Wright's Inoculation Department at St. Mary's Hospital, London, returned from holiday to his laboratory and found a contamination of mold (*Penicillium notatum*) in a plate culture of staphylococci. The growth of the bacterial colony was inhibited around the mold. The inhibition was later shown to be caused by the production of a substance by the mold to which Fleming gave the name "penicillin". His discovery of penicillin was published in 1929. Penicillin belongs to a group of antibiotics with a common β-lactam ring. Today, 69 years later, the understanding of penicillin (and other β-lactam compounds) biosynthesis has been reached not only at the chemical and biochemical level, but also at the genetic and molecular levels. The main interest is with *Penicillium chrysogenum* and *Acremonium chrysogenum* which have been the commercial sources of penicillin and other β-lactams. But in the late 1940s, screening of a wide range of fungi for new antibiotics led to the detection of penicillin produced by *Aspergillus nidulans* (Dulaney, 1947). Although this has never been of commercial significance, *A. nidulans* has been utilized as a model organism for genetic and molecular studies of penicillin biosynthesis.

The biosynthetic pathway for penicillin G in *A. nidulans* from amino acid precursors is shown in Figure 1-15. The primary metabolites which are the starting point for penicillin biosynthesis are the three amino acids L-α-aminoadipic acid, L-cysteine, and L-valine. The first step is the formation of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) catalyzed by the single large enzyme ACV synthetase (ACVS). The tripeptide is cyclized by isopenicillin N synthetase (IPNS), also called ACV cyclase, in an oxidative reaction in which four hydrogen atoms are removed from ACV, and one molecule of oxygen is consumed (Baldwin and Abraham, 1988). Isopenicillin N is modified by the enzyme acyltransferase (acyl CoA:6-amino-penicillanic acid acyltransferase, ACT). This enzyme removes the aminoadipyl side chain, and replaces it by phenoxyacetyl to form penicillin G.

Streptomyces clabvuligerus contains isopenicillin epimerase which converts isopenicillin to penicillin N. Certain fungi and many bacterial species further modify the lactam ring by the sequential action of expandase and hydroxylase activities to form decacetylcephalosporin C. This is converted into either cephalosporins or cephamycins, depending on the produing organism.

Delta-L-(α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) was first isolated from *Aspergillus nidulans* and studied by Van Liempt *et al.* (1989), and further analyzed by MacCabe *et al.* (1991). Isopenicillin synthetase (IPNS) was first isolated and studied by Baldwin and Abraham (1988) and was further studied by Roach *et al.* (1995) and Blackburn *et al.* (1995). Acyl CoA:6-amino-penicillanic acid acyltransferase (ACT) was purified from *A. nidulans* (Whiteman *et al.*, 1990; Montenegro *et al.*, 1990).

The first β-lactam biosynthetic gene to be cloned was the isopenicillin synthetase (IPNS) gene of *Aspergillus chrysogenum*. This gene was identified using oligonucleotides based on a partial sequence of the purified enzyme (Samson *et al.*, 1985). Availability of this gene quickly led to the isolation of the IPNS gene, *ipn*A, of *A. nidulans* (Ramon *et al.*, 1987) by heterologous hybridization. The discovery that a cephamycin biosynthetic gene cluster from the Gram-negative bacterium, *Flavobacterium* spp. SC1 and 154, hybridized to the IPNS gene of *P. chrysogenum*, enabled the identification of the region containing the *acvA* gene encoding the δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) in *A. nidulans* (Burnham *et al.*, 1989). Later the *acv*A gene was positively identified in *A. nidulans* by MacCabe *et al.* (1991). The isolation of the *pen*DE gene encoding acyl CoA:6-amino-penicillanic acid acyltransferase (ACT) was initially achieved in *P. chrysogenum* (Barredo *et al.*, 1989), using oligonucleotides designed from the N-terminal amino acid sequence of the purified enzyme. The equivalent gene, *acy*A, of *A. nidulans* was isolated using the *P. chrysogenum penDE* gene as a probe (Montenegro *et al.*, 1990). The *Aspergillus nidulans npe*A locus was identified by MacCabe *et al.* (1990). The locus consists of three contiguous genes in the order of *acv*A-*ipn*A-acyA and is required for penicillin biosynthesis. These three genes, which are clustered in chromosome VI (3.0 Mb), were cloned from *A. nidulans*. Eeach gene is expressed as a single transcript from a separate promoter (Martin and Gutierrez, 1995).

Biosynthesis Of Isoprenoid-Polyketide Mycotoxins

Some mycotoxins isolated from *Aspergillus* contain both isoprenoid and polyketide moieties. Turner and Aldridge (1983) listed a number of isoprenoid-polyketide mycotoxins produced by *Aspergillus*. Austin, fumagillin, and territrem B belong to this group of mycotoxins.

Austin. Austin, a polyketide-isoprenoid mycotoxin, was first isolated from *Aspergillus ustus* by Chexal *et al.* (1976). Austin was later found in *A. terreus* by Springer *et al.* (1979). The biosynthetic pathway of austin in *Aspergillus ustus* was initially studied by Wicnienski (1979) and proposed by Simpson *et al.* (1982). The major step in the biosynthesis is the preliminary coupling of the sesquiterpene precursor farnesyl pyrophosphate and the methylated polyketide. The three methyl groups come from methionine. Rearrangement of this hypothetical intermediate leads to the formation of austin (Figure 1-16).

Fumagillin. Fumagillin, first isolated from *Aspergillus fumigatus*, was recognized as an antibiotic agent (Hoza, 1966). At high doses fumagillin was lethal and caused toxic alteration in the liver, the kidney (Lauren *et al.*, 1989), and the respiratory epithelium (Amitani *et al.*, 1995). Fumagillin is a potent anti-angiogenic compound (Horsman *et al.*, 1995; Nishimura *et al.*, 1996). Fumagillin is another example of a secondary metabolite

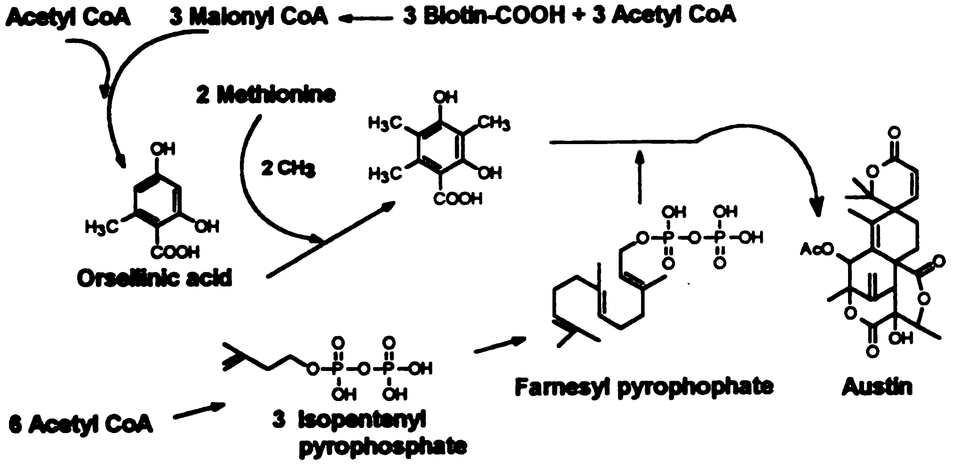


Figure 1-16. Biosynthesis of austin in *Aspergillus ustus.*

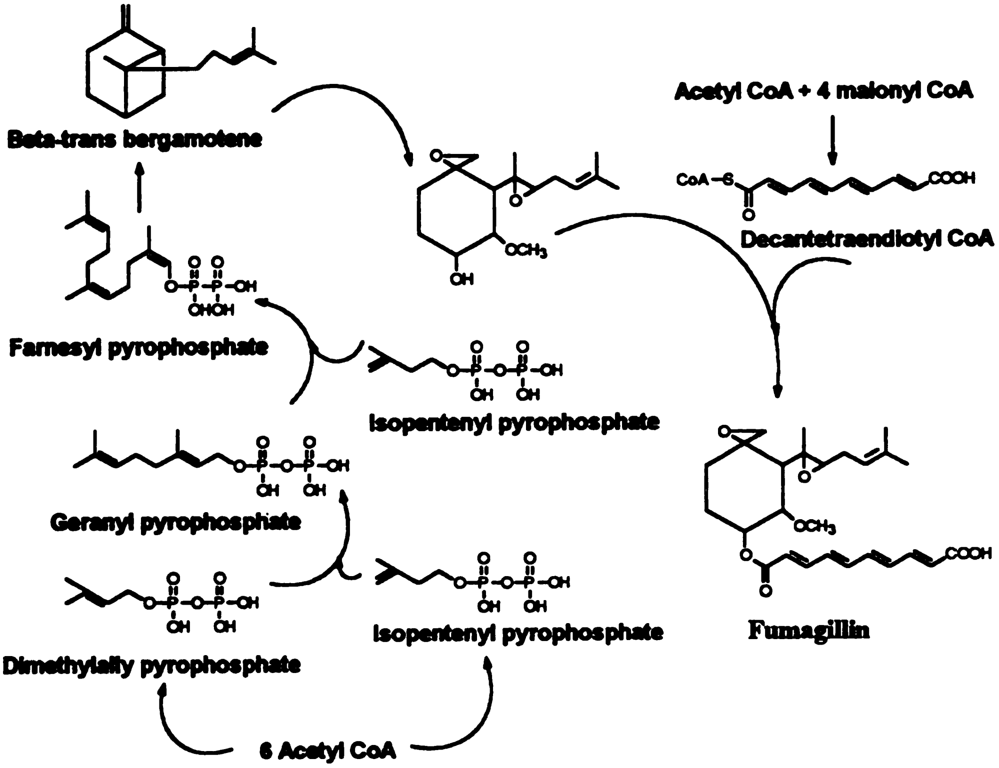


Figure 1-17. Biosynthesis of fumagillin in *Aspergillus fumigatus.*

assembled from two distinct pools of precursor intermediates, one is polyketide and the other is isoprenoid. Birch and Hussain (1969) showed that in *A. fumigatus* the decantetraendioic acid moiety, which is one of the two precursors of fumagillin, has a polyketide origin and that the terminal carboxyl group represents the original carboxyl end of the polyketide. They proposed an hypothesis for the origin of the unusual nucleus of fumagillin from farnesyl pyrophosphate via a bergamotene intermediate. This hypothesis was later supported by the detailed studies of Cane and Levin (1976) on the biosynthesis of ovalicin of *Pseudeurotium ovalis* Stolk. Fumagillin is an intermediate in the ovalicin biosynthetic pathway. The proposed biosynthetic pathway of fumagillin is shown in Figure 1-17.

Territrem B. Territrem A and territrem B were first identified in stored unhulled rice contaminated with *Aspergillus terreus* in Taiwan by Chung *et al.* (1971). Ling (1976) demonstrated that both toxins induce tremor and convulsions in mice. Territrems do not contain nitrogen. This is the most distinctive feature of territrems which sets them apart from the known N-containing tremorgenic mycotoxins. Other members of this family of compounds have been identified and characterized by Ling *et al.* (1984), Peng *et al.* (1985), and Ling (1994). For example, territrem C was isolated from the chloroform extract of rice cultures of *Aspergillus terreus* 23-1, which also produces territrems A and B. Ling *et al.* (1984) also studied acute toxicity and some physicochemical properties of territrems. Territrems contain isoprenoid and polyketide moieties. The biosynthetic pathways of both territrem A and territrem B have been studied in *Aspergillus terreus*

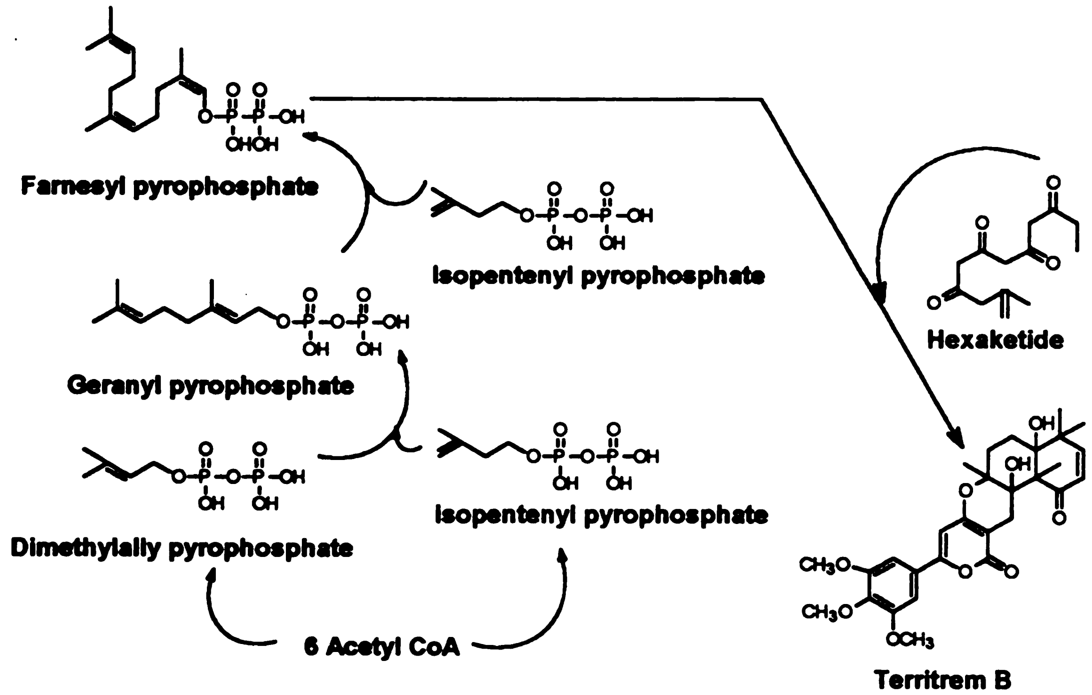


Figure 1-18. Biosynthesis of territrem B in *Aspergillus terreus*.

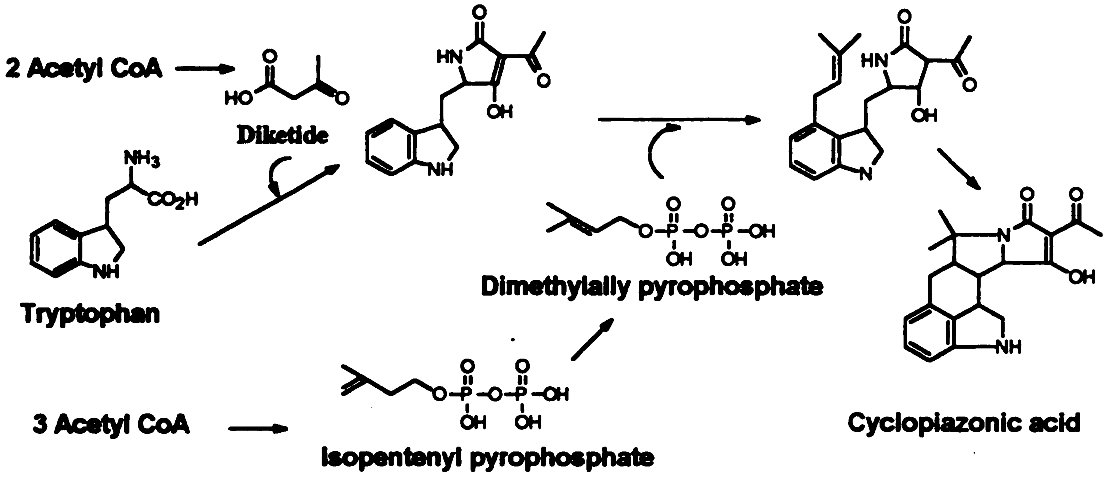


Figure 1-19. Biosynthesis of cyclopiazonic acid in some species of *Aspergillus*.

(Kuo and Yun, 1988; Lee *et al.*, 1992). The major step in the biosynthesis of territrem B is the combination of one molecule of hexaketide and one molecule of farnesyl pyrophosphate. Farnesyl pyrophosphate is formed from one molecule of isopentenyl pyrophosphate and one molecule of dimethylally pyrophosphate (Figure 1-18).

Biosynthesis Of Amino Acid-Isoprenoid-Polyketide Mycotoxins

This group of mycotoxins contains all the three basic precursors: amino acid, polyketide, and isoprenoid components. Cyclopiazonic acid belongs to this group of mycotoxins.

Cyclopiazonic acid. Cyclopiazonic acid is produced by strains of *Aspergillus flavus*, *A. oryzae* and *A. versicolor*, as well as several species of *Penicillium* (Luk *et al.*, 1977; Gallagher *et al.*, 1978; Richard and Gallagher, 1979; Dorner, 1983; Dorner *et al.*, 1984; Trucksess *et al.*, 1987; Lee and Hagler, 1991). The biosynthesis of cyclopiazonic acid was reviewed by Holzapfel (1980). The molecule is synthesized in some species of *Aspergillus* (Figure 1-19) possibly from tryptophan and an acetyl CoA-derived diketide to yield α-acetyl γ-(β-indolyl) methyltetramine acid to which is added a C_5 dimethylallyl group with subsequent cyclization to yield cyclopiazonic acid. Because the C_5 dimethyallyl group is derived from isopentenyl pyrophosphate, cyclopiazonic acid is composed of the three basic precursors: tryptophan an amino acid, a deketide, and an isopentenyl pyrophosphate. Two of the enzymes involved in the later steps of this pathway were isolated and partially characterized (Steenkamp *et al.*, 1973) and the stereochemistry of the cyclization was thoroughly studied (Steyn *et al.*, 1975a).

Epilog

The biosynthesis of mycotoxins in *Aspergillus* introduced in this review represents a small piece of the whole picture of the enormous chemical diversity and the complex biosyntheses of mycotoxins of this genus. In addition to the challenges still offered by the mycotoxins of *Aspergillus* to chemists and biochemists, their potential biological activity including mammalian toxicity also challenge the medical and toxicological fields. One important challenge now is to understand the molecular biology of the biosyntheses of this group of mycotoxins.

PART 2. AFLATOXINS

TOXICITY OF AFLATOXINS

Aflatoxins are secondary metabolites synthesized by certain strains of *Aspergillus flavus, A. parasiticus,* and *A. nomius* (Gorman *et al.*, 1992; Kurtzman *et al.*, 1987). These fungi are ubiquitous and frequently grow on a variety of food and feed crops and cause aflatoxin contaminaton in many areas in the world (Blount, 1961; Jelinek *et al.*, 1989; Ellis *et al.*, 1991). Peanuts, cotton seed, corn and treenuts are the major crops affected in the USA.

Aflatoxins are basically classified into the B or G series based on their color of fluorescence under long wave (360 nm) UV light (B for blue, and G for green). Aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2) are the four major aflatoxins produced by fungi in nature.

It is likely that aflatoxicosis existed for many years prior to the epizootic outbreak in Britain in 1960 known as Turkey X disease (Blount, 1961) in which more than 100,000 turkeys were destroyed with acute hepatotoxicity caused by aflatoxin-contaminated feed. However, that dramatic outbreak demonstrated the seriousness of aflatoxin contamination, and led to the recognition that aflatoxin contamination is both an economic and a public health problem in many areas of the world.

As observed in field outbreaks, acute structural and functional damage to the liver (such as fatty infiltration, necrosis, and extensive bile duct proliferation), the major target organ for aflatoxins, have been reported in most laboratory animals and in several domestic animal species (Busby and Wogan, 1984). Human exposure to aflatoxin occurred directly through ingestion of contaminated crops and resulted in fatal aflatoxicosis in India (Krishnamachari *et al.*, 1975) and in West Africa (Ngindu *et al.*, 1982).

In addition to acute hepatotoxicity, aflatoxins are strong mutagens and carcinogens. Aflatoxins have been demonstrated to be potent mutagens in a variety of test systems causing different mutations. For example, aflatoxins have been demonstrated to cause mutations in: bacteriophage (Goze *et al.*, 1975) and plasmids (Wood *et al.*, 1987; Courtemanche and Anderson, 1994); bacteria (Raina *et al.*, 1980; Lowery *et al.*, 1983; Refolo *et al.*, 1987; Foster *et al.*, 1988); *Drosophila melanogaster* (Lamb and Lilly, 1971; Graf *et al.*, 1984); cotton leaf-worm (Abdou *et al.*, 1984); the chick embryo (Bloom, 1984); monkeys (Adgigitov *et al.*, 1984); mice (Umeda *et al.*, 1977; Macgregor, 1979); and human cells (Kaden *et al.*, 1987). The types of mutations linked to aflatoxin exposure include substitutions, frameshifts, sister chromatid exchanges, chromosome aberrations, DNA single-strand breaks, micronucleus formation, and lethal mutations.

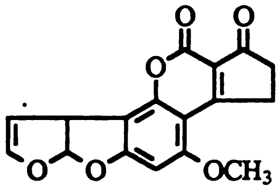
The experimental data, which have been obtained from different animals ingesting AFB_1 such as rat, rainbow trout, duck, rhesus monkey and tree shrew, demonstrate that AFB_1 is the most potent naturally occurring carcinogen (Adamson *et al.*, 1973; Carnaghan, 1965; Gopalan *et al.*, 1972; Reddy *et al.*, 1976; Sinnhuber *et al.*, 1968; Tilak, 1975) in these experimental systems. For example, early experiments with laboratory rats demonstrated that AFB_1 is a renal carcinogen (Epstein *et al.*, 1969). A small incidence of colon carcinomas resulted when AFB_1 was administrated to Fischer rats either orally or via the diet (Wogan and Newberne, 1967). Administration of AFB_1 elicited tumors in the central and peripheral nervous systems of pregnant rats (Goerttler *et al.*, 1980). Sieber *et al.* (1979) found that long term exposure of AFB_1 to monkeys induced hepatocelluar carcinomas, osteogenic sarcomas, bladder papillary carcinoma, gall bladder adeno-carcinomas, and pancreatic carcinomas.

Experimental evidence has linked AFB_1 to human lung cancer (Hayes *et al.*, 1984; Mace *et al.*, 1994). Epidemiologically, AFB_1 also has been suggested as a synergistic factor along with hepatitis B virus (HBV) in the generation of human primary liver cancer (PLC), and human hepatocellular carcinoma (HCC) (Groopman *et al.*, 1988). Very recently it was demonstrated that HBV and AFB_1 played a synergistic role in the development of hepatocellular carcinoma in tree shrews (Yan *et al.*, 1996).

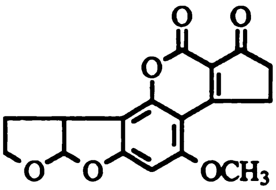
Aflatoxins can cause a variety of other toxic effects. AFB_1 experimentally has been shown to be a potent teratogen in rat, hamster, and chick embryo (Butler *et al.*, 1966; Elis and DiPaolo, 1967; Verrett *et al.*, 1973). Aflatoxicosis altered the reproductive efficiency of both male and female domestic animals, particularly poultry (Ottinger and Doerr, 1980). Aflatoxins have potential toxic effects on the immune system in animals resulting in susceptibility to diseases caused by bacteria, fungi, and viruses (Edds *et al.*, 1973; Richard *et al.*, 1973; Giambrone *et al.*, 1985). Herrold (1969) reported that AFB_1 caused megalocytosis in the proximal tubules in the kidney. Hemorrhagic kidney syndrome that commonly occurs in chickens in Africa has been linked to AFB_1 (Dafalla *et al.*, 1987). In cultured kidney cell lines, AFB_1 induced mitochondrial degeneration and loss of microvilli (Yoneyama *et al.*, 1987). The possible effects of AFB_1 on nervous system function were proposed by finding this toxin in tissue samples from patients with Reye's syndrome, a pediatric disease characterized by cerebral edema and neuronal degeneration (Chaves-Carballo *et al.*, 1976). In rats, AFB_1 caused a decrease of serotonin and its metabolite in brain, and also inhibited brainstem tryptophan metabolism (Coulombe *et al.*, 1985).

The generally accepted biochemical mechanism of AFB_1 toxicity is its biotransformation to the AFB_1-8,9 epoxide by cytochrome P_{450}-dependent epoxidation of the terminal furan ring double bond (Essigmann *et al.*, 1982; Stark, 1980; Soman and Wogan, 1993). AFB_1-8,9 epoxide is a potent electrophilic molecule which can form adducts with protein and DNA. The protein adducts may result in abnormal function and cause acute toxicity, and DNA adducts may cause mutagenicity and carcinogenicity. In addition, formation of free radicals from AFB_1 suggests a possible role of free radical metabolites in the carcinogenicity (Kodama *et al.*, 1990).

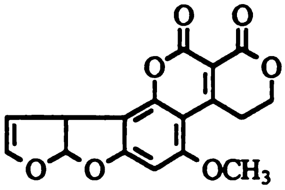
As early as the late 1970s, Autrup *et al.* (1979) reported that colon tissues from rat and human were capable of activating AFB_1 to form DNA adducts. Later it was demonstrated that AFB_1-8,9 epoxide combines mainly to the N-7 of guanine to form DNA adducts (Essigmann,



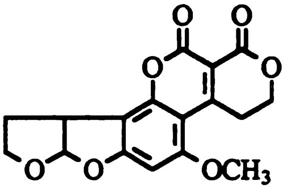
Aflatoxin B_1



Aflatoxin B_2



Aflatoxin G_1



Aflatoxin G_2

Figure 1-20. Structures of Aflatoxin B_1, B_2, G_1, an G_2.

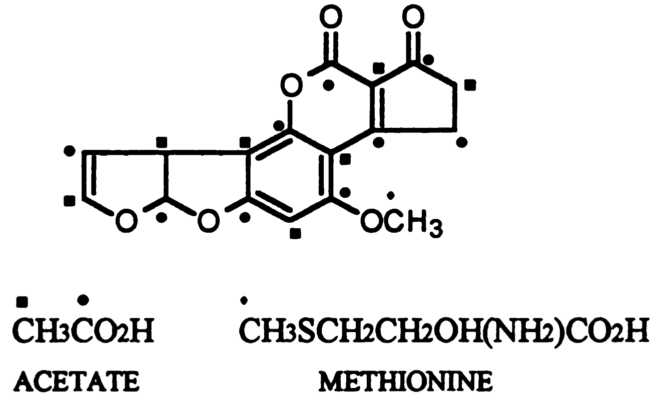


Figure 1-21. The origin of the 16 skeletal carbon atoms of aflatoxin B_1.

et al., 1982). These DNA adducts have been proven to be mutagenic in many *in vitro* mutagen-detecting systems such as bacteria, fungi, drosophila, and mammalian cells. For example, mutations in codon 61 of the Ha-*ras* proto-oncogene have been shown to occur with a high frequency in AFB$_1$-induced liver tumors in the CF1 mouse (Bauer-Hofman *et al.*, 1990). Another major mutational hot spot for DNA adduct formation by AFB$_1$ in human primary liver cancer (PLC) and human hepatocellular carcinoma (HCC) was demonstrated to be codon 249 in the p53 tumor suppresser gene (Puisieux *et al.*, 1991). Experimental data demonstrated that mutations in p53 occur later in tumor development, and therefore suggested that these mutations may act as tumor promoters not as tumor initiators (Nose *et al.*, 1993). AFB$_1$ can also induce specific mutations which activate another proto-oncogene c-ki-*ras* (Soman and Wogan, 1993).

Because of the threat of aflatoxin contamination to public health the US Food and Drug Administration set action levels of 20 ppb for human foods (except for milk which is 0.5 ppb) and 20 to 300 ppb in most animal feeds (Labuza, 1983).

BIOSYNTHESIS OF AFLATOXIN B$_1$

Biosynthesis Of Aflatoxin B$_1$

Aflatoxins are polycyclic, unsaturated compounds consisting of a coumarin nucleus flanked by a bisfuran system containing either a furan ring (B$_1$, G$_1$ series) or a dihydrofuran ring (B$_2$, G$_2$ series) on one side, and either a cyclopentanone (B series), or a six-member lactone (G-series)

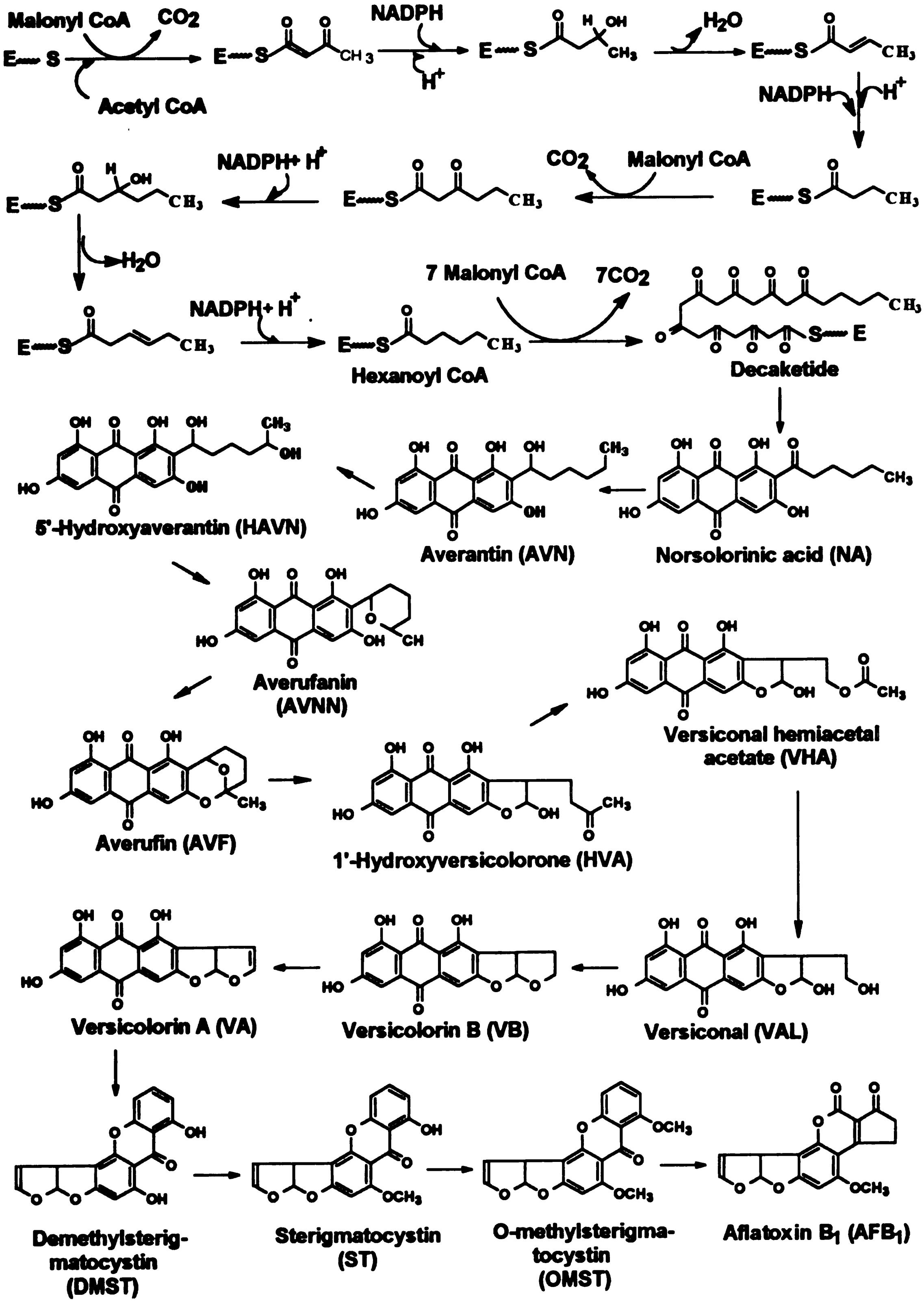


Figure 1-22. The aflatoxin B₁ biosynthetic pathway in *Aspergillus parasiticus*.

on the other side (Figure 1-20).

Most researchers in the field of aflatoxin biosynthesis have focused on aflatoxin B_1 (AFB_1) because it is the most toxic and abundant aflatoxin produced by fungi. The currently accepted AFB_1 biosynthetic pathway begins with acetyl CoA and malonyl CoA to form a proposed polyketide precursor, which is then converted sequentially to several pathway intermediates in the order of norsolorinic acid (NA), averantin (AVN), averufanin (AVNN), averufin (AVF), versiconal hemiacetal acetate (VHA), versiconal (VAL), versicolorin B (VB), versicolorin A (VA), demethylsterigmatocystin (DMST), sterigmatocystin (ST), O-methyl sterigmatocystin (OMT), and finally aflatoxin B_1 (AFB_1) (Figure 1-22) (Dutton 1988; Bhatnager *et al.*, 1992; Yabe and Hamasaki, 1993). The established pathway is the result of a combination of the use of *Aspergillus* mutants blocked in the AFB_1 pathway in certain steps, radiolabeled-precursor feeding experiments, enzyme isolation (purification) and activity determination, and chemical structure determination by nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy. Determination of the structure (especially the radiolabeled atom distribution and the stereochemical configuration) of any potential precursor and the final product is a very important requirement. Without accurate structure determination, all the other experimental results are not fully explainable. Therefore, any comprehensive review on AFB_1 biosynthesis should include structure analysis. However, in this brief review, only the most important experiments related to structure determination will be mentioned. Steyn *et al.*(1980) presented an excellent review on structure determination related to AFB_1 biosynthesis.

According to the chemical structure of the starter units, the intermediates and the final product, and the demonstrated or speculated nature of the biochemical reactions involved, the biosynthesis of AFB_1 can be divided into seven distinct stages: (1) **the fatty acid stage** in which it is proposed that a hexanoyl CoA is synthesized with one acetyl CoA and two malonyl CoA as the building units; (2) **the polyketide stage** in which a proposed decaketide is formed (by adding another seven malonyl CoA to the hexanoyl CoA in a manner similar to fatty acid synthesis but without the complete reduction, dehydration, and reduction cycles) and finished by formation of a stable anthraquinone norsolorinic acid; (3) **the internal ketal stage** in which the six-carbon side chain of norsolorinic acid is modified (through averantin, and averufanin) to form the internal ketal of averufin; (4) **the dihydrobisfuran stage** in which the ketal of averufin is further modified by oxidative reorganization (in the order of versiconal hemiacetal acetate, versiconal, versicolorin B) to form versicolorin A which contains an internal ketal structure; (5) **the anthraquinone-xanthone conversion stage** in which the anthraquinone versicolorin A undergoes a complex set of oxidative cleavage and deoxygenation reactions to form the xanthone demethylsterigmatocystin; (6) **the methylation stage** in which demethylsterigmatocystin is modified by methylation to form sterigmatocystin and in turn O-methylsterigmatocystin. (7) **the xanthone-coumarin conversion stage** in which the xanthone structure of O-methylsterigmatocystin undergoes oxidative cleavage, subsequent cyclization and loss of one carbon atom to form the coumarin nucleus of aflatoxin B_1.

Acetyl CoA, malonyl CoA, and C-1 of methionine are the carbon sources of AFB_1. The study of the sources of the carbons in aflatoxin B_1 was started in the late 1960's.

Among many studies, the experiments conducted by Biollaz *et al.* (1970) established conclusively that AFB_1 is synthesized with acetate units as the starter. AFB_1, prepared by separately feeding of [1-^{14}C]- and [2-^{14}C]-acetate to an *A. flavus* culture, was analyzed by step degradation. The origin of the 16 skeletal carbon atoms was determined (Figure 1-21). The nearly equal distribution of the labeled carbon atoms at all the centers suggested that AFB_1 is derived from a single, highly rearranged polyketide chain. This hypothesis was further supported (Hsieh and Mateles, 1970; Hsieh and Mateles, 1971). In the study by Biollaz *et al.* (1970), [methyl-^{14}C]-methionine was found to specifically label the methoxy carbon atom of AFB_1. Later, the development of [^{13}C] NMR spectroscopy, which reduced the time used in these studies, confirmed that acetate is the source of carbon for AFB_1 (Steyn *et al.*, 1975b; Pachler *et al.*,1976; Gorst-Allman *et al.*, 1978).

The first stage was proposed to occur via a fatty acid biosynthetic pathway to form a hexanoate (Townsend *et al.*, 1984; Brown *et al.*, 1996).

The second stage was proposed to occur through a polyketide synthetic process to form a decaketide (Lee *et al.*, 1971; Feng and Leonard, 1995; Chang *et al.*, 1995).

The third stage is the internal ketal stage in which a proposed decaketide goes through norsolorinic acid, averantin, and averufanin to form averufin (an internal ketal). The anthraquinones norsolorinic acid (NA) ($C_{20}H_{18}O_7$) (Lee *et al.*, 1971), averantin (AVN) ($C_{20}H_{20}O_7$) (Birkinshaw *et al.*, 1966), and averufin (AVF) ($C_{20}H_{16}O_7$) (Donkersloot *et al.*, 1972), are each produced by blocked mutant strains derived from wild-type *A. parasiticus*. Each compound is derived from a C_{20}-decaketide, and was demonstrated to be an intermediate in the AFB_1 pathway by feeding experiments. Bennett *et al.* (1980)

demonstrated the transformation of [^{14}C]-AVN into AVF, but not into NA in *A. parasiticus.* Therefore AVN was placed after NA and before AVF in the AFB$_1$ pathway. McCormick *et al.* (1987) found AVF in wild-type *A. parasiticus.* In studies with blocked mutants they demonstrated [^{14}C]-radiolabeled averufanin (AVNN) accumulated in AVF but not AVN. The results demonstrated that AVNN is a precursor in the AFB$_1$ pathway after AVN and before AVF. With improved HPLC, Yabe *et al.* (1991a) suggested that 5'-hydroxyaverantin may be an intermediate between AVN and AVNN.

The fourth stage is the dihydrobisfuran stage in which averufin goes through versiconal hemiacetal acetate, versiconal, and versicolorin B (C) to form versicolorin (a dihydrobisfuran). Lin *et al.* (1973) first demonstrated that averufin (AVF) was converted into AFB$_1$ by *A. parasiticus.* Yao and Hsieh (1974) treated wild-type *A. parasiticus* with the insecticide dichlorvos (dimethyl 2,2-dichlorovinyl phosphate) which presumably acts as a specific enzyme inhibitor, and found AFB$_1$ production was inhibited but versiconal hemiacetal acetate (VHA) accumulated. In the presence of dichlorvos, AVF was converted into VHA. Therefore AVF lies before VHA in the AFB$_1$ pathway. Several mechanisms have been proposed for the rearrangement of averufin (AVF) to versiconal hemiacetal acetate (VHA). Among them an epoxide rearrangement is consistent with experimental evidence. Nidurufin (2'-hydroxyaverufin) is a natural metabolite of *Aspergillus nidulans* (Aucamp and Holzapfel, 1970). Hydroxylation at C-2 of averufin could convert averufin into nidurufin. A rearrangement of nidurufin followed by a Baeyer-Villiger oxidation (Townsend *et al.*, 1982) was proposed to convert nidurufin into versiconal hemiacetal acetate.

In feeding studies without dichlorvos treatment, VHA was converted to AFB_1 in wild-type *A. parasiticus.* In feeding studies with dichlorvos, versiconal (VAL), versicolorin B (VB), and versicolorin A (VA), (but not VHA) were converted into AFB_1 (Yao and Hsieh, 1974; Fitzell *et al.*, 1977; Hsieh *et al.*, 1978). Therefore VHA was before VAL, VB and VA in the AFB_1 pathway. Hsieh *et al.* (1989) and Anderson and Chung (1990) found that VHA was converted to versiconal (VAL) which was further converted to versicolorin C (VC) by *A. parasiticus.* Lin and Anderson (1992) and Silva *et al.* (1996) demonstrated that a versiconal cyclase purified from *A. parasiticus* catalyzes the dehydration of VAL to VB or versicolorin C (VC). Indeed versicolorin C (VC) is the racemate of VB. Yabe *et al.* (1991b) found that VB was converted into VA in *A. parasiticus* by a desaturase activity.

Townsend *et at* (1988) suggested that 1'-hydroxy-versicolorone (HVN) may be an intermediate between AVF and VHA. Yabe and Hamasaki (1993) suggested that, in the AFB_1 pathway, the conversion of AVF to VA goes through a metabolic grid involving versiconal hemiacetal acetate, versiconol acetate, versiconol, and versiconal.

In the fifth stage, versicolorin A (an anthraquinone) is converted to demethylsterigmatocystin (a xanthone). Hsieh *et al.* (1978) found that [^{14}C]-labeled versicolorin A (VA) was efficiently converted to sterigmatocystin (ST) by *A. versicolor.* Thomas (1965) proposed that the conversion of versicolorin A to sterigmatocystin could occur by oxidative decarboxylation and elimination of an acetate-derived methyl carbon atom. This hypothesis was further supported by the [^{13}C]-NMR spectrum data of sterigmatocystin and aflatoxin B_1 (Pachler *et al.*, 1976). Jeenah and Dutton (1983) found that a VA-accumulating mutant of *A. parasiticus* was able to convert sterigmatocystin to both O-methylsterigmatocystin (OMST) and AFB_1 suggesting that VA is an earlier precursor than ST in the AFB_1 pathway. Yabe *et al.* (1989) found that demethylsterigmatocystin (DMST) was converted to AFB_1; and in the presence of S-adenosyl-[methyl-^{3}H]methionine, DMST was first converted into ST, and then to OMST in *A. parasiticus.* According to recent data, VA is the last anthroquinone and DMST is the first xanthone in the AFB_1 pathway. DMST is clearly an intermediate between VA and ST. At this stage the anthraquinone VA is converted into the xanthone DMST through a possible oxidative cleavage and deoxygenation (Yabe *et al.*, 1989).

In the sixth stage, demethylsterigmatocystin is methylated to form sterigmatocystin which in turn is methylated to form O-methylsterigmatocystin. Singh and Hsieh (1976) demonstrated that *A. parasiticus* was capable of converting [^{14}C]-sterigmatocystin (ST) into AFB_1. Bhatnagar *et al.* (1987) found an isolate of *A. parasiticus* which accumulated O-methylsterigmatocystin (OMST) without AFB_1. When [^{14}C]-OMST was fed to the mycelia of a mutant of *A. parasiticus,* [^{14}C]-AFB_1 was produced and when [^{14}C]-ST was fed to the same mutant, [^{14}C]-OMST was produced. Cleveland and Bhatnager (1987), and Bhatnagar and Cleveland (1988) demonstrated that, in *A. parasiticus,* a postmicrosomal activity (PMA) catalyzed the conversion of ST to OMST, and a microsomal activity (MA) catalyzed the conversion of OMST to AFB_1. These results suggest that OMST is an intermediate in the AFB_1 pathway between ST and AFB_1.

In the seventh stage, O-methylsterigmatocystin is converted to aflatoxin B_1. In this stage, the xanthone structure of O-methylsterigmatocystin undergoes oxidative cleavage, subsequent cyclization and loss of one carbon atom to form the coumarin nucleus of aflatoxin B_1. This is the complete current picture of AFB_1 biosynthesis which is generally accepted.

Based on the experimental data that vesicolorin A (VA) was a precursor of AFB_1 (not of AFB_2) and versicolorin B (VB) was a precursor of both AFB_1 and AFB_2, combined with the fact that VB was converted to VA, McGuire *et al.* (1989) and Yabe *et al.* (1991b) proposed that VB was the branch point leading both to AFB_1 and AFB_2 in the aflatoxin biosynthesis pathway.

Lee *et al.* (1975) found two distinct O-methyltransferase activities in aflatoxin biosynthesis. Based on their experiments, Cleveland *et al.*(1987) and Cleveland (1989) suggested that AFB_1/AFG_1 and AFB_2/AFG_2 are synthesized separately. This idea was further confirmed. Yabe *et al.* (1989) demonstrated that *A. parasiticus* NIAH-26, which does not normally produce aflatoxins, was able to convert demethylsterigmatocystin (DMST) to aflatoxins B_1 and G_1 (not to B_2 and G_2); and to convert dihydrodemethylsterigmatocystin (DHDMST) to aflatoxins B_2 and G_2 (not B_1 and G_1). They also demonstrated that O-methyltransferase I converts DMST and DHDMST into sterigmatocystin and dihydrosterigmatocystin, respectively; and O-methyltransferase II converts sterigmatocystin and dihydrosterigmatocystin into O-methylsterigmatocystin (OMST) and dihydro-O-methylsterigmatocystin (DHOMST), respectively. These data support the idea that AFB_1/AFG_1 and AFB_2/AFG_2 are synthesized separately.

Henderberg *et al.* (1988) demonstrated that [^{14}C]-sterigmatocystin was converted to AFG_1 in wild-type *A. flavus* and *A. parasiticus* but [^{14}C]-AFB_1 was not. These data suggested that AFG_1 may not be derived from AFB_1; while AFB_1 and AFG_1 may share a common pathway at least until sterigmatocystin formation.

The relationship between of AFB_1/AFG_1 and AFB_2/AFG_2 in aflatoxin biosynthesis then may be summarized as follows: the four aflatoxins share a common pathway in their biosynthesis up to versicolorin B. After versicolorin B, one branch leads to AFB_1/AFG_1, the other to AFB_2/AFG_2. AFB_1 and AFG_1 share a common pathway up to sterigmatocystin; while AFB_2 and AFG_2 share a common pathway up to dihydrosterigmatocystin.

Genes Involved In The Aflatoxin B_1 Pathway

In order to understand the regulatory mechanisms involved in aflatoxin biosynthesis, a very important approach is to clone and sequence the relevant pathway genes. Woloshuk *et al.* (1989), Horng *et al.* (1990), and Skory *et al.* (1990) developed transformation systems for *A. flavus* and *A. parasiticus*, respectively, which have been demonstrated to be useful tools to introduce exogenous DNA into these fungi to identify, isolate, and characterize the genes related to aflatoxin biosynthesis.

Genes can be isolated by the use of heterologous sequences to screen DNA libraries, by the use of PCR primers to amplify desired genes from genomic DNA, by the use of antibodies raised against purified proteins to probe DNA expression libraries, or by the use of complementation tests with relevant mutants and appropriate selectable markers.

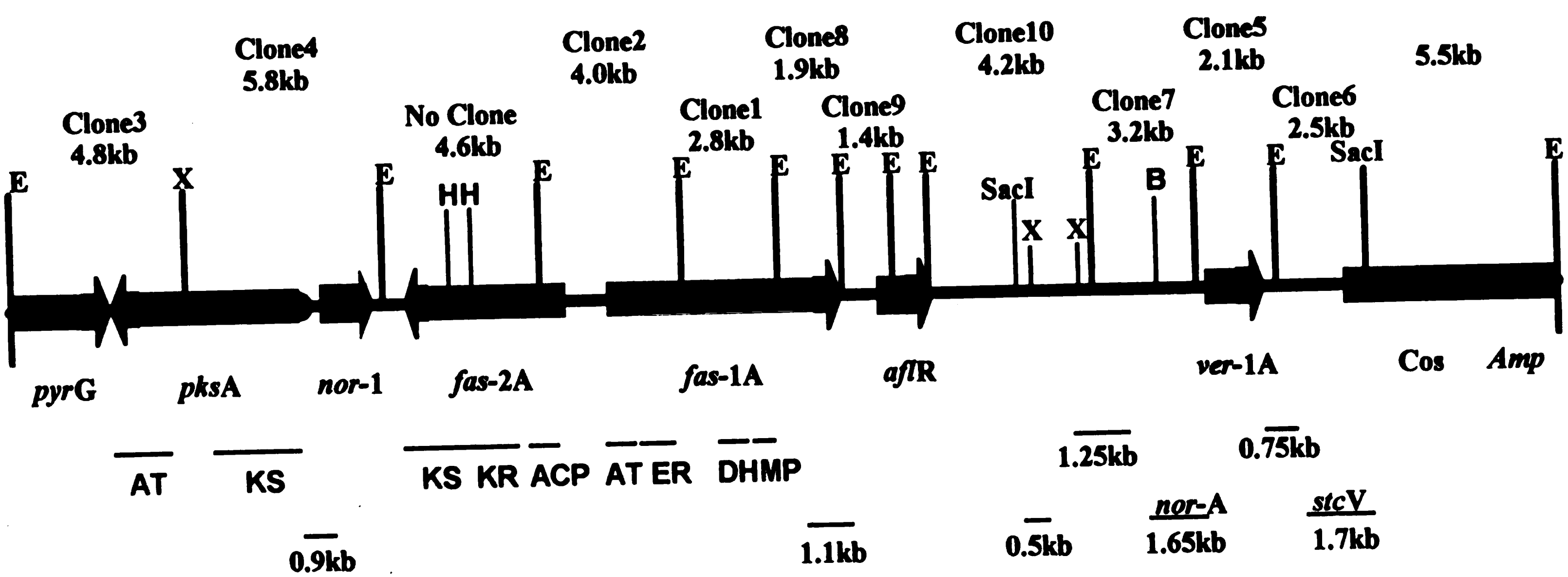
The first aflatoxin pathway gene isolated, named *nor*-1, is involved in the conversion of norsolorinic acid (NA) to averantin (AVN) in the aflatoxin pathway, and was cloned by genetic complementation of the aflatoxin blocked mutant strain, *A. parasiticus* B62 (*nor*-1, *nia*D, *brn*-1), with a cosmid library which was constructed by inserting wild type *A. parasiticus* genomic DNA into a vector containing a nitrate reductase gene (*nia*D) as a selectable marker (Chang *et al.*, 1992). Another gene, named *ver*-1 which encodes an activity associated with the conversion of versicolorin A to sterigmatocystin, was cloned similarly by genetic complementation of another aflatoxin blocked mutant, *A. parasiticus* CS 10 (*ver*-1, *pyr*G, *wh*-1), with a cosmid genomic DNA library and the homologous gene (*pyr*G) encoding orotidine monophosphate decarboxylase for the selection of transformants (Skory *et al.*, 1992). Payne *et al.* (1993) cloned a gene called *afl*-2 from *A. flavus* by using an aflatoxin defective strain and a genomic DNA library from aflatoxigenic *A. flavus* in a cosmid vector containing the *pyr*-4 gene of *Neurosprora crassa*. Complementation of the mutated *afl*-2 gene in a double mutant containing *afl*-2 and a lesion resulting in accumulation of norsolorinic acid suggested that the product of the gene has a regulatory function (Payne *et al.*, 1993). Another regulatory gene (*apa*-2, which is very similar to *afl*-2) has been cloned from *A. parasiticus* by transformation of strains with a cosmid containing both *nor*-1 and *ver*-1 (Chang *et al.*, 1993). The two regulatory genes, *apa*-2 and *afl*-2, are both called *afl*R. Yu *et al.* (1993) isolated a methyltransferase-related gene (*omt*-1) by the use of a polyclonal antibodies raised against a 40-kDa methyltransferase to screen a cDNA library from wild type aflatoxigenic *A. parasiticus*.

Even though there are no data showing accumulation of a stable intermediate (e.g. a polyketide between acetate and norsolorinic acid), recent molecular data support the hypothesis that AFB_1 originates from a polyketide.

Figure 1-23. Restriction endonuclease and transcript map of cosmid Nor-A. Cosmid Nor-A was cloned from genomic DNA of the wild-type *Aspergillus parasiticus*. **Restriction enzymes:** E, *Eco*RI; X, *Xba*I; H, *Hind*III; B, *Bam*HI; **Genes:** *pyr*G (selective marker in the cosmid), encoding orotidine monophosphate decarboxylase; *pks*A, encoding a polyketide synthase (including acyl carrier protein, β-ketoacyl carrier protein synthase, acyltransferase, and thioesterase functional domains); *nor*-1, encoding norsolorinic acid reductase; *fas*-2A, encoding a fatty acid synthetase α-subunit (including β-ketoreductase, enoylreductase, and acyl carrier protein functional domains); *fas*-1A(*uvm8*), encoding a fatty acid synthetase β-subunit (including enoyl reductase and malonyl/palmityl transferase functional domains); *afl*R, involved in regulation of AFB_1 biosynthesis; *ver*-1A, encoding an activity associated with the conversion of versicolorin A to sterigmatocystin; *Amp* (selective marker), encoding β-lactamase; *nor*-A, encoding a reductase activity; *stc*V, encoding a cytochrome P-450 protein. **Symbols:** narrow straight line, detected transcript with unknown function; narrow straight line with abbreviation below, deduced functional domain from DNA sequence; narrow straight line with number below and gene name above, detected transcript with possible known function; **Abbreviations:** ACP, acyl carrier protein; KS, β-ketoacyl ACP synthase; KR, β-ketoreductase; DH, dehydratase; ER, enoylreductase; MP, malonyl-palmityl transferase.

Figure 1-23.

Cosmid Nor-A



Symbols:——, 2.0 kb DNA;——, 2.0 kb transcript.

Disruption of the *pks*-A gene in *A. parasiticus* resulted in a strain which lost both the ability to produce aflatoxins and the ability to accumulate norsolorinic acid and all other intermediates in the aflatoxin pathway (Feng and Leonard, 1995; Chang *et al.*, 1995; Trail *et al.*, 1995). Sequence analysis showed four functional domains in this gene, acy carrier protein, β-ketoacyl carrier protein synthase, acyltransferase, and thioesterase, all of which are usually present in polyketide synthases and fatty acid synthetases. Disruption of a gene (*fas*-1A) physically located between *nor*-1 and *ver*-1 in *A. parasiticus* B62 (*nor*-1, *nia*D, *brn*A), which normally accumulates norsolorinic acid, resulted in a new set of mutants which were unable to accumulate norsolorinic acid and AFB_1 (Mahanti *et al.*,1996). The predicted amino acid sequence of this gene showed a high level of identity with extensive regions in the enoyl reductase and malonyl/palmityl transferase functional domains in the β-subunit of yeast fatty acid synthetase suggesting that *fas*-1A encodes a novel fatty acid synthetase which synthesizes part of the polyketide backbone of AFB_1. Therefore, *fas*-1A and *pks*A are clearly involved in AFB_1 biosynthesis, function before *nor*-1 in the AFB_1 pathway, and possibly work together at the fatty acid stage and polyketide stage in the AFB_1 pathway.

A gene named *nor*-A was identified when an *Aspergillus parasiticus* cDNA library was screened with monoclonal antibody raised against a purified 43-kDa protein demonstrating norsolorinic acid reductase activity (Cary *et al.*, 1996). Northern blot and Western blot analyses showed that *nor*A transcript and protein are present only when the fungus was grown in medium conducive to aflatoxin biosynthesis. *nor*A is located between *ver*-1 and *nor*-1 in the aflatoxin gene cluster, a region which is in part located in a cosmid named

Oliver Yu, Ph.D.
Associate Member
Principal Investigator
oyu@danforthcenter.org
975 North Warson Road
St. Louis, Missouri 63132
p 314 587 1441
f 314 587 1541

DONALD DANFORTH
PLANT SCIENCE CENT

Nor-A (Figure 1-23), isolated from *A. parasiticus*. The deduced amino acid sequence of *nor*A had 49% amino acid identity with an aryl-alcohol dehydrogenase gene from *Phanerochaete chrysosporium*. The function of *nor*A in the AFB$_1$ pathway is not clear.

Purification Of Enzymes Involved In The Aflatoxin B$_1$ Pathway

Purifying enzymes that catalyze aflatoxin synthesis from *A. flavus* or *A. parasiticus* crude mycelial extracts is not easy because the enzymes are present in relatively low concentrations and are extremely short lived (Dutton, 1988). Mashaly *et al.* (1988) purified an enzyme (70 kDa) showing activity in converting sterigmatocystin to aflatoxin B$_1$. Bhatnagar *et al.* (1988) and Keller *et al.* (1993) have purified two methyltransferases (168 kDa and 40 kDa) to homogeneity; both enzymes catalyze conversion of sterigmatocystin (ST) to O-methylsterigmatocystin (OMST). A 38 kDa reductase that catalyzes the reduction of NA to AVN was purified by Bhatnagar and Cleveland (1990); an isozyme (43 kDa) of the reductase also has been purified to homogeneity (Bhatnagar, unpublished observation). Another NA reductase (140 kDa) was also isolated (Chuturgoon and Dutton, 1991). Lin and Anderson (1992) purified versiconal cyclase (72 kDa) catalyzing the dehydration of versiconal to versicolorin B or versicolorin C. Studies on protein isolation, purification, and enzymatic reaction suggest that different enzymes with the same catalytic function may be isolated from pertinent cells (Bhatnagar *et al.*, 1991).

Regulation Of Aflatoxin Biosynthesis

The regulation of aflatoxin biosynthesis has been studied at different levels by many researchers. Many environmental factors can influence aflatoxin biosynthesis. The three most important factors controlling aflatoxin formation in field crops are relative humidity, moisture, and temperature (Russell, 1979; Wicklow, 1990; Gorman *et al.*, 1992). At the biochemical level, studies on the regulation of aflatoxin biosynthesis started in the 1970's. One of the early important studies was conducted by Shih and Marth (1974). They investigated the influence of sodium azide on AFB_1 biosynthesis and found that, at low concentration, sodium azide selectively inhibits the respiratory system of the mold, enhances production of AFB_1 and total lipid, and promotes [1-^{14}C]-glucose incorporated into aflatoxin. Shih and Marth concluded that the decrease in oxidative respiration during late logarithmic growth favored an accumulation of acetate (acetyl CoA) and NADPH via the Embden-Meyerhof pathway. Later, aflatoxin biosynthesis was found to be influenced by many other biochemical factors, such as the cellular energy status (Buchanan *et al.*, 1987), the intracellular level of cyclic AMP (Khan and Venkitasubramanian, 1986), the cellular redox state (Bhatnagar *et al.*, 1986), and glycolytic activity (Gupta *et al.*, 1977). Aflatoxin biosynthesis was also influenced by the level of glucose (Buchanan and Lewis, 1984), the levels of other carbohydrates (Abdollahi and Buchanan, 1981), and the pH value of the growth medium (Cotty, 1988).

In the 1990's, aflatoxin biosynthesis was found to be regulated at the transcriptional level (Skory *et al.*, 1993; Trail *et al.*, 1995). A regulatory gene, *afl*R, for aflatoxin biosynthesis was characterized (Woloshuk *et al.*, 1994). DNA sequence analysis showed that the *afl*R gene possesses a binuclear zinc finger DNA-binding domain suggesting that the product of *afl*R is a regulatory protein (Chang *et al.*, 1995). Overexpression of *afl*R relieved nitrate inhibition of aflatoxin biosynthesis (Chang *et al.*, 1995).

BIOCONTROL OF AFLATOXIN CONTAMINATION

Possible Function Of Aflatoxins

Aflatoxins may provide a mechanism for a toxigenic fungus to survive in the environment. Aflatoxins were found to be concentrated in both the conidium and the sclerotium of aflatoxigenic strains (Wicklow and Cole, 1982; Cotty, 1988). The presence of aflatoxins in sclerotia and conidia may have a long term survival value, because aflatoxins are toxic to a variety of predators of fungi, especially insects (Willetts and Bullock, 1992).

Aflatoxins may have survival value only for the producing fungus in the field. There is not a clear cut relationship between sclerotia and aflatoxin production. Some fungal strains produce aflatoxins but not sclerotia and vice versa (Bennett *et al.*, 1979). Aflatoxin production is highly variable within the overall *A. flavus* group. Aflatoxin production can vary by as much as a million-fold among isolates from the same species (Clevstrom and Ljunggren, 1985). *A. parasiticus* and *A. nomius* produce both the B-series and G-series of aflatoxins (Kurtzman *et al.*, 1987). *A. flavus* is observed to produce only the B-series of aflatoxins (Samson and Frisvad, 1991). The ability to produce aflatoxin is highly conserved among most wild type strains of *A. parasiticus* and *A. flavus* (Dorner *et al.*, 1984). This ability is easily lost in culture (Cotty, 1989). Therefore, aflatoxin may only have survival value for the aflatoxigenic fungi in the field.

Species Of The *Aspergillus Flavus* Group

One primary requirement for studies on the field populations of members of *Aspergillus* *flavus* group is the ability to classify species of the *A. flavus* group. There are many taxonomic schemes used to classify species in the *A. flavus* group (Klich and Pitt, 1988; Samson and Frisvad, 1991). The standard for classifying a species proposed by Papa (1986) may be the best one. He proposed that a species represents a collection of strains which behave as clonal organisms with the exception of occasional parasexuality between members of the same vegetative compatibility group (VCG). Parasexuality can be observed in the laboratory only in the same VCG. According to King and Stransfield (1985), clonal organisms are a group of identical organisms descended from a single common ancestor by mitosis. How to determine if a group of organisms is originated from a single common ancestor? One standard is based on DNA polymorphism.

Analyzing DNA polymorphism, Bayman and Cotty (1993) proposed that the *A. flavus* group consists of four species which are *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, and *A. tamarii*. Among the four, no aflatoxigenic isolates have been obtained from *A. tamarii*. Within each of the three aflatoxin producing species, there is great variability. Bayman and Cotty (1991) further classified each species into Vegetative Compatibility Groups (VCGs). Each species is composed of several Vegetative Compatibility Groups (VCGs) (Papa, 1986). Physiological and morphological traits such as enzyme production, plant virulence and sclerotial morphology, are much more consistent within a VCG (Bayman and Cotty, 1993).

By using this classification scheme as a tool, scientists now know the vast diversity of strains within the *Aspergillus flavus* group (Papa, 1986; Bayman and Cotty, 1991), the vast diversity in ecological niches (Brown *et al.*, 1991), the vast diversity of different regional population structures (Bayman and Cotty, 1991), and the vast diversity of changing populations caused by the agricultural practices (Shearer *et al.*, 1992). The distributions of the *A. flavus* group fungi are very complex. Almost all species from the *A. flavus* group are better adapted to a soil environment, but certain species are better adapted to niches above the soil. For example, *A. parasiticus* occurs more frequently on peanuts than on corn (Hill *et al.*, 1985). Sometimes all species within the group may occur on the same crop or in the same field (Cotty, 1992b).

Biocontrol Of Aflatoxin Contamination

Even though aflatoxins are produced only by certain species of *Aspergillus flavus, A. parasiticus,* and *A. nomius*, these fungi are ubiquitous and frequently grow on a variety of food and feed crops and cause aflatoxin contamination in many areas in the world (Blount, 1961; Ellis *et al.*, 1991; Jelinek *et al.*, 1989).

Aflatoxin contamination is difficult to eliminate from the food chain without novel forms of treatment. These compounds are resistant to normal food processing such as milling and cooking, and some of their bio-metabolites are also very toxic and mutagenic such as AFM_1 in cow milk. Therefore aflatoxin contamination causes serious health problems, and in turn results in economic problems. There have been several strategies currently in use or proposed for reducing or eliminating aflatoxin from contaminated food and feed such as screening/detection, removal/decontamination, or altered AFB_1 metabolism/DNA adduct formation (Chu, 1991; Ellis *et al.*, 1991; Park and Liang, 1993). On the other hand, strategies for reducing/eliminating aflatoxin contamination at the preharvest level may be better approaches. These strategies include improving agronomic practices (such as irrigation and fungicide application) and classical breeding programs (such as selecting aflatoxin-resistant strains of crops). These strategies of aflatoxin decontamination and contamination prevention are still very useful and are widely used. With the development of modern molecular techniques, studies on the mechanism of aflatoxin biosynthesis at the molecular level has been an active focus area. Such studies not only can provide information for improving agronomic practices and breeding programs, for developing safe and specific 'aspergicides', for developing genetically engineered crops, but also can provide new biocontrol agents by knocking out genes involved specifically in aflatoxin biosynthesis to generate genetically stable nontoxigenic strains of *Aspergillus*. These strains in theory may be safely utilized in the field to reduce or eliminate aflatoxin contamination of food or feed crops by biological exclusion (biocontrol). Therefore the following brief review will focus on the possibility of developing such biocontrol agents.

The idea of using biocontrol agents to control aflatoxin contamination is that nonaflatoxigenic strains or mutants of the *A. flavus* group can compete with the toxigenic strains in the field for growing space and nutrition, and/or by the production of interfering compounds to reduce or eliminate aflatoxin contamination.

It is possible to obtain nonaflatoxigenic strains for use as biocontrol agents. Aflatoxin production is highly variable (up to a million-fold) within the overall *A. flavus* group. Many strains cannot produce detectable aflatoxins (Clevstrom and Ljunggren, 1985). Over the centuries, industrial strain selection has reduced fungal toxicity and increased fungal traits associated with both product quality and efficient fermentation in products using *Aspergilli*. Therefore, it is also possible to identify nonaflatoxigenic isotates in the field. Alternatively, nonaflatoxigenic strains can be created by the use of molecular techniques to genetically disrupt specific genes only in aflatoxin biosynthesis. However, not all nonaflatoxigenic strains are capable of reducing aflatoxin contamination when inoculated in the field (Cotty, 1992a). Following selection, the co-inoculation of both the producing and nonproducing strains should be tested both in the greenhouse and in the field.

It is safe to use nonaflatoxigenic strains as biocontrol agents. The inoculation of nonaflatoxigenic strains or mutants in the field may cause some concern about the danger of those potential pathogens to humans. Indeed, in many areas people respire high concentrations of *Aspergillus* spores but without noticeable disease. For example, in the koji, baking, and brewing industries workers over several generations have been exposed to very high concentrations of spores throughout their working years with a very low incidence of disease (Barbesgaard *et al.*, 1992). It is important to note that inoculation of nonaflatoxigenic strains in the field can result in *A. flavus* population with altered composition, but without increased population size (Cotty, 1992c). Indeed, inoculation of nonaflatoxigenic strains in the field may provide the opportunity to improve the overall safety of fungal populations by reducing human exposure to aflatoxin through both dietary and respiratory routes. Aflatoxins can cause several physiological effects on the host crops and on domestic animals (Mclean *et al.*, 1992; Robens and Richard, 1992). Reducing the aflatoxigenic population of fungi in the field can also reduce the physiological or toxic effects on aflatoxins of crops and domestic animals.

Normal agricultural practices generate a very large quantity of organic materials contaminated with *A. flavus* group fungi in the field. Because those materials consist of crop remnants, corn cobs, gin trash, inoculation of the *A. flavus* group fungi occurs naturally without our notice. The main difference between natural inoculation and artificial inoculation is that in the latter only nonaflatoxigenic strains are inoculated.

Inoculation of nonaflatoxigenic strains in the field generally will not increase the infection level of crops. Crop infection by the *A. flavus* group often happens when crops are wounded or under the stress of nutrition limitation. Aflatoxins are not required for the producing fungi to infect the host crops (Cotty, 1989). Infection is more heavily dependent on host predisposition and the environment than on the number of *A. flavus*. Therefore nonaflatoxigenic strains can compete with the aflatoxigenic strains for areas which are infection-predisposed in the crops. In three-year tests on cotton, inoculation of nonaflatoxigenic strains in the field has not resulted in an increase of infection rates (Cotty, 1992c).

It is effective to use nonaflatoxigenic strains as biocontrol agents. The advantage of inoculation of nonaflatoxigenic strains of *A. flavus* over other types of microbial biocontrol agents is that nonaflatoxigenic strains are adapted to similar environmental conditions as aflatoxigenic strains. Other potential agents, such as bacteria, may be inactive under the hot, dry conditions associated with aflatoxin contamination (Brown *et al.*, 1992). The *A. flavus* group fungi are associated with crops in the field during crop development and remain associated with the crop during harvest, storage and processing.

Inoculation of nonaflatoxigenic strains in the field both before and after harvest has been shown to provide protection from aflatoxin contamination of corn (Brown *et al.*, 1991).

Nonaflatoxigenic strains or mutants of the *A. flavus* group have been proven to reduce aflatoxin production and crop contamination by competition for growing space and nutrition, and/or by the production of interfering compounds (Cotty, 1990; Shantha *et al.*, 1990).

Perspectives On The Use Of Fungal Biocontrol Agents

Based on the above discussion, the possibility of using nontoxigenic fungi as biocontrol agents to reduce or eliminate aflatoxin contamination is promising. However there are still some questions which need to be answered. One is the biology of a fungal population in the field and the other is the function of aflatoxin to the producing organism.

Because of the tremendous reproductive and dispersal abilities of the *A. flavus* group and the influence of different crops and different soils on the population structure of the fungus, scientists still do not know the change in population structure over time, or the mechanism of natural selection in the fungal population. Therefore studies on one group of fungi have often turned out to have limited application to other groups (Cotty and Bayman, 1993).

Even though there is much research left to be done, the preliminary results of using the nonaflatoxigenic strains to control the aflatoxigenic strains are very positive. For example, in greenhouse and field experiments, in which cotton bolls and corn ears were inoculated with combinations of different strains of the *A. flavus* group, nonaflatoxigenic strains reduced aflatoxin contamination by 80 to 90% (Cotty, 1990; Brown *et al.*, 1991). Dorner *et al.* (1992) demonstrated that nonaflatoxigenic *A. parasiticus* strains reduced aflatoxin contamination when concentrated mycelia/spore suspensions were applied to developing peanuts. Those procedures are both reliable and economic, and are active in the field under hot, dry conditions that are near optimal for the aflatoxin production by the *A. flavus* group.

As discussed above, it is possible to identify nontoxigenic isolates in the field as biocontrol agents. Alternatively, nontoxigenic biocontrol agents (strains) can be created by the use of molecular techniques to genetically disrupt specific genes (gene-knock-out strategy) involved in aflatoxin biosynthesis. The gene-knock-out biocontrol agents may share the same niche with toxigenic strains in the ecosystem, and therefore, may be more effective in the field than the natural nontoxigenic isolates.

An understanding of the regulation of aflatoxin biosynthesis and the general biology of the toxigenic fungus (e.g. regulatory mechanisms involved in hyphal growth, hyphal differentiation, and sporulation) may also lead to the development of new fungicides, the improvement of agronomic practices, the development of resistant crops (by genetic engineering or classical breeding), and the development of biocontrol agents (such as nontoxigenic competitive fungi) for control of aflatoxin contamination.

In the following chapters, the enzymatic function of the Nor-1 protein, the pattern of Nor-1 protein accumulation in *A. parasiticus* grown in liquid media and on solid media, the localization of Nor-1 protein accumulation and the localization of *nor*-1 promoter activity during aflatoxin biosynthesis, and the function of the *flu*p gene which is associated with hyphal development and the process of sporulation and indirectly influences the accumulation of aflatoxin in *A. parasiticus* are presented.

CHAPTER 2

ENZYMATIC FUNCTION OF THE NOR-1 PROTEIN INVOLVED IN AFLATOXIN B_1 BIOSYNTHESIS IN *ASPERGILLUS PARASITICUS*

ABSTRACT: To study the function of the *nor*-1 gene, which is directly involved in aflatoxin B_1 (AFB_1) biosynthesis *Aspergillus parasiticus*, a *nor*-1 cDNA was expressed in *Escherichia coli*. The resulting Nor-1c protein was used in an enzyme activity assay to test the function of the *nor*-1 gene in the AFB_1 pathway. The results confirmed that the Nor-1c protein is a ketoreductase which converts the AFB_1 pathway intermediate norsolorinic acid (NA) to averantin (AVN) in the presence of NADPH and a supernatant fraction (105,000 × *g*) obtained from *E. coli* DH5α. The results suggest that an unidentified cofactor(s) may be necessary for the function of the native Nor-1 protein and that the *nor*-1 gene is involved in only one of multiple proposed pathways for the conversion of NA to averufin (AVF) in the AFB_1 pathway in *A. parasiticus*.

INTRODUCTION

The anthraquinones norsolorinic acid (NA) ($C_{20}H_{18}O_7$), averantin (AVN) ($C_{20}H_{20}O_7$), and averufin (AVF) ($C_{20}H_{16}O_7$) (Figure 2-1) are each produced by blocked mutant strains of the wild-type *A. parasiticus*, and were demonstrated to be intermediates in the aflatoxin

B_1 (AFB_1) pathway by feeding experiments (Lee *et al.*, 1971; Birkinshaw *et al.*, 1966; Donkersloot *et al.*, 1972; Dutton, 1988). NA is converted to AVF via one or more proposed alternative pathways by converting NA to either AVN or to averufanin (AVNN) initially (Figure 2-1) (Bennett and Christensen, 1983; Button, 1988; McCormick *et al.*, 1987; Yabe *et al.*, 1991b).

The *nor*-1 gene is physically located in the AFB_1 pathway gene cluster (Figure 1-23) in the genome of *A. parasiticus* suggesting that *nor*-1 is involved in AFB_1 biosynthesis. Many genes physically arranged in a cluster in the genome of fungi are found to be involved in the same biosynthetic pathway (Timberlake and Barnard, 1981; Feitelson *et al.*, 1985; Chater, 1992; Kimura and Tsuge, 1993; Hohn *et al.*, 1993; Rainey *et al.*, 1993; Ueda *et al.*, 1993; Keller and Adams, 1995) for efficient expression (Feitelson *et al.*, 1985). Disruption of the *nor*-1 gene in a toxigenic strain of *A. parasiticus* (SU-1) resulted in significant NA accumulation and significant reduction of aflatoxin accumulation (Trail *et al.*, 1994) suggesting that *nor*-1 is directly involved in AFB_1 biosynthesis in *A. parasiticus*.

Analysis of the proposed amino acid sequence of *nor*-1 provided evidence that this gene encodes a ketoreductase (Trail *et al.*, 1994). Comparison of the chemical structure of NA and AVN (Figure 2-1) suggests that the 5'-keto group of NA can be converted to the 5'-hydroxyl group of AVN by a ketoreductase. Therefore it is reasonable to hypothesize that the Nor-1 protein encoded by *nor*-1 can convert NA to AVN in the AFB_1 pathway and that the *nor*-1 gene is involved in one branch of proposed alternative pathways for conversion of NA to AVF. To prove this hypothesis requires isolation of the native Nor-1 protein.

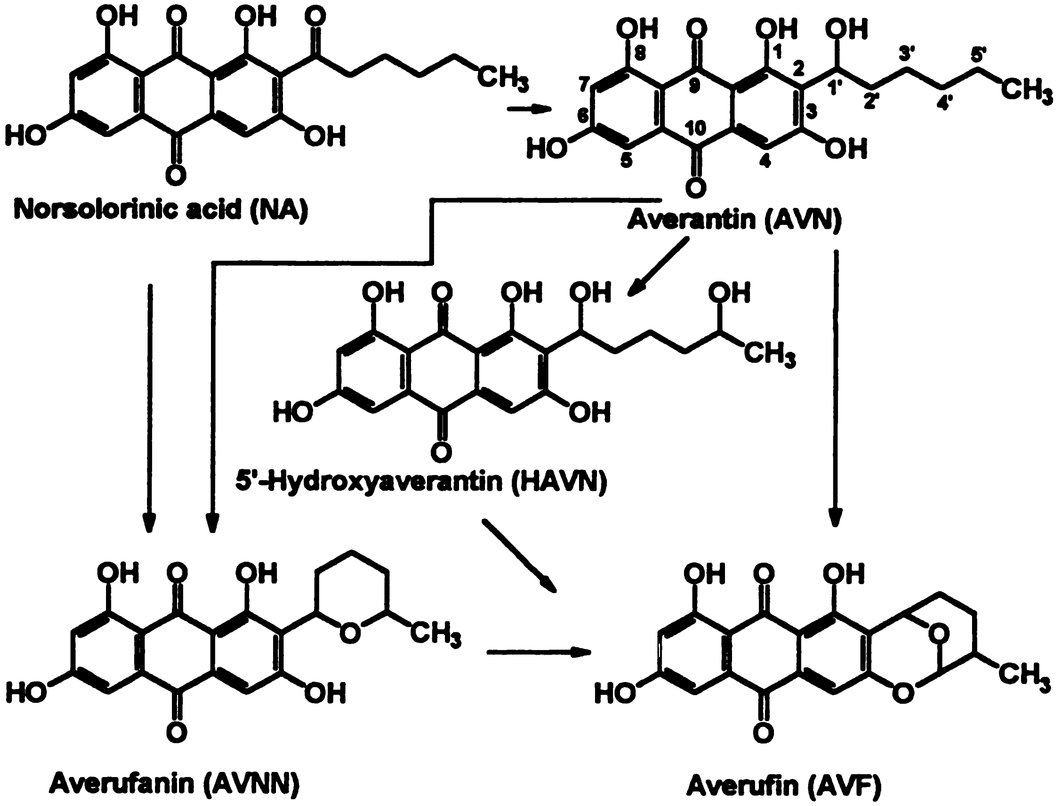


Figure 2-1. Proposed multiple alternative pathways which convert NA to AVF (Bennett and Christensen, 1983; Button, 1988; McCormick *et al.*, 1987; and Yabe *et al.*, 1991).

Several NA reductase (38, 140, and 43 kDa) activities have been purified to homogeneity from toxigenic *A. parasiticus* (Bhatnagar and Cleveland, 1990; Chuturgoon and Dutton, 1991; Bhatnagar, unpublished data) which can convert NA to AVN. But these three NA reductases do not have the same molecular mass as the Nor-1 protein (31 kDa deduced from the DNA sequence of *nor*-1). These data suggest that: (1) the Nor-1 protein is extremely unstable; (2) the fungal cell contains extremely low levels of the Nor-1 protein; and/or (3) the Nor-1 protein needs a cofactor(s) (in addition to NADPH) to perform the reductase activity and the cofactor(s) was lost during purification. These data also suggest that an alternative strategy is required to determine the function of the Nor-1 protein. A *nor*-1 cDNA cloning and expression strategy was chosen in this study.

The goal of this study was to determine the enzymatic function of the Nor-1c protein. The Nor-1c protein was produced in a host bacterium transformed with a *nor*-1 cDNA expression vector. The enzymatic function of the Nor-1c protein was tested to analyze the function of the *nor*-1 gene in the AFB_1 pathway.

The results confirmed that the Nor-1c protein is a ketoreductase which converts NA to AVN in the presence of NADPH and a supernatant fraction (105,000 × *g*) obtained from *Escherichia coli* DH5α. The data also suggest that an unidentified cofactor(s) may be necessary for the native Nor-1 protein function and that the *nor*-1 gene is involved in only one of multiple proposed pathways of NA conversion to AVF.

MATERIALS AND METHODS

Bacterial Strain, Fungal strains, and Culture Conditions. Plasmids were amplified in *Escherichia coli* DH5α F ^{e}[F*endA1 hsdR17*(r_K^- m_K+) *upE44 thi-1 recA1 gyrA* (Nal^r) *relA1Δ*(*lacZYA-argF*)$_{U169}$ (*m80lacZ*ΔM15)] using standard methods (Ausubel *et al.*, 1987). *Aspergillus parasiticus* NRRL 5862 (ATCC 56775, SU-1) (American Type Culture Collections) was used as the aflatoxin-producing wild-type strain. *Aspergillus parasiticus* ATCC 56774, which accumulates averantin, was used as a source of averantin. A *nor*-1 disrupted strain of *A. parasiticus* (Trail *et al.*, 1994) (designated ΔNor-1) was used for NA preparation. An aflatoxin-inducing liquid growth medium consisting of 2% (w/v) yeast extract and 6% (w/v) sucrose (pH 5.8) (YES broth) was used to grow mycelia at 29°C in the dark with constant shaking (150 rpm) for protein preparation and aflatoxin extraction. YES solid medium [YES broth plus 1.5% (w/v) Bacto-agar] was used for spore preparation. Fungal strains were maintained as frozen spore stock suspensions at -80°C. Spore stock suspensions were prepared by suspending spores in 20% (v/v) glycerol.

Nucleotide Sequence Analysis. A *nor*-1 cDNA (with an *Eco*RI adapter at the 5'-end and an *Xho*I adapter at the 3'-end) was cloned into the *Eco*RI/*Xho*I sites of pBluescriptSKII$^-$ (Stratagene Cloning Systems, La Jolla, CA) resulting in pNOR which was kindly provided by Dr. Perng-Kuang Chang (USDA. ARS. SRRC). Nucleotide sequence analysis of the *nor*-1 cDNA was performed by the dideoxy chain termination method with Sequenase II (Biochemical Corp., Cleveland, Ohio) as described in the manufacturer's instructions. The cloned insert was sequenced on both strands with the T7 and T3 primers

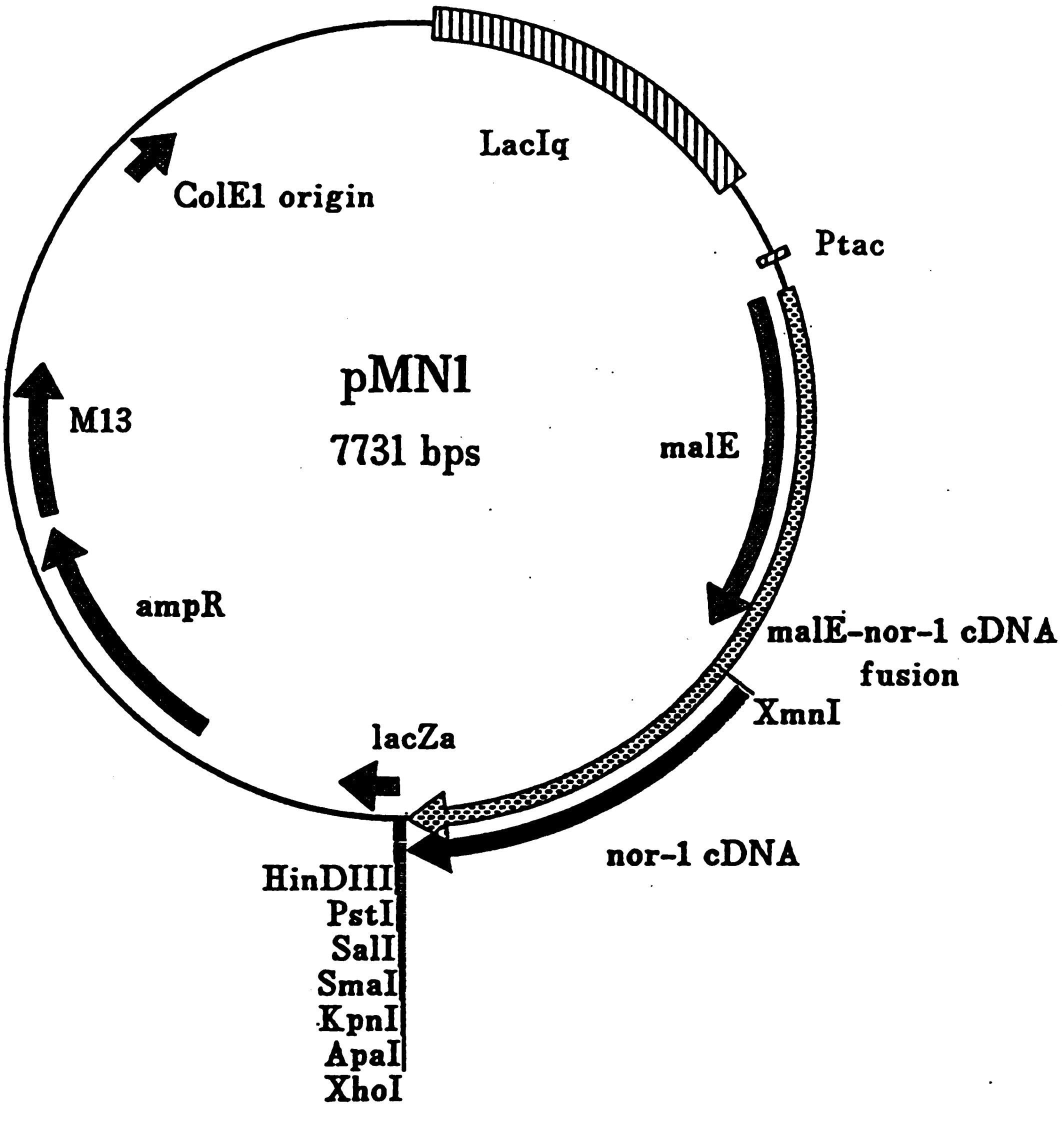


Figure 2-2. Construction of expression vector pMN1. An *Eco*R1/*Sal*I fragment (1040 bp) containing a *nor*-1 cDNA was cloned into expression vector pMAL-c2. The *nor*-1 cDNA in the resulting expression vector pMN1 was fused with the *mal*E gene encoding maltose biding protein (MBP) in the same reading frame to form a *mal*E/*nor*-1-cDNA fusion which was expressed as a Nor-1c/MBP fusion protein.

supplied in the kit. In order to resolve the ambiguities in the initial sequence data, one internal 17-nucleotide primer (5'GTATTTGGTCACCGGGG3') was also used for sequencing.

Construction of pMN1, a pMAL-c2-*nor*-1-cDNA Expression Vector. The *nor*-1 cDNA fragment was removed from pNOR using the restriction enzymes *Bam*HI and *Kpn*I and cloned into the *Bam*HI/*Kpn*I sites of the expression vector pQE31 (QIAexpress, QIAGEN Inc. Chatsworth, CA). The *nor*-1 cDNA was removed again from pQE31 with the restriction enzymes *Eco*RI and *Sal*I and cloned into the *Eco*RI/*Sal*I sites of the expression vector pMAL-c2 (New England Biolabs Inc., Beverly, MA). The resulting vector was cut with *Eco*RI, and the protruding ends were filled with dNTPs by the Klenow fragment of *E. coli* DNA polymerase I to create blunt ends. Blunt-end ligation created a new expression vector called pMN1 (Figure 2-2) in which the *nor*-1 cDNA was fused in frame with the 3'-end of the *mal*E gene (encoding the maltose-binding protein) in pMAL-c2.

Preparation of the Nor-1c/MBP Fusion Protein and the Nor-1c Protein. The expression vector pMN1 was transformed into *E. coli* DH5α using standard methods (Ausubel *et al.*, 1987). The Nor-1c/MBP fusion protein and the Nor-1c protein were prepared following the instructions of the Protein Fusion & Purification System (New England Biolabs Inc. Beverly, MA). The resulting proteins were concentrated to approximately 1 mg/ml in an Amicon Centriprep concentrator (Amicon Inc., Beverly, MA). Cleavage of the fusion protein with protease factor Xa and separation of the Nor-1c protein from MBP by affinity chromatography were carried out according to the manufacturer's instructions (New England Biolabs Inc., Beverly, MA).

Fungal Culture and Crude Protein Extract Preparation. For culture of the fungus in liquid media, a stock spore suspension was inoculated into liquid media with a final spore concentration of 1.5×10^5 spores/ml, and grown at 29°C in the dark with constant shaking (150 rpm). Mycelia were collected by filtration, and were either used immediately, or frozen under liquid nitrogen and stored at -80°C until use. The dry weight of mycelia growing in liquid media was determined after drying the mycelium in a preweighed flask overnight at 80°C. To generate a crude cell extract, mycelia were ground under liquid nitrogen with a mortar and pestle to produce a fine powder. The frozen mycelial powder was quickly transferred to a 50-ml centrifuge tube containing precooled extraction buffer (PEB; a volume approximately 5 times the mass of cell material) consisting of 0.01 M Tris·HCl (pH 8.0), 0.14 M NaCl, and 0.025% (w/v) NaN_3. Just before mixing with mycelia, the following chemicals were added to PEB: 20 mM phenylmethylsulfonyl fluoride (PMSF), 0.4 TIU (trypsin inhibition unit)/ml aprotinin, 10 mM iodoacetamide, 4 mM 2-mercaptoethanol, 10 mM EDTA, and 5% (v/v) glycerol. Cell debris was removed by centrifugation two times at 1,000 × *g* for 10 min. The resulting supernatant fraction was further centrifuged at 10,000 × *g* for 1 h to generate a crude extract (10,000 × *g* supernatant). For a protein concentration assay, sodium dodecyl sulfate (SDS) was added to the crude extract [to a 2% (w/v) final concentration] and was boiled at 100°C for 5 min. The protein concentration was then determined with the Bio-Rad Protein Assay reagent (Bio-Rad Lab., Hercules, CA) according to the manufacturer's instructions.

Immunoaffinity Purification of the Native Nor-1 Protein. Affinity column preparation using CNBr-activated Sepharose 4B (Sigma, St. Louis, MO) and polyclonal antibody (PAb) raised against the Nor-1c/MBP fusion protein (Chapter 3. Preparation and Purification of Polyclonal Antibodies) was carried out according to published standard methods (Ausubel *et al.*, 1995c). The basic procedure for affinity purification of the Nor-1 protein was established by Dr. R. Mehigh in our laboratory. Briefly, an affinity column (1.2 x 5.5 cm) containing 4.5 ml Sepharose-4B resin conjugated with 1 mg PAb was equilibrated with high salt phosphate-buffered saline (HS-PBS) consisting of 390 mM NaCl, 10 mM Na_2HPO_4, and 10 mM NaH_2PO_4 (pH 7.2). Twenty mg of crude extract (5 mg/ml) from *A. parasiticus* SU-1 or ΔNor-1 (*nor*-1 disrupted strain) cultured in YES liquid medium for 60 h was loaded onto the column. The column was washed with HS-PBS until the OD_{280nm} returned to the baseline. The native Nor-1 protein was eluted with 0.1 M glycine·HCl (pH 2.5) and collected with a fraction collector. Each fraction (2 ml) was neutralized immediately with 0.3 ml 1 M Tris·HCl (pH 8.0). Fractions were pooled and dialyzed against three changes of low salt phosphate-buffered saline (LS-PBS) consisting of 130 mM NaCl, 10 mM Na_2HPO_4, and 10 mM NaH_2PO_4 (pH 7.2) at 4°C over a 36 h period.

Western Blot Analysis. Proteins were resolved by SDS-PAGE (polyacrylamide gel electrophoresis) and blotted to a polyvinylidene difluoride (PVDF) membrane (Du Pont Co., Boston, MA) using published standard methods (Ausubel *et al.*, 1995b). Immunodetection was carried out with a chemiluminescent detection kit (Schleicher & Schuell, Keene, NH) according to the manufacturer's instructions.

Norsolorinic Acid (NA) Preparation. Conidia (about 1x10^8) from a *nor*-1 disrupted strain, *A. parasiticus* ΔNor-1, were inoculated into 2 L of YES liquid medium and cultured for 2 days at 29°C with constant shaking (150 rpm) in the dark. The culture was then incubated under stationary conditions at 30°C in the dark for an additional 13-day period of time. The mycelia (red color) were collected by filtration and a portion (250 g wet weight) was extracted with 300 ml acetone three times. The red purple extract solution was dried with a Rotovapor R110 (Brinkmann Instruments Inc. Westbury, NY) at 65°C. The resulting solid materials were extracted with 100 ml chloroform. The chloroform extract was concentrated with a Rotovapor R110 at 72°C (5-6 ml final volume left) and extracted again with 100 ml acetone. The NA in the acetone-extract (purple-red) was further purified by running preparative thin layer chromatography (TLC) (PKF SILICA GEL 60 Å, 20 x 20 cm, Whatman Inc. Clifton, NJ) with chloroform-acetone (9:1) as the developing system. The NA spot (brown color) on the TLC plate was scraped off with a razor blade and the NA was extracted with chloroform.

Averantin (AVN) Preparation. Averantin was prepared following the method developed by Bennett *et al.* (1980) with modifications. A freeze-dried hyphal culture of *A. parasiticus* ATCC 56774, which accumulates averantin, was reconstituted according to ATCC's instructions. Conidia (about 1x10^8) of strain ATCC 56774 were inoculated into 2 L of YES liquid medium, and cultured with constant shaking (150 rpm) for 2 days at 29°C in the dark followed by incubation without shaking for an additional 13-day period of time at 29°C in the dark. Approximately 210 g (wet weight) of orange mycelia were collected by filtration and extracted with acetone until colorless. Water was added to make a 30% (v/v) acetone solution. The acetone solution was washed with hexane (1:1 ratio), and the orange pigments were then partitioned from the acetone into chloroform (1:1 ratio). The chloroform extract was concentrated to 5 ml at room temperature, and then further purified by preparative TLC (HPK SILICA GEL 60 Å, 20 x 20 cm, Whatmam Inc. Clifton, NJ) with chloroform-acetone (9:1) as the developing system. The AVN spot (yellow-brown color) on the TLC plate was scraped off with a razor blade and the AVN was extracted with chloroform.

Enzyme Activity Assay of the Nor-1c/MBP Fusion Protein and the Nor-1c Protein. The enzymatic assay was conducted according to a published method (Yabe *et al.*, 1991b) with modifications. The reaction mixture included an appropriate quantity of protein (70 to 105 µg), 90 µM NA, 0.23 mM NADPH, 90 mM KH_2PO_4 (pH 7.5), and 10% (v/v) glycerol in a total volume of 100 µl. Reactions were conducted at 37°C in the dark for appropriate periods of time (30 to 90 min), and then stopped by adding 900 µl ethyl acetate. The ethyl acetate extract was air dried and the residue was extracted by adding 400 µl chloroform. The reaction products in chloroform extract were resolved by TLC (HPK SILICA GEL 60 Å, 10 x 10 cm, Whatmam Inc. Clifton, NJ) with benzene-ethyl acetate (7:3) as the developing system.

UV/VIS, Mass, and NMR Spectroscopy of NA and AVN. UV/VIS spectroscopy was conducted using a UV/Visible light spectrophotometer (CARY 3E, Varian, Australia Pty. Ltd., Australia). Mass spectrometry was carried out on a double-focusing mass spectrometer (JEOL AX-505H, Varian, Australia Pty. Ltd., Australia) with the help of Dr. B. Chamberlin (Department of Biochemistry, Michigan State University, East Lansing, MI) under the following conditions: electron energy, 70 eV; scan range, *m/z* 45-600. The ^{1}H proton nuclear magnetic resonance (NMR) spectroscopy was performed with a NMR spectrometer (VX12-500, Varian, Australia Pty. Ltd., Australia) with the help of Dr. K. Johnson (Department of

Chemistry, Michigan State University, East Lansing, MI) under the following conditions: proton probe at 80 mHz; range, 0 to 12 ppm; solvent, deuterated dimethyl sulfoxide (d6DMSO).

Aflatoxin Analysis by TLC and ELISA. Aflatoxins were extracted with 10 ml of chloroform from mycelial liquid culture medium (not including mycelium) (1 ml) or from mycelial solid culture medium (including mycelium) (1 mg). Chloroform extracts were tested either semiquantitatively by TLC (for total aflatoxins) with chloroform-acetone (95:5) as the developing system, or quantitatively by enzyme-linked immunosorbent assay (ELISA) (for AFB_1). The direct competitive ELISA was carried out according to the method of Pestka *et al.* (1980) using anti-AFB_1 antibodies kindly provided by Dr. J. Pestka (Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI).

RESULTS

Sequence Analysis of the *nor*-1 cDNA. Nucleotide sequence analysis (Figure 2-3) showed that the *nor*-1 cDNA was missing 6 nucleotides (ATGAAC) from the protein-coding region at 5'-end of the proposed transcript beginning with the translation initiation codon (ATG) based on a comparison with the *nor*-1 genomic DNA sequence (Trail *et al.*, 1994). It was thought that this short deletion might not have a significant effect on the Nor-1c protein activity. One of the three reading frames of the *nor*-1 cDNA encoded a complete open reading frame and had three in-frame stop codons at the 3'-end which in theory could generate proteins with predicted molecular mass of 31 kDa, 34 kDa, or 38 kDa if read through of the primary translation termination codon occurred.

Factor Xa cutting site ↓ (*nor*-1 cDNA 5'... ATG AAC) ↓

DNA 5': *mal* E....G GAG GGA AGG ATT T*CA GAA TTA ATT CGG CAC GAG* GGA TCA CTT AGC CAG CAC GAT CAA
N-AA 001 E G R I S E L I R H E G S L S Q H D Q

DNA 5': GAG AGG CTC TCT ACG CCA TAC CGG GAT GGA CCG CCT GAG GAG ACG GTG TAT TTG GTC ACC
N-AA: 021 E R L S T P Y R D G P P E F T V Y L V T

N-AA: 041— ———————————————————————— 280

DNA5': TGG TAG GAC GCT AGT GAC GAC GAA GCG CAG TCA TAT GTT TCT GAG ACG AGC AGA AAC GTG
N-AA: 281 W #1 D A S D D E A Q S Y V S E T S R N V

DNA5': CTG GCG TGG AGC TGG TCC AGA AAG GCG CGG TGA GGT CCT GGG TTC GTG TCG CGG CGG CTT
N-AA: 301 L A W S W S R K A R #2 G P G F V S R R L

DNA5': TCG CTC GAT CAG TTC GTG TAT GAC CTT TTC GGT CTT TTC CGT TCC GTC TGT TTA GGC TCT
N-AA: 321 S L D Q F V Y D L F G L F R S V C L G S

DNA5': CAC AAG ATA AAA CCA AAT TGA AAC CTA ACG TTC GTT TTC AAA AAA AAA AAA AAA AAA AAC
341 H K I K P N #3 N L T F V F K K K K K K T

DNA5': *TCG AGG GGG GGC CCG GTA CCC CGG GTC GAC CTG CAG GCA AGC TTG ... lacZa*
N-AA: 361 S R G G P V P R V D L Q A S L

Figure 2-3. Nucleotide and predicted amino acid sequence of the 5' and 3'-ends of *nor*-1 cDNA in expression vector pMN1. The underlined nucleotides represent the carboxyl terminus of the maltose binding protein encoded by *mal*E (in the expression vector pMAL-c2) which is fused in-frame with the *nor*-1 cDNA. The nucleotides in italics were introduced during vector construction. The nucleotides in bold represent the *nor*-1 cDNA. The nucleotides in parentheses (ATGAAC) were missing from the protein-coding region at 5'-end of the proposed transcript beginning with the translation initiation codon (ATG) based on a comparison with the *nor*-1 genomic DNA sequence. The factor Xa cutting site is indicated by an arrow. The two Nor-1c proteins (starting from the factor Xa cutting site, 32 and 36 kDa) are predicted to be derived from translation termination at the first two stop codons (marked #1 at residue 282, and #2 at residue 311), respectively. The two Nor-1c proteins are therefore predicted to lack two amino acids (M, and T) which are replaced by 8 amino acid (I, S, E, L, I, R, H, and E) at the amino terminus. N-AA indicates the N-terminus of the amino acid sequence.

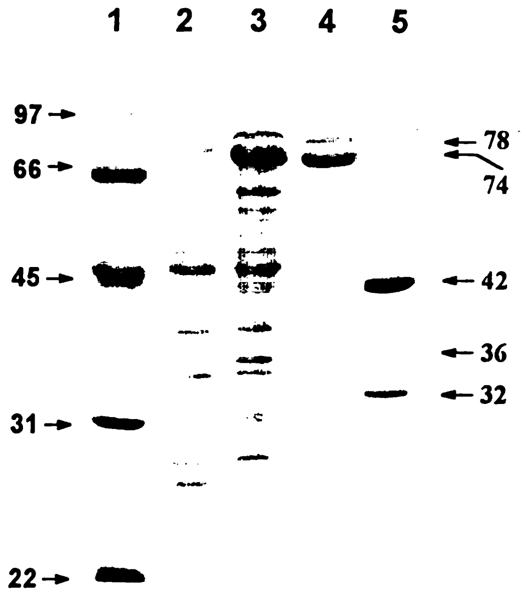


Figure 2-4. SDS-PAGE detection of the Nor-1c/MBP fusion protein and the Nor-1c protein. Expression vector pMN1 containing the *nor*-1 cDNA fused with *mal*E encoding maltose binding protein (MBP) was transformed into *Escherichia coli* DH5α. Under isophenylthiogalactoside (IPTG) induction, two Nor-1c/MBP fusion proteins were produced in the bacterial host. Lanes: **1**, molecular mass standard (kDa); **2**, total crude extract (10,000 × *g* supernatant) from *E. coli* DH5α; **3**, total crude extract from *E. coli* DH5α transformed with expression vector pMN1; **4**, two Nor-1c/MBP fusion proteins (74 kDa, and 78 kDa) purified by affinity chromatography; and **5**, two Nor-1c proteins (32 kDa, and 36 kDa) and MBP (42 kDa) obtained by factor Xa cutting of the two Nor-1c/MBP fusion proteins.

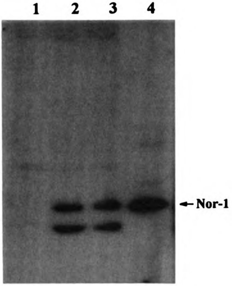


Figure 2-5. Western blot analysis of the native Nor-1 protein in *A. parasiticus* SU-1. Each lane contained 10 μg protein. The primary antibody was the IgG fraction of the antiserum raised against the Nor-1c/MBP fusion protein (Chapter 3) (10 μg/ml). Lanes: **1**, total crude extract (10,000 × *g* supernatant) from the *nor*-1-disrupted strain *A. parasiticus* ΔNor-1 cultured in YES liquid medium for 60 h; **2** and **3**, total crude extract from the wild-type *A. parasiticus* SU-1 cultured in YES liquid medium for 48 h and 60 h, respectively; **4**, the native Nor-1 protein (31 kDa) purified with anti-Nor-1c/MBP fusion protein PAb affinity column from the total crude extract of *A. parasiticus* SU-1 cultured in YES medium for 60 h.

Preparation of the Nor-1c/MBP Fusion Protein and the Nor-1c Protein. The expression vector pMN1 was transformed into *E. coli* DH5α. After 2 h induction with isophenylthiogalactoside (IPTG), two Nor-1c/MBP fusion proteins (approximately 74, and 78 kDa) were produced in the host cell and were detected by SDS-PAGE (Figure 2-4). The fusion proteins were purified by maltose resin affinity chromatography and cut with protease factor Xa resulting in the appearance of the maltose binding protein (42 kDa) and two Nor-1c proteins (approximately 32 kDa, and 36 kDa) (Figure 2-4).

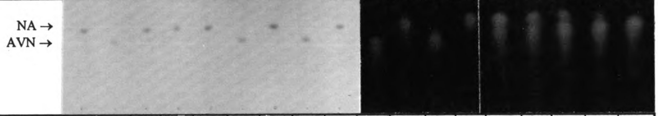
Immunoaffinity Purification of the Native Nor-1 Protein. Twenty mg of crude extract obtained from the aflatoxin-producing strain *A. parasiticus* SU-1, or from the *nor*-1 disrupted strain *A. parasiticus* ΔNor-1, was used to purify the native Nor-1 protein by immunoaffinity chromatography. The concentrated Nor-1 protein, resolved by SDS-PAGE, was detected by Western blot analysis. Only one major protein band corresponding to the size predicted for the Nor-1 protein was detected by the IgG fraction of the anti-Nor-1c/MBP fusion protein serum (Chapter 3) in the wild-type *A. parasiticus* SU-1 (Figure 2-5, lane 4). This protein band was not detected using the same antibodies in the *nor*-1 disrupted strain *A. parasiticus* ΔNor-1 (Figure 2-5, lane 1). Using the same antibodies, in addition to the expected Nor-1 protein, several other proteins were detected in the crude extract of *A. parasiticus* SU-1 (Figure 2-5, lanes 2, 3) suggesting that cross-reactivity occurred.

Enzyme Activity of the Nor-1c/MBP Fusion Protein and the Nor-1c Protein. The immunoaffinity purified Nor-1c/MBP fusion protein, the Nor-1c/MBP fusion protein in the crude extract (10,000 × *g* supernatant) of *E. coli* cells containing expression vector pMN1, and the immunoaffinity purified Nor-1c protein were assayed for their ability to convert NA to

Figure 2-6. Enzyme activity assay of the Nor-1c/MBP fusion protein and the Nor-1c protein. All reactions were conducted at 37°C in the dark. The reaction products were resolved by TLC with benzene-ethyl acetate (7:3) as the developing system. The photo of reactions 1 through 9 was taken under white light. The photo of reactions 10 through 18 was taken under UV light. **Reactions:** 1, NA standard; 2, AVN standard; 3, NADPH plus NA control. *, crude extract (10,000 × *g* supernatant) of *E. coli* DH5α; ♥, pellet (105,000 × *g*) of crude extract from *E. coli* DH5α; ♠, supernatant (105,000 × *g*) of crude extract from *E. coli* DH5α; ♦, 35 μg anti-Nor-1c/MBP fusion protein PAb; •, Nor-1c/MBP fusion protein was stored for 4 months at -80°C; + or −, containing or not containing the component in the same row in the "Reaction components" column.

Figure 2-6.

Reaction number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Reaction time (min)	30	30	30	30	30	30	30	30	30	30	50	30	90	10	20	30	40	50



Reaction components																		
0.9 mM NA	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.9 mM AVN	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
NADPH	–	–	+	+	+	+	–	+	–	+	+	+	+	+	+	+	+	+
70μg *E.coli* [•]	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
70μg *E.coli* containing pMAL-c2	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
70μg *E.coli* containing pMN1	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
70μg *E.coli* containing pMN1	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
35μg Nor-1c, 35μg *E.coli*	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
35μg Nor-1c, 35μg *E.coli*	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
35μg Nor-1c /MBP, 35μg *E.coli*	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
35μg Nor-1c /MBP, 35μg *E.coli* P[♥]	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
35μg Nor-1c /MBP, 35 μg *E.coli* S.[♠]	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
35 μgNor-1c /MBP, 35μg *E.coli* S., Ab[♦]	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
35μg Nor-1c /MBP O.[•], 35μg *E.coli*	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
35μg Nor-1c /MBP O., 35 μg *E.coli*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
35μg Nor-1c /MBP O., 35μg *E.coli*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
35μg Nor-1c /MBP O., 35μg *E.coli*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
35μg Nor-1c /MBP O., 35μg *E.coli*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+

AVN (Figure 2-6). In the presence of NADPH, only the crude extract from *E. coli* DH5α containing the Nor-1c/MBP fusion protein (reaction 6), the mixture of the affinity purified Nor-1c protein and the *E. coli* cell crude extract (reaction 8), and the mixture of the affinity purified Nor-1c/MBP fusion protein and the *E. coli* cell crude extract (reaction 10) completely converted NA (90 μM) to a compound that co-migrated with AVN upon TLC analysis. Neither the affinity purified Nor-1c protein (not shown) nor the control *E. coli* extract (reaction 4) converted NA to AVN in the presence of NADPH. These data suggested that *E. coli* provided some essential cofactor(s) required for NA reductase activity. The cofactor(s) was found in the 105,000 × *g* supernatant (reaction 12) and not in the 105,000 × *g* pellet of the *E. coli* extract (reaction 11). NADPH, *E. coli* cell extract, and *E. coli* cell extract containing MBP were unable to convert NA to AVN (reactions 3, 4, and 5). Anti-Nor-1c/MBP fusion protein PAb inhibited the Nor-1c/MBP activity (reaction 13) suggesting that the enzymatic activity originated from the Nor-1c protein. The freshly prepared Nor-1c/MBP fusion protein finished the NA-AVN conversion in 30 min and the stored (at -80°C for 4 months) Nor-1c/MBP fusion protein finished the same conversion in 50 min (reactions 14 to 18) suggesting that storage reduced the Nor-1c protein activity.

UV/VIS, Mass, and NMR Spectra of NA. To confirm the identity of the compound used as the substrate in the enzyme activity assay, the compound was purified in two consecutive TLC steps. The purified compound, which had the same Rf value (0.45) with benzene-ethyl acetate (7:3) as the developing system and the same color (brown under white light) as the standard NA, was scraped from the TLC plate and then analyzed by UV/VIS (UV

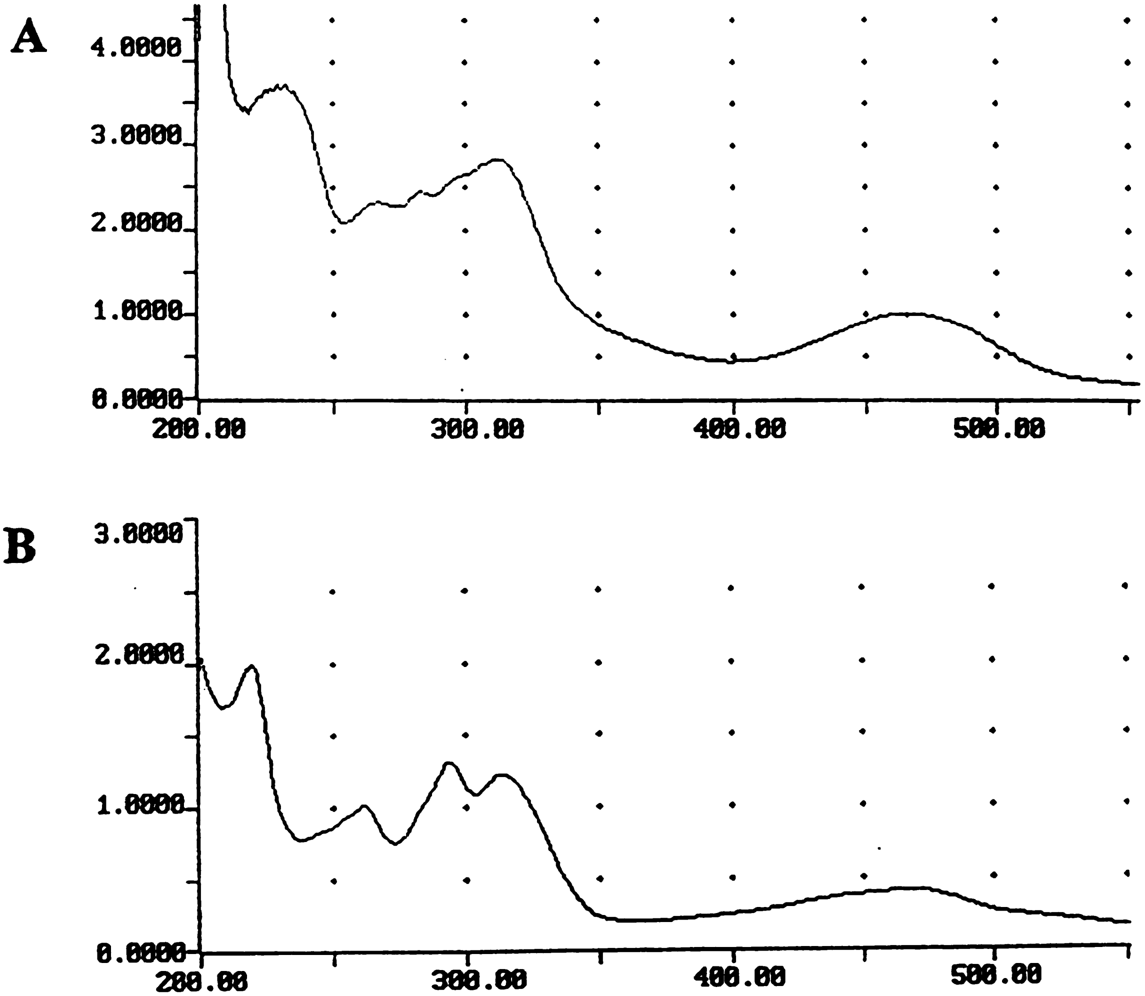


Figure 2-7. UV/VIS spectroscopy of norsolorinic acid (NA) and averantin (AVN). **A:** UV/VIS spectrum (200-600 nm) of NA (in ethanol). λ_{max} (EtOH)(ε): 235nm(24,500), 269nm(16,900), 284nm(18,600), 297nm(19,900), 314nm(22,900), and 465nm(7,760). **B:** UV/VIS spectrum (200-600 nm) of AVN (in ethanol). λ_{max}(EtOH)(ε): 222nm(26,784), 262nm(15,810), 298nm(20,386), 315nm(22,022), 453 nm(6,658).

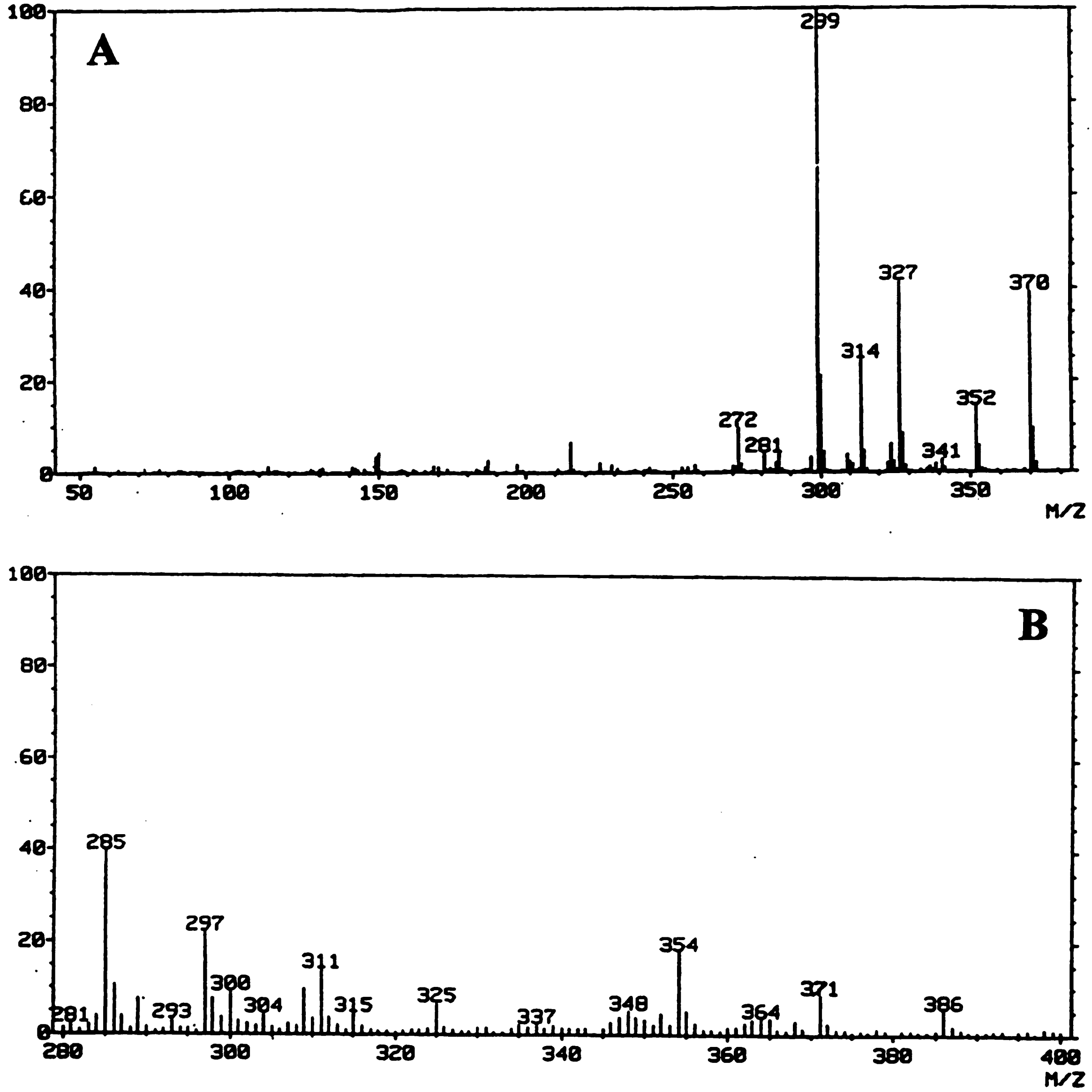
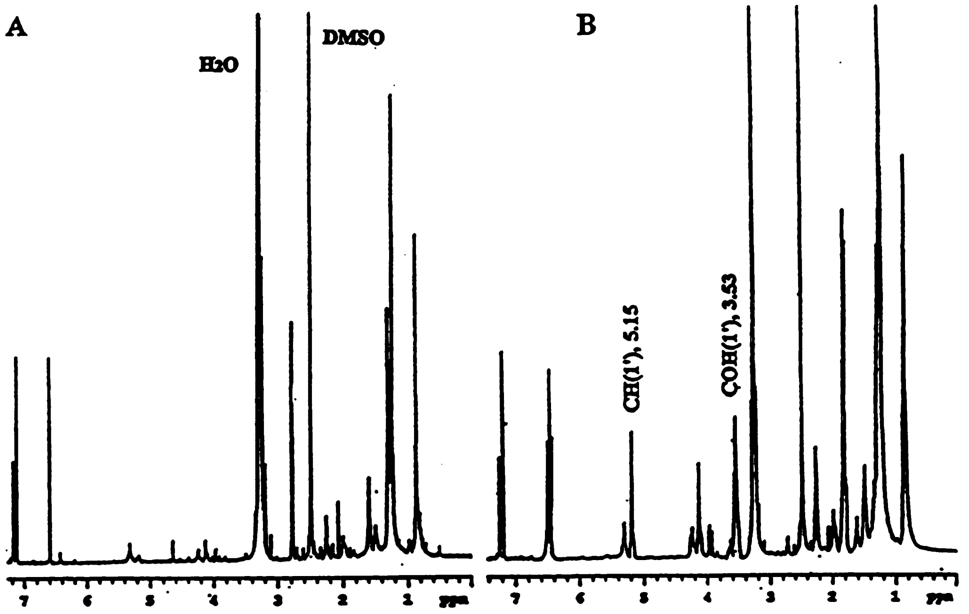
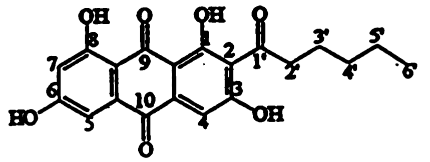
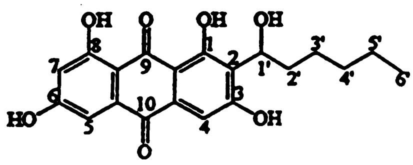


Figure 2-8. Mass spectroscopy of norsolorinic acid (NA) and averantin (AVN). A: The mass spectrum of the purified NA [relative intensity (%): molecular ion at *m/z* 370; fragments at *m/z* 327, 314, 299, 272]. B: The mass spectrum of the enzymatic end product AVN [relative intensity (%): molecular ion at *m/z* 371; fragments at *m/z* 354, 325, 311, 297, and 285].





Norsolorinic Acid (NA)



Averantin (AVN)

Figure 2-9. NMR spectroscopy of norsolorinic acid (NA) and averantin (AVN). **A:** NMR spectrum of NA (in d6DMSO) (δppm): CH_3(6'), 0.88(3H); CH_2(4'5'), 1.30(2H); CH_2(3'), 1.60(2H); CH_2(2'), 2.81(2H); CH(7), 6.61(1H); CH(5), 7.13(1H); CH(4), 7.22(1H). **B:** NMR spectrum of the end product AVN (in d6DMSO) (δppm): CH_3(6'), 0.85(3H); CH_2(3',4',5'), 1.30(6H); CH_2(2'), 1.84(2H); COH(1'), 3.53(1H); CH(1'), 5.15(1H); H(7), 6.53(1H); H(5), 7.19(1H); H(4), 7.22(1H).

and visible light), mass, and NMR spectroscopy. The six maximum absorbence values of the UV/VIS spectrum (200-600 nm) of the purified compound in ethanol (Figure 2-7A) were consistent with the data for NA reported by Cole and Cox (1981) [λ_{max} (EtOH)(ε): 235nm(24,500), 269nm(16,900), 284nm(18,600), 297nm(19,900), 314nm(22,900), and 465nm(7,760)]. The mass spectrum (Figure 2-8A) and the NMR spectrum (Figure 2-9A) of the purified compound were also consistent with the published data for NA (Cole and Cox, 1981) [mass spectrum (relative intensity (%): molecular ion at *m/z* 370; fragments at *m/z* 327, 314, 299, 272; NMR spectrum (in d6DMSO, δppm): CH3(6'), 0.88(3H); CH2(4',5'), 1.30(2H); CH2(3'), 1.60(2H); CH2(2'), 2.81(2H); CH(7), 6.61(1H); CH(5), 7.13(1H); CH(4), 7.22(1H)].

UV/VIS, Mass, and NMR spectra of AVN. To identify the end product in the conversion of NA by the Nor-1c/MBP fusion protein, the enzymatic reaction was scaled up 10 fold. The enzymatic end product, which had the same Rf value (0.37) with benzene-ethyl acetate (7:3) as the developing system and the same color (yellow under white light) as the standard AVN on TLC, were scraped from the TLC plate and then analyzed by UV/VIS (UV and visible light), mass, and NMR spectroscopy. The five maximum absorbence values of the UV/VIS spectrum (200-600 nm) of the end product in ethanol (Figure 2-7B) were consistent with AVN data reported by Bennett *et al.* (1980) [λ_{max}(EtOH) (ε): 222nm(26,784), 262nm(15,810), 298nm(20,386), 315nm(22,022) 453 nm(6,658)]. The mass spectrum (Figure 2-8B) of the end product was consistent with AVN data published by Bennett *et al.* (1980) [relative intensity (%): molecular ion at *m/z* 372; fragments at *m/z* 354, 325, 311, 309, 297, and 285] with one exception (molecular ion at *m/z* 371, not at *m/z* 372 as expected). The NMR spectrum of the end product in d6DMSO (Figure 2-9B) was consistent with the data for AVN published by Bennett *et al.* (1980) [(δppm): CH3(6'), 0.85(3H); CH2(3',4',5'), 1.30(2H); CH2(2'), 1.74(2H); COH(1'), 3.53(1H); CH(1'), 5.15(1H); CH(7), 6.53(1H); CH(5), 7.02 (1H); CH(4), 7.02(1H)]. Two "new peaks" appeared in the NMR spectrum of AVN. One was COH(1'), 3.53(δppm); another CH(1'), 5.15(δppm). These two new peaks resulted from keto-reduction by the addition of two more hydrogen atoms. The standard AVN purified from the fungal strain *A. parasiticus* ATCC 56774 was shown to have the same UV/VIS, mass, and NMR spectra as AVN generated in the NA reductase reaction.

AFB$_1$ Accumulation in *A. parasiticus* SU-1 and *A. parasiticus* ΔNor-1. Direct competitive ELISA analysis was utilized to quantify AFB$_1$ accumulation in the wild-type *A. parasiticus* SU-1 and the *nor*-1 disrupted transformant, *A. parasiticus* ΔNor-1, cultured in YES liquid medium. After 2, and 5 days of culture, approximately 25 fold more AFB$_1$ was produced by the wild-type *A. parasiticus* SU-1 (4.55 ± 0.02 mg, and 6.8 ± 0.03 mg of AFB$_1$ per gram of dry mycelial mass, respectively) when compared with the *nor*-1 disrupted transformant *A. parasiticus* ΔNor-1 (0.18 ± 0.02 mg, and 0.27 ± 0.03 mg, respectively).

DISCUSSION

The data presented in this study demonstrated that the Nor-1c protein (or the Nor-1c/MBP fusion protein) is able to convert the aflatoxin B$_1$ pathway intermediate NA to AVN but only in the presence of NADPH and a cofactor(s) obtained from *E. coli* DH5α. This suggests that the native Nor-1 protein is an NADPH dependent NA ketoreductase requiring an unidentified cofactor(s) and that *nor*-1 encodes an activity involved in one of multiple proposed pathways for the conversion of NA to AVF in the AFB$_1$ pathway in *A. parasiticus* SU-1.

The Nor-1c protein has a ketoreductase activity capable of converting NA to AVN in vitro. The UV/VIS, mass, and NMR spectrum data confirmed that the substrate was NA and the end product was AVN. The conversion of the 1'-keto group of NA to the 1'-hydroxyl group of AVN and the requirement of NADPH in the enzymatic conversion confirmed that the activity involved in this conversion is a NADPH dependent NA ketoreductase activity. The conclusion that only the Nor-1c protein has this ketoreductase activity in the enzyme activity assay was deduced from the following three observations: (1) the conversion of NA to AVN was inhibited by the addition of the anti-Nor-1c/MBP fusion protein PAb when the Nor-1c protein was involved in the enzyme activity assay; (2) NADPH, the *E. coli* protein plus NADPH, and the MBP plus the *E. coli* protein and NADPH were unable to convert NA to AVN; and (3) analysis of the proposed amino acid sequence of *nor*-1 provided evidence that the Nor-1c protein was a ketoreductase (Trail *et al.*, 1994).

Bhatnagar and Cleveland (1990) proved that the conversion of NA to AVN is reversible in the presence of a 38-kDa reductase plus NADP. This may also be true for the Nor-1c protein, but the reverse reaction was not conducted in the current study.

The Nor-1c protein showed NA ketoreductase activity only in the presence of a cofactor(s) from *E. coli* DH5α suggesting that a cofactor(s) may be also necessary for the native Nor-1 protein to show the NA ketoreductase activity. A 38-kDa reductase (Bhatnagar and Cleveland, 1990), a 43-kDa isozyme of the reductase (Bhatnagar, unpublished observation), and a 140-kDa reductase (also called dehydrogenase) (Chuturgoon and Dutton, 1991) have been purified to homogeneity, and are reported to convert NA to AVN. Surprisingly the purification of the 31-kDa native Nor-1 protein encoded by *nor*-1 has not been reported. This observation may be explainable with the data presented in this study (see below).

Western blot analysis using PAb raised against the Nor-1c/MBP fusion protein showed that: the accumulation of a 31-kDa protein had a pattern consistent with AFB_1 accumulation in *A. parasiticus* SU-1 (Chapter 3); the 31-kDa protein purified from a crude extract of *A. parasiticus* SU-1 by affinity chromatography was detected as a single band; and the 31-kDa protein was not detected in a crude extract from the *nor*-1 disrupted strain ΔNor-1. These observations suggest that the 31-kDa protein is the native Nor-1 protein encoded by the *nor*-1 gene. But, the nor-1c protein alone does not show the reductase activity. Therefore, it is possible that a cofactor(s) is needed for the activity of the native Nor-1 protein. The cofactor(s) may have been lost during purification step in previous studies resulting in inability to purify Nor-1. Such a cofactor(s) was possibly provided by *E. coli* DH5α in this study.

Alternatively, because polyketide synthase (PKS) and fatty acid synthetase (FAS) enzymes often function in multisubunit complexes, it is possible that the native Nor-1 protein is a part of a multiple polypeptide complex required for AFB_1 biosynthesis. Therefore, only when it is included and held in a functional configuration directly associated with the complex, does the native Nor-1 protein have the reductase activity. It is hypothesized that certain low mass element(s) in an *E. coli* supernatant (105,000 × *g*) substitute for a required cofactor or eliminate the requirement for protein/protein interaction to help the Nor-1 protein to show reductase activity. Further research is warranted.

The *nor*-1 gene is confirmed to be directly involved in AFB1 biosynthesis in *A. parasiticus*, an observation which was reported previously (Skory *et al.*, 1993; Trail *et al.*, 1994). ELISA data in this study showed that the *nor*-1 disrupted strain ΔNor-1 reduced the accumulation of AFB1 by approximately 25 fold. The Nor-1c protein encoded by the *nor*-1 cDNA was able to convert NA to AVN, and both NA and AVN have been proven to be the intermediates in AFB1 pathway (Bennett *et al.*, 1980; Yabe *et al.*, 1993).

The mass and NMR spectroscopic data strongly suggest that NA (370 Da) is converted to AVN (372 Da) (the 1'-keto group is reduced to hydroxyl group), even though the theoretical molecular mass of AVN is 372 Da, but the observed mass spectrum of AVN had a base peak at *m/z* 371, not at *m/z* 372 as expected. Often ions $(M-1)^+$ and sometimes $(M-2)^+$, $(M-3)^+$ are observed in the process of mass spectroscopy due to losses of hydrogen atom(s) from the molecular ion M^+. Sometimes the ion $(M-1)^+$ is much more abundant than the molecular ion M^+ (Rose and Johnstone, 1982) as occurred here with AVN. The NMR spectrum data of NA was also consistent with the literature (Cole and Cox, 1981), with one exception. Norsolorinic acid in this research was prepared without a crystallization step. Therefore, the NMR spectrum of NA in this study not only had a major peak at 2.5 δppm which originated from DMSO, but also had a peak at 3.4 δppm which originated from water. The NMR data of AVN were more complicated. The enzymatic end product, AVN, and the AVN prepared from *A. parasiticus* ATCC 56774 in this study had the same NMR spectrum. This NMR spectrum was consistent with the data reported by Bennett *et al.* (1980), but was less consistent with the data reported by Birkinshaw *et al.* (1966) [(δppm): $CH_3(6')$, 1.00(3H); $CH_2(3',4',5')$, 1.40(2H); $CH_2(2')$, 1.90(2H); CH(7), 6.15(1H); CH(5), 6.80(1H); CH(4), 6.82(1H)]. These observed differences could result from different quality of solvents and different instruments.

In summary, the data presented in this study confirmed that the Nor-1c protein is a ketoreductase which converts the AFB_1 pathway intermediate norsolorinic acid (NA) to averatin (AVN) in the presence of NADPH and a supernatant fraction (105,000 × *g*) obtained from *E. coli* DH5α. This suggests that an unidentified cofactor(s) may be necessary for the native Nor-1 protein function and that the *nor*-1 gene is involved in only one of multiple proposed pathways for the NA conversion to averufin (AVF) in the AFB_1 pathway in *A. parasiticus*.

CHAPTER 3

THE ACCUMULATION OF THE NOR-1 PROTEIN AND THE ACTIVITY OF THE *NOR*-1 PROMOTER DURING AFLATOXIN B_1 BIOSYNTHESIS IN *ASPERGILLUS PARASITICUS*

ABSTRACT: Two different procedures were utilized to study the expression of the *nor*-1 gene during aflatoxin B_1 biosynthesis in *Aspergillus parasiticus*. First, polyclonal antibodies, which were raised against the Nor-1c protein (expressed in *Escherichia coli* from a *nor*-1 cDNA), were used to monitor the accumulation of the native Nor-1 protein. Second, a *nor*-1/GUS reporter construct was used to monitor the activity of the *nor*-1 promoter. The results showed that the accumulation of the Nor-1 protein (in liquid media and on solid media) and the activity of the *nor*-1 promoter (on solid media) are regulated in the same pattern as the accumulation of aflatoxin and are also closely correlated to the development of sporulation in *A. parasiticus*. The results also suggested that the expression of *nor*-1 is regulated in a temporal and spatial pattern in *A. parasiticus* grown on sold media.

INTRODUCTION

A major focus of current studies on aflatoxin biosynthesis in *Aspergillus parasiticus* is the regulation of the expression of the genes involved in this complex pathway. Northern blot analysis demonstrated that the expression of *nor*-1, which is directly involved in aflatoxin B1 biosynthesis, is regulated at the level of transcript accumulation in the toxigenic strain *A. parasiticus* SU-1 grown in liquid media (Skory *et al.*, 1993; Trail *et al.*, 1994; Trail *et al.*, 1995). However, the expression of *nor*-1 can also be studied at the level of the accumulation of the Nor-1 protein and at the level of the activity of the *nor*-1 promoter in *A. parasiticus.*

Conditions of toxigenic fungi growing in liquid media are very different from the natural environmental conditions of the same fungi growing in soil or on host plants in the field. For example, sporulation usually cannot be observed in liquid media. Therefore, studies conducted in liquid media may not completely reflect the regulation of the genes involved in aflatoxin biosynthesis in toxigenic fungi grown on solid media.

Aflatoxins have been found in high concentrations in spores (Wicklow and Cole, 1982; Wicklow and Shotwell, 1983; Cotty, 1988; Bayman and Cotty, 1990). Mycelial growth rate was reduced and sporulation and aflatoxin accumulation were totally inhibited in *A. flavus* and *A. parasiticus* by addition of 2.0% (v/w) phosphates to Sabouraud dextrose agar (Lebron *et al.*, 1989). These data suggest that the regulation of aflatoxin biosynthesis may be correlated to sporulation.

Based on these data and observations, the major hypothesis in this study is that the accumulation of the Nor-1 protein (in liquid media and on solid media) and the activity of the *nor*-1 promoter activity (on solid media) are regulated in the same pattern as the accumulation of aflatoxin and are correlated to the development of sporulation in *A. parasiticus.*

The data presented in Chapter 2 demonstrated that the Nor-1c protein was expressed in *E. coli* from a *nor*-1 cDNA, the Nor-1 protein had the same molecular mass as that predicted from the *nor*-1 gene sequence, and the Nor-1 protein had the enzymatic activity necessary to convert norsolorinic acid (NA) to averantin (AVN), two intermediates in the AFB_1 pathway. These data suggested that the Nor-1c protein and the Nor-1 protein may have very similar, if not identical, amino acid sequences, and therefore, have the same immunogenicity and the same anitgenicity. Hence, a subsidiary hypothesis is that polyclonal antibodies (PAb) raised against the Nor-1c protein would be useful to monitor the accumulation of the Nor-1 protein.

The *Escherichia coli uid*A gene encoding β-glucuronidase (GUS) has been used in fungi as a reliable reporter for gene expression at the level of promoter activity (Monke and Schafer, 1993). Therefore, the activity of *nor*-1 promoter can be monitored by a *nor*-1 promoter/GUS construct (*nor*-1/GUS) providing another tool to address the major hypothesis.

Accordingly, two experimental procedures are proposed to address the major hypothesis in this study. First, polyclonal antibodies (PAb) will be raised against the fusion of the Nor-1c protein and the maltose binding protein (MBP) (Nor-1c/MBP). The PAb will be used to detect the accumulation of the Nor-1 protein. Second, a GUS reporter construct will be used to monitor the activity of the *nor*-1 promoter. The accumulation of the Nor-1 protein and the activity of the *nor*-1 promoter will be compared with the pattern of aflatoxin accumulation and the development of sporulation during aflatoxin biosynthesis.

The data presented in this study showed that the accumulation of the Nor-1 protein (in liquid media and on solid media) and the activity of the *nor*-1 promoter (on solid media) are regulated in the same pattern as the accumulation of aflatoxin and are closely correlated to the development of sporulation in *A. parasiticus*. The data also suggested that the expression of *nor*-1 is regulated in a temporal and spatial pattern in *A. parasiticus* grown on sold media.

MATERIALS AND METHODS

Bacterial Strain, Fungal Strains, and Growth Media. Plasmids were amplified in *Escherichia coli* DH5α F' ^{e}[F'*endA1 hsdR17*(rK^{-}mK^{+})*supE44 thi*-1 *recA1 gyrA* (Nal^{r}) *relA1* (*lacZYA-argF*) $_{U169}$ (*m80lacZ* ΔM15)] using standard methods (Ausubel *et al.*, 1987). *Aspergillus parasiticus* NRRL 5862 (ATCC 56775, SU-1) was used as the aflatoxin-producing wild-type strain. An *A. parasiticus* transformant (designated *nor*-1/GUS) containing pAPGUSNN, a reporter construct containing the *nor*-1 promoter fused with the *E. coli uid*A gene encoding β-glucuronidase (kindly provided by David Wilson in our laboratory) was used to monitor the activity of *nor*-1 promoter. In this transformant, pAPGUSNN was inserted at the 5'-end of the chromosomal *nor*-1 locus. Fungal strains were maintained as frozen spore stock suspensions [in 20% (v/v) glycerol] at -80^{o}C. Aflatoxin-inducing media, YES broth [2% (w/v) yeast extract, 6% (w/v) sucrose, pH 5.8] and YES agar [YES broth containing 1.5% (w/v) Bacto-agar] were used to grow mycelia for protein preparation and aflatoxin extraction.

Fungal Culture, Dry Weight Determination, and Analysis of Fungal Morphology. For growth of fungi on solid medium, 5 µl of a spore stock suspension (1.5 x 10^6 spores/ml) was inoculated onto the center of YES agar media and grown at 29°C in the dark. Mycelia were collected using a spatula to scrape the mycelia from the surface of agar. The collected mycelia were either used immediately or frozen under liquid nitrogen and stored at -80°C until use. The dry weight of mycelia growing on solid media was determined by a filtration/drying method described by Olsson and Jennings (1991).

For growth of fungi in liquid media, a stock spore suspension was inoculated into liquid media with a final spore concentration of 1.5 x 10^5 spores/ml and grown at 29°C with constant shaking (150 rpm) in the dark. Mycelia were collected by filtration and were either used immediately or frozen under liquid nitrogen and stored at -80°C until use. The dry weight of mycelia grown in liquid media was determined by drying the mycelial pad in a preweighed flask overnight at 80°C before weighing. A nutritional shift culture assay using nonaflatoxin-inducing peptone-mineral salts (PMS) medium and aflatoxin-inducing glucose-mineral salts (GMS) medium was performed according to Wiseman and Buchanan (1987). Briefly, spores were inoculated into PMS medium (1.5 x 10^5 spores/ml) and incubated for 60 h and then shifted into GMS medium for a 60-h period of incubation.

The morphological characteristics of a colony grown on YES agar were examined under a dissection microscope.

Temporal and Regional Collection of Samples From Solid Growth Medium. Conidia (7.5 x 10^3) were inoculated at the center of YES agar in Petri dishes. Colonies with the same growth rate were selected. For collecting samples from the central region, at least 4 cultures were needed. Two days after inoculation, the area covered by the mycelium (in four Petri dishes) was marked with a circle and designated the 'central region'. Two days (4 days, 5 days, or 6 days) after inoculation, the mycelium which covered the central region of the first (second, third, or fourth) Petri dish was collected as the '2-day' ('4-day', '5-day', or '6-day') 'central-region' sample C2 (C4, C5, or C6). For collecting samples from the middle region, at least 3 cultures were needed. Two days after inoculation, the area covered by the mycelium (in three Petri dishes) was marked with a circle (the 2-day mark). Four days after inoculation, the area covered by the mycelium (in three Petri dishes) was marked with a second circle (the 4-day mark). The area between the 2-day mark and the 4-day mark was designated the 'middle region'. Four days (5 days, or 6 days) after inoculation, the mycelia which covered the middle region of the first (second, or third) Petri dish was collected as the '4-day' ('5-day', or '6-day') 'middle-region' sample M4 (M5, or M6). For collecting samples from the peripheral region, at least 2 cultures were needed. Four days after inoculation, the area covered by the mycelium (in two Petri dishes) was marked with a circle (the 4-day mark). The area between the 4-day mark and the circular edge of the agar was designated the 'peripheral region'. Five 5 days (or 6 days) after inoculation, the mycelia which covered the peripheral region of the first (or second) Petri dish was collected as the '5-day' (or '6-day') 'peripheral region' sample P5 (or P6). The sampling procedure is shown schematically in Figure 3-5B.

Crude Protein Extract Preparation. Crude protein extract preparation from fungal cells was conducted as described in Chapter 2.

Preparation and Purification of Polyclonal Antibodies. A *nor*-1 cDNA was cloned into the expression vector pMAL-c2 resulting in pMN1. The Nor-1c/MBP (fusion protein) was produced in *E. coli* transformed with pMN1. The Nor-1c/MBP was cut with factor Xa to yield the Nor-1c protein and the MBP. The Nor-1c/MBP and the Nor-1c protein were purified by affinity chromatography as described in Chapter 2.

The antigen-adjuvant mixture (100 μl of *Hunter's* TiterMax™ #R-1 adjuvant plus 120 μg of the Nor-1c/MBP in 50 μl of 10 mM phosphate-buffered saline) was prepared according to the manufacturer's instructions (CytRx Corporation, Atlanta, GA) and was used for primary injection and boosts. The anti-Nor-1c/MBP serum of the rabbit was prepared according to Ausubel *et al.* (1995a). Briefly, the antigen-adjuvant emulsion was injected into the rabbit subcutaneously at four different sites on the rabbit's back. The rabbit was boosted subcutaneously 4 weeks later after the primary injection and reboosted 2 weeks after the initial boost. The titer of the antiseum was estimated by indirect ELISA according to Ausubel *et al.* (1995a) using the Nor-1c/MBP and the Nor-1c protein as coating antigens respectively. Briefly, a microtiter plate was coated with 10 μg/ml antigen and blocked with 0.25% (w/v) bovine serum albumin (BSA). The antigen-coated and BSA-blocked wells of the microtiter plate were incubated with serial dilutions of the antiserum (10 to 10^6 dilution). The wells were then incubated with goat anti-rabbit-IgG antibodies conjugated with alkaline phosphatase (Sigma, St. Louis, MO). The activity of the captured alkaline phosphatase was determined according to the manufacturer's instructions. The dilution of the antiserum which showed 2 times the alkaline phosphatase activity of the preimmune serum in ELISA assay was defined as the titer.

The IgG fraction of the antiserum was precipitated with 33% (v/v) saturated ammonium sulfate (SAS). The double-purified polyclonal antibodies (PAb) were obtained by passing a portion of the IgG fraction twice through an affinity column containing MBP plus crude protein (10,000 × *g* supernatant) of *E. coli* DH5α as ligands. The triple-purified PAb were obtained by passing a portion of the double-purified PAb through a second affinity column containing the Nor-1c protein as the ligand. Affinity column preparation using CNBr-activated Sepharose 4B (Sigma, St. Louis, MO) and ligand proteins (either a mixture of MBP and *E. coli* DH5α protein in a 1:1 ratio, or Nor-1c protein alone) was carried out according to a published standard method (Ausubel *et al.*, 1995c). The procedure for affinity purification of PAb was carried out according to the method established by Dr. R. Mehigh in our lab with modifications (Chapter 2, Immunoaffinity purification of the Nor-1 protein). Five mg of the IgG fraction (or the double purified PAb) (5 mg/ml) was loaded onto the affinity column for purification of the double purified PAb (or the triple purified PAb).

Western Blot Analysis of the Native Nor-1 Protein. Protein, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), was blotted to a polyvinylidene difluoride (PVDF) membrane (Du Pont Co., Boston, MA) using a standard published method (Ausubel *et al.*, 1995b). Immunodetection was carried out with a chemiluminescent detection kit (Schleicher & Schuell, Keene, NH) according to the manufacturer's instructions.

Aflatoxin Analysis by TLC and ELISA. Aflatoxins were extracted for 1 h at room temperature with chloroform (10 ml) either from mycelial liquid growth media (not including mycelia) (1 ml) or from mycelial solid growth media (including mycelia) (1 g). Chloroform-extracted aflatoxins were tested either semiquantitatively by thin layer chromatography (TLC) with chloroform-acetone (95:5) as the developing system, or quantitatively by enzyme-linked immunosorbent assay (ELISA). For ELISA, only aflatoxin B_1 (AFB_1) was tested according to the method of Pestka *et al.* (1980) using anti-AFB_1 antibodies kindly provided by Dr. J. Pestka (Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI).

Quantitative Assay of β-Glucuronidase Activity. Quantitative analysis of β-glucuronidase activity was performed by spectrophotometry as described by Jefferson *et al.* (1986), and Tada *et al.* (1991) using *p*-nitrophenylglucuronide as the substrate. One unit was defined as the amount (nanomole) of *p*-nitrophenol produced in one min at 37°C by 1 mg protein (nmol/min per mg protein).

RESULTS

Titer and Specificity of Polyclonal Antibodies. The titer of the anti-Nor-1c/MBP serum was determined by ELISA. The titers of antisera from the first and second boost were similar (450,000 and 500,000). The immunogen used for antibody production in this study was the Nor-1c/MBP composed of the MBP (42 kDa) and the Nor-1c protein (31 kDa). Because the serum contained antibodies against both portions of the fusion protein, the titer which was determined using the Nor-1c/MBP as the antigen was higher than the titer which was determined using the Nor-1c protein as the antigen.

In Western blot analysis of crude fungal cell extracts, the IgG fraction detected at least 7 protein bands (Figure 3-1). The double-purified PAb detected two protein bands (Figure 3-2). The triple-purified PAb detected only one major protein band (Figure 3-1) with a

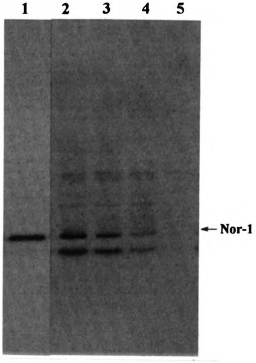


Figure 3-1. Western blot analysis demonstrating the specificity of PAb from different levels of purification. All crude extracts (except lane 5) were obtained from *A. parasiticus* SU-1 grown in YES liquid medium. Each lane contained 10 µg crude protein. **Lanes: 1,** crude extract (from the 60-h sample) was probed by the triple-purified PAb (10 µg/ml); **2, 3,** and **4,** crude extracts from the 60-h, 54-h, and 48-h samples, respectively, were probed with the IgG fraction (10 µg/ml); **5,** crude extract from *nor*-1 disrupted strain of *A. parasiticus* grown in YES liquid medium (for 60 h) was probed with the IgG fraction (10 µg/ml). The molecular mass of the Nor-1 protein (top band) was 31 kDa.

Table 3-1. Titers and specificities of anti Nor-1c/MBP PAb.

Samples	Titer against the Nor-1c/MBP	Titer against the Nor-1c protein	Specificity (cross reaction)
Antiserum from primary injection	200,000	50,000	> 7 bands
Antiserum from first boost	450,000	110,000	> 7 bands
Antiserum from second boost	500,000	120,000	> 7 bands
Double-purified PAb			2 bands
Triple-purified PAb			1 band

Notes:

(1). The antiserum from primary injection was obtained 4 weeks after the primary immunization. The antiserum from the first boost and the second boost were obtained 2 weeks after the first boost and the second boost respectively.

(2). The titers of antisera were estimated using either the Nor-1c/MBP, or the Nor-1c protein as the antigen.

(3). The relative specificity was estimated by Western blot analysis.

(4). The specificity of the antiserum was estimated using the IgG fraction in Western blot analysis.

size of 31 kDa which was the expected size of the Nor-1 protein. The 31-kDa protein band was also detected by both the IgG fraction and the double-purified PAb. The relative specificities of the IgG fraction, the double-purified PAb, and the triple-purified PAb (as measured by the degree of cross reactivity) are summarized in Table 3-1.

The Accumulation of the Nor-1 Protein and the Accumulation of AFB$_1$ in *A. parasiticus* SU-1 Grown in YES Liquid Medium. The pattern of the accumulation of the Nor-1 protein was analyzed in *A. parasiticus* SU-1 (an aflatoxin-producing strain) grown in YES liquid medium (an aflatoxin-inducing medium). Western blot analysis of crude extracts from cells harvested at the appropriate time points showed that the Nor-1 protein was not

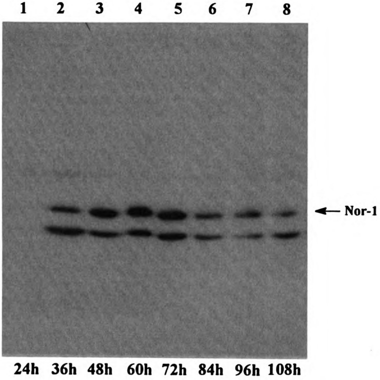


Figure 3-2. Western blot analysis of the accumulation of the Nor-1 protein in *A. parasiticus* SU-1 grown in YES liquid medium. Each lane contained 10 µg crude protein. **Lanes: 1** through **8**, crude extract from fungal cells grown for 24 h, 36 h, 60 h, 72 h, 84 h, 96 h, and 108 h, respectively, and probed by the double-purified PAb (10 µg/ml). The molecular mass of the Nor-1 protein (top band) was 31 kDa.

detected at 24 h of incubation, but was detected from 36 h to 108 h of incubation (Figure 3-2). The maximum level of the Nor-1 protein appeared between 36 h and 72 h of incubation and decreased from 72 h to 108 h of incubation. ELISA analysis of the growth medium showed that AFB_1 began to accumulate after 24 h of incubation (Figure 3-3). The maximum rate of AFB_1 accumulation occurred between 36 h to 60 h of incubation (from 19 to 121 mg/L). AFB_1 accumulation remained at almost the same level from 60 h to 108 h of incubation. The maximum rate of accumulation of the Nor-1 protein occurred at the same time as the maximum rate of AFB_1 accumulation.

The Accumulation of the Nor-1 Protein and the Accumulation of AFB_1 in *A. parasiticus* SU-1 in Nutritional Shift Assay. A nutritional shift assay was conducted to measure the accumulation of the Nor-1 protein in *A. parasiticus* SU-1 grown in a nonaflatoxin-inducing PMS medium and then shifted into aflatoxin-inducing GMS medium. After 60 h of incubation in PMS no Nor-1 protein was detected by Western blot analysis (Figure 3-4). The Nor-1 protein was detected as early as 6 h after a shift from PMS to GMS. The corresponding accumulation of AFB_1 in the growth medium was determined by ELISA (Table 3-2). After the nutritional shift, AFB_1 increased more slowly in the fresh PMS (from 0.13 to 2.42 mg/L) than in the fresh GMS (from 0.13 to 129.80 mg/L). In the nutritional shift assay, the pattern of the accumulation of the Nor-1 protein was consistent with the pattern of the accumulation of AFB_1.

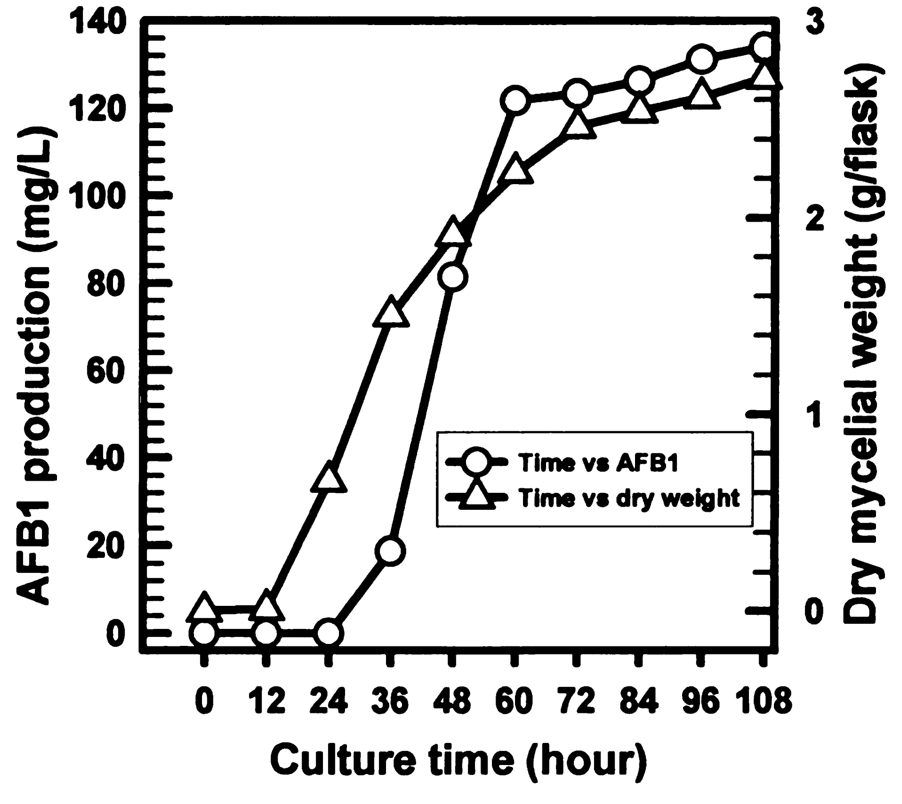


Figure 3-3. The accumulation of AFB_1 and mycelial dry weight in *A. parasiticus* SU-1 grown in YES liquid medium. The concentration of AFB_1 was assayed by ELISA in samples taken 12 h, 24 h, 36 h, 60 h, 72 h, 84 h, 96 h, and 108 h after inoculation. The mycelial dry weight was estimated at the same time points.

Table 3-2. The accumulation of aflatoxin B_1 in *A. parasiticus* SU-1 in nutritional shift assay.

Incubation time after a shift from PMS (h)	AFB_1 in PMS after shift: mean ± SE (mg/L)	AFB_1 in GMS after shift: mean ± SE (mg/L)
0	0.13 ± 0.09	0.13 ± 0.09
6	0.16 ± 0.07	7.89 ± 0.16
12	0.59 ± 0.06	25.36 ± 0.25
24	1.06 ± 0.07	45.47 ± 0.28
48	2.12 ± 0.08	115.36 ± 1.23
60	2.42 ± 0.11	129.80 ± 1.26

Note: *A. parasiticus* SU-1 was grown in a nonaflatoxin-inducing PMS medium for 60 h and then shifted into aflatoxin-inducing GMS medium. Each value represents the mean ± standard error (SE) of triple tests.

Morphology of *A. parasiticus* SU-1 Grown on YES Agar. The morphology of *A. parasiticus* SU-1 grown on YES agar was observed under a dissection microscope. Immediately after inoculation, the area occupied by the inoculated spores was irregular. After one day of incubation, the germination of individual spores was randomly oriented. After two days of incubation, a white round colony (0.5-1 cm in diameter) appeared with a white circular band of substrate hyphae (0.1- 0.2 cm in width) at the margin and a white round area of aerial hyphae (0.3 - 0.6 cm in diameter) at the central region. After three days of incubation, the white circular band of substrate hyphae (at the margin of the colony) moved outwards followed by an adjacent white circular band of aerial hyphae (0.2 - 0.4 cm in width). A yellow-green round area of immature spores (1.4 - 1.6 cm in diameter) appeared at the central region. After four days of incubation, the white circular band of substrate hyphae and the adjacent white circular band of aerial hyphae continued to move outwards followed by a yellow-green circular

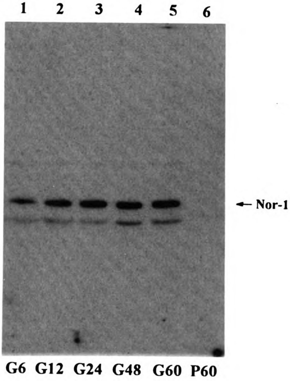


Figure 3-4. Western blot analysis of the accumulation of the Nor-1 protein in *A. parasiticus* SU-1 in PMS-GMS nutritional shift assay. Each lane contained 10 μg crude protein and was probed by the double-purified PAb (10 μg/ml). **Lanes: 1, 2, 3, 4,** and **5**, crude extract from cells collected after incubation in GMS for 6 h, 12 h, 24 h, 48h, and 60 h (G6, G12, G24, G48, and G60), respectively, after a shift from a 60-h incubation in PMS (P60); **6**, crude extract from cells collected after a 60-h period of incubation in PMS (0 h in GMS). The molecular mass of the Nor-1 protein (top band) was 31 kDa.

band of immature spores (1.0 - 2.0 cm in width). A brown-green round area of mature spores (1.0 - 2.0 cm in diameter) appeared at the central region of the Petri dish. After five days of incubation, the white circular band of substrate hyphae reached the edge of the agar, the adjacent white circular band of aerial hyphae and the yellow-green circular band of immature spores continued to move outwards followed by a brown-green circular band of mature spores (1.5 - 2.5 cm in width). The hyphae in the central region started to degenerate. After six days of incubation, the band of substrate hyphae and the band of aerial hyphae disappeared. The band of immature spores and the band of mature spores moved outwards and a brown round area of degenerated hyphae (0.2- 0.5 cm in diameter) appeared at the center.

Morphological Development and the Accumulation of AFB$_1$ at the Colony Level in *A. parasiticus* Grown on YES Agar. Spores were inoculated onto the center of YES agar in a Petri dish and samples (C2, C4, C5, C6, M4, M5, M6, P5, and P6) were collected as described (Materials and Methods) and shown schematically in Figure 3-5B. The accumulation of AFB$_1$ in each sample was determined by ELISA. The morphological characteristics and the accumulation of AFB$_1$ in each samples were summarized in Table 3-3. By comparing the morphological characteristics with the accumulation of AFB$_1$, a pattern was found. In the growing colony, the concentration of AFB$_1$ increased from the region with substrate hyphae plus aerial hyphae (3.2 mg/g), to the region with substrate hyphae plus aerial hyphae plus immature spores (4.3-4.7 mg/g), to the region with immature spores plus mature spores (5.8-7.4 mg/g), to the region with mature spores (7.4-9.0 mg/g), and to the region with mature spores plus degenerated hyphae (7.9 mg/g) in a colony. The pattern of the accumulation

Table 3-3. The morphological characteristics and the accumulation of AFB$_1$ in *A. parasiticus* SU-1 grown on YES agar.

Samples	Collecting region	Incubation time (day)	Morphological characteristics	AFB$_1$ (mg/g)
C2	Central	2	Substrate and aerial hyphae	3.2 ± 0.8
C4	Central	4	Immature and mature spores	5.8 ± 0.4
C5	Central	5	Mature spores	7.4 ± 0.8
C6	Central	6	Mature spores, and degenerated hyphae	7.9 ± 0.7
M4	Middle	4	Substrate and aerial hyphae, and immature spores	4.3 ± 0.5
M5	Middle	5	Immature and mature spores	7.4 ± 0.6
M6	Middle	6	Mature spores	9.0 ± 1.2
P5	Peripheral	5	Substrate and aerial hyphae, and immature spores	4.7 ± 0.4
P6	Peripheral	6	Immature and mature spores	6.9 ± 0.3

Notes:

(1) Conidia were inoculated at the center of YES agar in a Petri dish. **Samples:** Samples **C2, C4, C5,** and **C6** were collected from the central region 2 days, 4 days, 5 days, and 6 days after inoculation; samples **M4, M5,** and **M6** were collected from the middle region 4 days, 5 days, and 6 days after inoculation; samples **P5**, and **P6** were collected from the peripheral region 5 days, and 6 days after inoculation. A schematic of the sample collection procedure is shown in Figure 3-5B.

(2) The accumulation of AFB1 (mg/g dry fungal mass) was determined by ELISA (Materials and Methods).

(3) Each value represents the mean ± standard error (SE) of three independent tests.

of AFB$_1$ in the growing colony was correlated to the morphological development of sporulation in *A. parasiticus* SU-1.

The Accumulation of the Nor-1 Protein, the Activity of the *nor*-1 Promoter, and the Accumulation of Aflatoxin in *A. parasiticus* SU-1 Grown on YES Agar. Fungal spores were inoculated onto the center of YES agar in a Petri dish. Samples (C2, C4, C5, C6, M4, M5, M6, P5, and P6) were collected as described (Materials and Methods) and shown schematically in Figure 3-5B. Western blot analysis of crude mycelial extracts

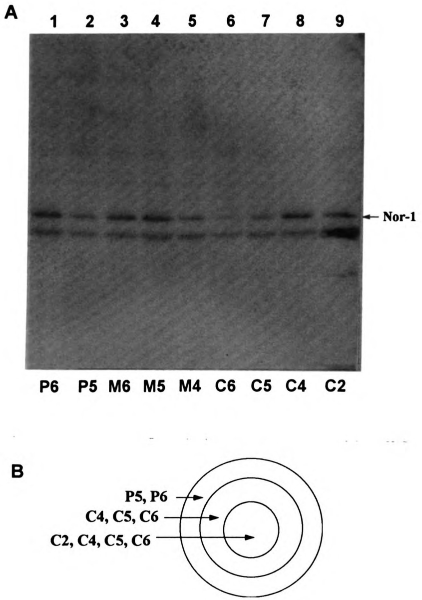


Figure 3-5. Western blot analysis of the accumulation of the Nor-1 protein in *A. parasiticus* SU-1 grown on YES agar. Each lane contained 10 µg crude protein and was probed with the double-purified PAb (10 µg/ml). **A: Lanes: C2, C4, C5,** and **C6,** samples collected from the central region 2 days, 4 days , 5 days , and 6 days after inoculation; **M4, M5,** and **M6,** samples collected from the middle region 4 days, 5 days, and 6 days after inoculation; **P5,** and **P6,** samples collected from the peripheral region 5 days , and 6 days after inoculation. **B:** A schematic of the sample collection procedure.

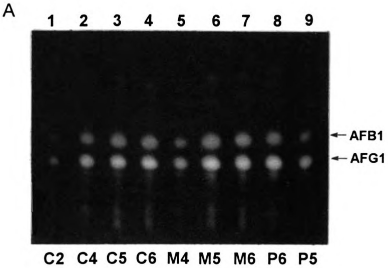


Figure 3-6. TLC analysis of the accumulation of aflatoxins in *A. parasiticus* SU-1 grown on YES agar. Aflatoxins from each sample (mycelia and agar) were extracted with chloroform and assayed by TLC with chloroform-acetone (95:5) as the developing system. Fluorescence was detected with long wavelength UV light. **A.** Lanes: **C2, C4, C5, C6, M4, M5, M6, P5, and P6** are samples collected as explained in the legend of Figure 3-5.

Table 3-4.

Table 3-4. The accumulation of the Nor-1 protein, the activity of the *nor*-1 promoter (GUS activity), and the accumulation of AFB_1 in *A. parasiticus* SU-1 grown on YES agar.

Samples	C2	C4	C5	C6	M4	M5	M6	P5	P6
Nor-1 protein (relative intensity on Western blot)	71	85	42	23	67	100	91	58	92
GUS activity (nmol/min /mg)	54 ± 12	185 ± 13	85 ± 11	25 ± 5	124 ± 7	187 ± 6	179 ± 11	100 ± 6	197 ± 9
AFB_1 accumulation (mg/g)	3.2 ± 0.8	5.8 ± 0.4	7.4 ± 0.8	7.9 ± 0.7	4.3 ± 0.5	7.4 ± 0.6	9.0 ± 1.2	4.7 ± 0.4	6.9 ± 0.3
AFB_1 (relative intensity on TLC)	12 ± 4	56 ± 5	73 ± 5	88 ± 8	35 ± 7	91 ± 8	100	53 ± 8	64 ± 9

Notes:

(1) Conidia were inoculated at the center of YES agar in a Petri dish. **Samples:** Samples **C2, C4, C5,** and **C6** were collected from the central region 2 days, 4 days, 5 days, and 6 days after inoculation; samples **M4, M5,** and **M6** were collected from the middle region 4 days, 5 days, and 6 days after inoculation; samples **P5,** and **P6** were collected from the peripheral region 5 days, and 6 days after inoculation (details in Materials and Methods). A schematic of the sample collection procedure is shown in Figure 3-5B.

(2) Each value represents (except Nor-1 protein data) the mean ± standard error (SE) of three independent tests.

(3) GUS activity was determined by spectrophotometry as described by Jefferson *et al.*(1986), and Tada *et al.*(1991) using *p*-nitrophenylglucuronide as the substrate. One unit was defined as the amount (nanomole) of *p*-nitrophenol produced in one min at 37°C by 1 mg protein (nmol/min per mg protein).

(4) AFB_1 accumulation was determined by ELISA (Materials and Methods).

(5) The relative intensity of AFB_1 on TLC and the relative intensity of the Nor-1 protein on Western blot were determined as described in Materials and Methods.

showed that the Nor-1 protein (31 kDa) was present in all the samples (Figure 3-5A). In the central region, the Nor-1 protein appeared 2 days after inoculation (C2), reached the highest level 4 days after inoculation (C4), and declined 5 days and 6 days after inoculation (C5 and C6). In the middle region, the Nor-1 protein appeared 4 days after inoculation (M4), and increased 5 days and 6 days after inoculation (M5 and M6). In the peripheral region, the Nor-1 protein appeared 5 days after inoculation (P5), and increased 6 days after inoculation (P6). For each sample, the GUS activity was measured spectrophotometrically. The accumulation of aflatoxins was determined by TLC (Figure 3-6). The relative intensities of the Nor-1 protein in Western blot and AFB_1 on TLC were determined with an EPSON ES-1000C scanner (EPSON Accessory, Singapore) and quantitated with Sigma Gel software. These results (along with the accumulation of AFB_1 measured by ELISA) are listed in Table 3-4 to facilitate comparison. The quantity of AFB_1 in ELISA analysis and the relative quantity of AFB_1 in TLC analysis in each sample was consistent with the relative quantity of Nor-1 protein in Western blot analysis (Figure 3-5), and was consistent with the level of *nor*-1 promoter activity in the GUS assay.

DISCUSSION

The data presented in this study demonstrated that the PAb raised against the Nor-1c/MBP were sufficiently specific for detection of the (native) Nor-1 protein by Western blot analysis. The data showed that the accumulation of the Nor-1 protein (in liquid media and on solid media) and the activity of the *nor*-1 promoter (on solid media) are consistent with the pattern of the accumulation of aflatoxin and are closely correlated to the morphological development of sporulation in *A. parasiticus*. The data also suggested that the expression of *nor*-1 is regulated in a temporal and spatial pattern in *A. parasiticus* grown on solid media.

The first step in this study was to prove that anti-Nor-1c/MBP PAb were sufficiently specific to detect the Nor-1 protein in Western blot analysis. This step was necessary not only because the immunogen used in this study was a fusion protein composed of the Nor-1c protein (instead of the Nor-1 protein) and MBP, but also because the fusion protein was likely contaminated with the bacterial protein. It was not surprising that the antiserum specificity was low (indicated by the cross reactivity in Western blot analysis). Therefore the antiserum could not be used in Western blot analysis of the Nor-1 protein without validation. The only major protein band detected by the triple-purified PAb not only had the expected size of the Nor-1 protein (31 kDa) but also was detected by the IgG fraction and the double-purified PAb in crude mycelial extracts from the wild-type *A. parasiticus* SU-1. The same protein band was not detected in crude mycelial extracts from the *nor*-1 disrupted strain. Therefore the double-purified PAb (or even the IgG fraction) could be used to monitor the accumulation of the Nor-1 protein in Western blot analysis.

A 28-kDa protein band was also detected by the IgG fraction and the double-purified PAb, but was not detected by the triple-purified PAb. One possible explanation is that the 28-kDa protein and the Nor-1 protein have similar antigenicity. One fraction of the total PAb (raised against the Nor-1c/MBP) may have a higher affinity to the 31-kDa Nor-1 protein and a lower affinity to the 28-kDa protein. Another fraction may have a lower affinity to the 31-kDa Nor-1 protein but a higher affinity to the 28-kDa protein. Therefore, if an excess of the double-purified PAb was used for further purification using affinity chromatography, the binding sites for the Nor-1c protein on the affinity column could be saturated by the PAb fraction which had the higher affinity to the 31-kDa Nor-1 protein. This fraction was then purified (triple-purified PAb). This could be one possible reason that the double-purified PAb detected both the Nor-1 protein and the 28-kDa protein, but the triple-purified PAb detected only the Nor-1 protein in Western blot analysis.

The pattern of the 28-kDa protein accumulation was consistent with the pattern of the Nor-1 protein accumulation. It is possible that the 28-kDa protein is also involved in the AFB_1 pathway. The 28-kDa protein and the Nor-1 protein may be synthesized at the same time and combine with each other in order to increase the efficiency of aflatoxin synthesis. The combination of the two proteins may stabilize the 28-kDa protein. Therefore, when the *nor*-1 gene is disrupted, there is no Nor-1 protein existing and the 28-kDa protein is rapidly degraded.

The temporal pattern of the accumulation of the Nor-1 protein and the accumulation of AFB_1 are closely correlated in *A. parasiticus* grown in liquid medium. In batch fermentation (YES liquid medium), the accumulation of the Nor-1 protein and the accumulation of AFB_1 increased simultaneously and quickly during late log-phase of the fungal growth and reached a maximum level at stationary phase. The level of the accumulation of the Nor-1 protein declined at approximately the same time that the accumulation of AFB_1 reached its peak level.

In the nutritional shift assay, the accumulation of the Nor-1 protein and the accumulation of AFB_1 began simultaneously and increased quickly a short time after the shift from the nonaflatoxin-inducing PMS medium to the aflatoxin-inducing GMS medium. The Nor-1 protein accumulation data were consistent with the *nor*-1 transcript accumulation data previously reported (Skory *et al.*, 1993; Trail *et al.*, 1994; Trail *et al.*, 1995).

In PMS medium, no Nor-1 protein was detected, but AFB_1 was detected. This apparently inconsistency may be explained in two possible ways. In the nutritional shift culture, the AFB_1 concentration in PMS medium was much lower than in GMS medium after the shift. Therefore, the corresponding Nor-1 protein in PMS could be too low to be detectable in Western blot analysis in this study. The second possible explanation may come from the result of *nor*-1 disruption. The disruption of the *nor*-1 gene resulted in an accumulation of norsolorinic acid (NA) and a significant decrease in aflatoxin accumulation (Trail *et al.*, 1994). This result suggests that a small quantity of AFB_1 could be synthesized and accumulated without the function of the Nor-1 protein in PMS medium.

The data from liquid batch fermentation and the nutritional shift assay confirmed the observed expression pattern of the *nor*-1 gene (at the level of transcript accumulation) in *A. parasiticus* grown in liquid media previously reported (Skory *et al.*, 1993; Trail *et al.*, 1994; Trail *et al.*, 1995) and demonstrated that the accumulation of the Nor-1 protein can provide an alternative measurement for the expression of *nor*-1. Therefore, it is also reasonable to predict that the accumulation of the Nor-1 protein can be used as a measurement for the expression of *nor*-1 in *A. parasiticus* grown on solid media.

The accumulation of the Nor-1 protein was higher at 72 h than at 36 h in YES liquid medium, but the rate of AFB_1 accumulation between 60 h and 72 h was much lower than between 36 h and 48 h. One possible explanation is that at 36 h most of the Nor-1 protein was active, while at 72 h most of the Nor-1 protein was inactive possibly because of the absence of NADPH (Chapter 1, Regulation of aflatoxin biosynthesis) or other unknown factors (Chapter 2, Discussion).

An alternative explanation comes from the observation that the *A. flavus* group is not very efficient at degrading aflatoxins under normal conditions (Doyle and Marth, 1978). It is possible that when aflatoxin concentrations reach high levels in the medium, fungal cells are forced to efficiently degrade aflatoxins to survive. AFB_1 could be synthesized at the same rate in the period from 60 h to 70 h and in the period from 36 h to 48 h because the similar accumulation of the Nor-1 protein was observed in the two periods of time. But in the period from 60 h to 70 h, a certain amount of AFB_1 was possibly degraded. This degradation could result in the much lower rate of AFB_1 accumulation.

The morphological development at the colony level of *A. parasiticus* grown on solid media in a Petri dish appears to be controlled by genetics and environmental influences. Here, morphological development at the colony level is defined as the development of the shape and color of a colony and distributions and colors of substrate hyphae, aerial hyphae, immature spores, mature spores, and degenerated hyphal cells in the colony. Regardless of the irregular geometrical shape of the area occupied by the inoculated spores and the random orientation of the germination of individual spores, the resulting colony and the concentric regions of distinct morphology in the colony are almost always nearly circular. Nutrients in YES agar are evenly distributed. Nutrients in coconut agar are not always evenly distributed because it may contain large pieces of coconut flakes with different sizes (Chapter 4). The nearly circular shape of the colony and the concentric regions of distinct morphology in the colony appeared on both media. But these two media do have different influences on the colors and widths of concentric circular regions of distinct morphology in the colony. These observations suggested that the morphological development of *A. parasiticus* is controlled by genetic and environmental influence at the colony level. In another words, morphological development appears to be regulated at the colony level.

Sporulation is one example of morphological development. The *Aspergillus nidulans flu*G gene is necessary for the synthesis of a small diffusible factor which may be an extracellular signal directing asexual sporulation and perhaps other aspects of colony growth (Lee and Adams, 1994a, 1994b, and 1996). This observation suggests that sporulation is regulated at the colony level by cell-cell communication.

The accumulation of the Nor-1 protein, the activity of the *nor*-1 promoter, and the accumulation of aflatoxin are consistent with each other and are possibly regulated at the colony level through an unknown mechanism which is closely related to the morphological development of sporulation in *A. parasiticus* grown on YES agar. Because of the inherent difficulties encountered in the separation of substrate hyphae, aerial hyphae, immature spores, and mature spores, the procedure of collecting samples from YES agar used in this study was a crude approach to achieve this separation. Nevertheless, the accumulation of the Nor-1 protein and the activity of the *nor*-1 promoter (indicated by GUS activity) increased and decreased at the same time (temporal level) in *A. parasiticus* SU-1 grown on YES agar. Higher levels of the Nor-1 protein accumulation and the *nor*-1 promoter activity were detected in regions containing conidiophores and immature conidia than in regions containing only substrate hyphae and aerial hyphae (spatial regulation). Decreased levels of the Nor-1 protein accumulation and the *nor*-1 promoter activity were observed in regions containing only mature spores and degraded hyphal cells. In the center of the colony, the accumulation of AFB_1 increased (per unit of dry weight) at the same time that the accumulation of the Nor-1 protein (or GUS protein) decreased. This phenomenon may result from the fact that AFB_1 is much more stable than the Nor-1 protein. The value for AFB_1 accumulation likely represents a true measurement of accumulative. The value for Nor-1 protein accumulation represents synthesis and degradation. In the center of the colony, when degenerated hyphae were not able to synthesize new Nor-1 protein, the accumulation of the Nor-1 protein decreased but the accumulation of AFB_1 still increased. These patterns were consistent with the pattern of the relative intensity and distribution of blue color (indicating the activity of *nor*-1/GUS) shown on a colony using the same *nor*-1/GUS reporter construct with 5-bromo-4-chloro-3-indolyl glucuronide as the substrate (Chapter 4). Also consistent with these observations, immunolocalization data presented later in this dissertation (Chapter 4) showed that more Nor-1 protein accumulated in conidiophores and conidia than in vegetative cells. Further, when a sporulation related gene (*flu*P) was disrupted in an aflatoxin-producing strain of *A. parasiticus*, fungal hyphae grown on solid media did not sporulate and AFB_1 remained at the same low level during a 6-day period of incubation (Chapter 5).

Because sporulation appeared to be regulated at the colony level and a close correlation between sporulation and the accumulation of aflatoxin (as well as the accumulation of the Nor-1 protein and the activity of the *nor*-1 promoter) was observed, the accumulation of the Nor-1 protein and the activity of the *nor*-1 promoter may also be regulated at the colony level.

The correlation between aflatoxin biosynthesis and sporulation in toxigenic *A. parasiticus* needs further study. Certain sporulation-related pigments with the basic structure of norsolorinic acid were detected in the *Aspergillus* genus (Steyn *et al.*, 1980). Spore pigments from *A. nidulans* and *A. parasiticus* also appear to be produced via polymerization of polyketide precursors (Brown *et al.*, 1993; Brown and Salvo, 1994). A development-related gene *w*A in *A. nidulans* possibly encodes a polyketide (Mayorga and Timberlake, 1992). The *A. parasiticus* polyketide synthase gene *pks*A is required for AFB_1 biosynthesis (Chang *et al.*, 1995). Therefore, it is possible that a common signal(s) at an early stage of development triggers both aflatoxin synthesis and sporulation. When sporulation is inhibited in liquid media, only aflatoxin synthesis occurs in *A. parasiticus*. When the mechanism of aflatoxin synthesis is absent in nontoxigenic fungi or mutation(s) of the genes involved in aflatoxin synthesis occurs in toxigenic fungi, only sporulation happens.

In summary, the data presented in this study showed that the accumulation of the Nor-1 protein (in liquid media and on solid media) and the activity of the *nor*-1 promoter (on solid media) are regulated in the same pattern as the accumulation of aflatoxin and are closely correlated to the morphological development of sporulation in *A. parasiticus*. These results also suggested that the expression of *nor*-1 is regulated in a temporal and spatial pattern in *A. parasiticus* grown on solid media.

CHAPTER 4

LOCALIZATION OF NOR-1 PROTEIN ACCUMULATION AND LOCALIZATION OF *nor*-1 PROMOTER ACTIVITY DURING AFLATOXIN B1 BIOSYNTHESIS IN *ASPERGILLUS PARASITICUS*

ABSTRACT: Anti-Nor-1c protein polyclonal antibodies were used in immunolocalization experiments to localize the accumulation of the Nor-1 protein (at the cellular and hyphal level) and a *nor*-1 promoter/GUS (*nor*-1/GUS) reporter construct was used to localize the activity of the *nor*-1 promoter (at the hyphal and colony level) during aflatoxin B1 biosynthesis in *Aspergillus parasiticus* grown in liquid and/or on solid growth media.

The Nor-1 protein was mainly localized in the cytosol of vegetative hyphae (although a significant quantity appeared to be associated with particles of unknown composition) either in liquid growth medium using cell fractionation/Western blot procedure and in situ immunolocalization procedures or from solid growth medium using an in situ immunolocalization procedure. On solid growth media, the highest level of the Nor-1 protein was associated with immature conidia, higher levels were associated with conidial stalks, lower levels were associated with vegetative hyphae, and none was associated with mature conidia (at the hyphal level). On solid growth medium, the highest level of the activity of the *nor*-1 promoter was detected in conidial heads, higher levels in conidial stalks, and lower levels in vegetative hyphae (at the hyphal level). At the colony level, the activity of the *nor*-1 promoter

was not detected in regions containing only substrate hyphae or mature conidia, but was detected in regions containing aerial hyphae (higher levels) and/or immature conidia (the highest level). These data suggest that localization of Nor-1 protein accumulation and localization of *nor*-1 promoter activity are closely correlated to the process of sporulation in a colony. Therefore the regulation of *nor*-1 gene expression may be regulated at the colony level during aflatoxin biosynthesis in *A. parasiticus* on solid media.

INTRODUCTION

Many studies have been conducted with a goal of understanding the genes and the proteins involved in aflatoxin biosynthesis (Chapter 1). Little effort, however, has been directed toward defining where aflatoxin biosynthesis occurs at the cellular level, at the hyphal level (vegetative hyphae, conidial heads, conidial stalks, and conidia), and at the colony level (regions in a colony containing substrate hyphae, aerial hyphae, immature conidia, and/or mature conidia in a colony).

Several enzyme activities involved in aflatoxin B_1 (AFB_1) synthesis have been detected in the cytosol while others are associated with organelles (Bhatnagar *et al.*, 1989; Yabe *et al.*, 1989; Yabe *et al.*, 1993; Yabe and Hamasaki, 1993; Matsushima *et al.*, 1994). These data suggest that the biosynthesis of aflatoxin is carried out in different cellular compartments. Therefore, aflatoxin pathway enzymes or intermediates may be transferred from one cellular compartment to another during aflatoxin biosynthesis.

Western blot analyses and *nor*-1/GUS assays presented in Chapter 3 suggest that the regulation of Nor-1 protein accumulation and the regulation of *nor*-1 promoter activity are closely correlated to the process of sporulation at the colony level. An understanding of the localization of Nor-1 protein accumulation and the localization of *nor*-1 promoter activity may result in a better understanding of the correlation between these two localizations and the process of sporulation during aflatoxin biosynthesis. This information may directly or indirectly lead to the development of safe, specific fungicides or natural plant products which can block the intracellular transfer of AFB_1 pathway enzymes or intermediates from one compartment to another, and/or interfere with the regulation of aflatoxin biosynthesis at the colony level and in turn inhibit AFB_1 biosynthesis in *A. parasiticus.*

Two hypotheses are proposed in this study: (1) the Nor-1 protein resides locally in certain organelles or in the cytosol; (2) localization of Nor-1 protein accumulation and localization of *nor*-1 promoter activity are closely correlated to the process of sporulation during aflatoxin biosynthesis in *A. parasiticus.*

The data presented in Chapter 3 also demonstrated that the polyclonal antibodies (PAb) raised against the Nor-1c protein were sufficiently specific to detect the native Nor-1 protein and that regulation of Nor-1 protein accumulation and regulation of *nor*-1 promoter activity were consistent with the pattern of AFB_1 accumulation. Therefore, two goals are proposed in this study: (1) localize the accumulation of the Nor-1 protein at the cellular level and at the hyphal level using the PAb raised against the Nor-1c protein; (2) localize the activity of the *nor*-1 promoter at the hyphal level and at the colony level using a *nor*-1/GUS reporter construct.

The localization data presented in this study demonstrated that the Nor-1 protein was mainly localized in the cytosol of vegetative hyphae. The highest level of the Nor-1 protein was associated with immature conidia, higher levels were associated with conidial stalks, lower levels were associated with vegetative hyphae, and none was associated with mature conidia. The highest level of *nor*-1 promoter activity was detected in conidial heads, higher levels in conidial stalks, and lower levels in vegetative hyphae. At the colony level, the activity of the *nor*-1 promoter was not detected in regions of a colony containing only substrate hyphae or mature conidia, but was detected in regions containing aerial hyphae (higher levels) or immature conidia (highest level). These data suggest that localization of Nor-1 protein accumulation and localization of *nor*-1 promote activity are closely correlated to the process of sporulation and hence these process may be regulated at the colony level during aflatoxin biosynthesis in *A. parasiticus* on solid media.

MATERIALS AND METHODS

Fungal Strains and Culture Media. *Aspergillus parasiticus* NRRL 5862 (ATCC 56775, SU-1) was used as the aflatoxin-producing wild-type strain. An *A. parasiticus* strain, *nor*-1/GUS, which contains the *nor*-1/GUS reporter construct (Chapter 3, Materials and Methods), was used to monitor *nor*-1 promoter activity. Fungal strains were maintained as frozen spore stock suspensions in 20% (v/v) glycerol at -80°C (Chapter 2, Materials and Methods). Aflatoxin-inducing liquid growth medium consisting of 2% (w/v) yeast extract, and 6% (w/v) sucrose (pH 5.8) (YES broth), and solid growth medium YES agar [YES broth plus 1.5% (w/v) Bacto-agar] were used to grow fungal cells for localization of Nor-1 protein accumulation and for localization of *nor*-1 promoter activity.

Fungal Culture. For culture of fungal colonies on solid medium, 5 µl of a stock spore suspension (1.5×10^6 spores/ml) was inoculated onto the center of YES agar and incubated at 29°C in the dark for appropriate periods of time. For fungal growth in liquid medium, a spore stock suspension was inoculated into YES broth to a final spore concentration of 1.5×10^5 spores/ml. The culture was incubated at 29°C with constant shaking (150 rpm) in the dark for appropriate periods of time. Mycelia were collected by filtration. For immunolocalization, the collected mycelia were used immediately. For Western blot analysis, the collected mycelia were either used immediately or frozen under liquid nitrogen and stored at -80°C until use. For fungal slide culture, the method according to Harris (1986) was modified as follows. A microscope slide was placed on the center of a Petri dish containing YES agar. Two blocks of YES agar (approximately 200 mm^3) were placed at two separate locations on the slide. A volume of 2 µl of spore stock suspension (1.5×10^6 spores/ml) was inoculated on each side of the YES agar block except the bottom and the top sides followed by placement of a cover glass on top of the YES agar block. The slides were incubated at 29°C for appropriate periods of time in the dark. The slides and the cover glasses with hyphae attached were used immediately for immunolocalization studies.

Cell Fractionation. Two protocols were used for cell fractionation. **Protocol 1. Cell breakage by Potter homogenization of protoplasts followed by differential centrifugation.** This protocol was carried out at 4°C according to Muller *et al.* (1991) with certain modifications. Briefly, mycelia, grown in YES liquid medium, were harvested by filtration and the resulting hyphal mat was washed twice with washing buffer consisting of 0.01 M Tris·Cl (pH 8.0), 0.14 M NaCl, and 0.025% (w/v) NaN_3 (TSA). Portions (20 g wet weight) of the washed hyphal mat were resuspended in 100 ml of cell-wall-digestion buffer consisting of 1.5% (w/v) Novozyme 234, 0.1 M citric acid (pH 6.0), and 0.6 M KCl for 4 h at 30°C with gentle shaking. The protoplasts were collected by passage through a 29 mm nylon mesh and washed three times with protoplast-washing buffer consisting of 2-(N-morpholino) ethane sulphonic acid (pH 7.5) and 0.6 M KCl (MES). The resulting protoplasts were resuspended with modified MES supplemented with 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 0.4 trypsin inhibition unit/ml aprotinin, 5 mM dithiothreitol, and 0.6 M KCl. The resuspended protoplasts were disrupted by a Potter homogenizer (Auther H. Thomas Co.). Cell debris was removed by centrifugation twice at 1,000 x *g* for 10 min. The resulting cell lysate was designated the PH extract (obtained by Potter homogenization). The PH extract was fractionated into different particle fractions and a final supernatant fraction by differential centrifugation. The 1,000 x *g* pellet fraction (PL) was obtained by centrifugation of the PH extract at 1,000 x *g* for 20 min followed by three washes with MES. The nuclear fraction (NU) was obtained from the post-1,000 x *g* supernatant fraction by centrifugation at 6,000 x *g* for 20 min followed by three washes with MES. The mitochondrial fraction (MT) was obtained from the postnuclear supernatant fraction by centrifugation for 30 min at 20,000 x *g* followed by three washes with MES. The lysosomal fraction (LS) was obtained from the postmitochondrial supernatant fraction by centrifugation for 30 min at 50,000 x *g* followed by three washes with MES. The microsomal fraction (MC) was prepared from the postlysosomal supernatant fraction by centrifugation for 1 h at 100,000 x *g* followed by three washes with MES. The ribosomal fraction (RS) was prepared from the postmicrosomal supernatant fraction by centrifugation at 140,000 x *g* for 1 h followed by three washes with MES. The postribosomal supernatant from this step was saved as the 140,000 x *g* cytosol fraction (CS). For protein concentration determination, a known volume of each fraction was mixed with an equal volume of 4% (w/v) sodium dodecyl sulfate (SDS) buffer and boiled for 5 min. Then the concentration was determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Protocol 2. Separation of organelles and the cytosol fraction by protoplast permeabilization. Protoplasts were prepared as described above. Permeabilization of protoplasts and separation of the organelle and cytosol fractions separation were conducted at 4°C according to the methods of Vida *et al.* (1993). Briefly, protoplasts were washed using the following scheme: a 5 min wash with buffer 1 [250 mM sorbitol, 20 mM HEPES-KOH (pH 6.8), 150 mM potassium acetate, and 5 mM magnesium acetate]; a 3 min wash with buffer 2 (50 mM sorbitol, and 5 mM magnesium acetate); a 5 min wash with buffer 3 (2.0 M potassium acetate, 250 mM sorbitol, and 5 mM magnesium acetate); and a 5 min wash with buffer 4 (250 mM sorbitol, and 5 mM magnesium acetate). After each wash, the cells were harvested by centrifugation for 45 sec at 13,000 x *g*. The combined supernatants from the four washes were the cytosol fraction and the resulting pellets were the organelle fraction. The protein concentration of each fraction was determined as described above. These two fractions were called the PP extracts (obtained from permeabilized protoplasts).

Western Blot Analysis of the Nor-1 Protein in Cell Fractions. Proteins in each cell fraction were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted to a polyvinylidene difluoride (PVDF) membrane (Du Pont Co., Boston, MA) using standard published methods (Ausubel *et al.*, 1995b). The double-purified anti-Nor-1c protein polyclonal antibodies (PAb) (Chapter 3) were used as the primary antibody in Western blot analysis. Immunodetection of the primary antibody was carried out with a chemiluminescent detection kit (Schleicher & Schuell, Keene, NH) according to the manufacturer's instructions. The relative intensity of Nor-1 protein in each lane in Western blot analysis was determined by scanning the X-ray autoradiograph with an EPSON ES-1000C scanner (EPSON Accessory, Singapore) and quantitated with Sigmagel software.

Analysis of Marker Enzyme Activities. The measurement of the activity of succinate dehydrogenase was carried out according to Singer *et al.* (1965) with modifications. This method was originally used to quantitate the total amount of succinate by the use of an excess unknown amount of succinate dehydrogenase. In the current study, the reaction was used to determine succinate dehydrogenase activity by the use of an excess known amount of succinate. The reaction was optimized and carried out for 30 and 60 min at room temperature in a total volume of 3 ml consisting of 0.5 ml of 0.3 M Tris buffer (pH 7.6), 0.1 ml of 0.1 M potassium ferricyanide, 0.3 ml of 0.01M succinate, and 0.05 mg of protein. The optical density of the reaction mixture was measured at 450 nm. One unit of succinate dehydrogenase was defined as the amount of enzyme causing a decrease of 1 mmole succinate in 1 min.

The measurement of catalase activity was conducted as described by Cohen *et al.* (1970). The reaction was optimized and carried out at 4°C for 1, 5, and 20 min in a total volume of 9 ml consisting of 1 ml of 2% (v/v) H_2O_2 solution in 0.01 M phosphate buffer (pH 7.0), and 0.02 mg of protein. The reaction was stopped by addition of 1 ml of 6 N H_2SO_4 followed by addition of 7 ml of 0.01 N $KMnO_4$. The residual H_2O_2 was measured by measuring absorbance at 480 nm. One unit of catalase was defined as the amount of protein required to liberate half the peroxide oxygen from a hydrogen peroxide solution in 100 s (Lück, 1965).

The activity of acid phosphatase was measured with a commercial assay kit (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's instructions. The reaction was carried out for 30 min at 37°C in a total volume of 5 ml consisting of 0.5 ml of 4 mg/ml *p*-nitrophenyl phosphate, 5.0 ml of 0.1 N NaOH, and 0.02 mg of protein. The measurement is based on the hydrolysis of *p*-nitrophenyl phosphate by the acid phosphatase yielding *p*-nitrophenol. Under alkaline conditions, *p*-nitrophenol is converted to a yellow complex readily measured by absorbance at 410 nm. The activity of acid phosphatase (Sigma units/ml) was calculated according to the manufacturer's instructions.

The activity of glucose-6-phosphatase was measured according to the method of Harper (1965) with modifications to optimize the reaction conditions. The reaction was carried out at 37°C for 30 min in a total volume of 0.3 ml consisting of 0.1 ml of 0.1 M citrate buffer (pH 6.5), 0.1 ml of 0.1 M glucose-6-phosphate, and 0.03 mg of protein. The reaction was stopped by addition of 1 ml of 10% (w/v) trichloroacetic acid. After centrifugation, 1 ml of supernatant was mixed with 5 ml of 2 mM molybdate solution and 1 ml of reducing agent (40 mM 1-amino-2-naphthol-4-sulphonic acid) for 15 to 30 min at room temperature. The optical density was measured at 660 nm. One unit of glucose-6-phosphatase was defined as the quantity of enzyme required to liberate 1 mmole phosphate from glucose-6-phosphate in 1 min.

The activity of glucose-6-phosphate dehydrogenase was carried out according to Niehaus and Dilts (1984). The reaction was carried out at 30°C from 1 to 30 min in a total volume of 2.5 ml consisting of 2.2 ml of 0.5 mM glucose-6-phosphate, 25 μl of 25 mM NADP, 25 μl of

25 mM phenazine methosulfate, 200 μl of 5 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and 0.5 mg of protein. The reaction was monitored by measuring absorbance at 578 nm. One unit was defined as the amount of enzyme that catalyzes the NADP-dependent oxidation of 1 mmole of glucose-6-phosphate in 1 min.

Nor-1 Protein Localization by Immunofluorescence Microscopy. Hyphae grown in YES liquid medium were collected by filtration and resuspended in fresh YES liquid medium (1 mg wet hyphae in 5 ml liquid medium). A volume of 50 μl of the hyphal suspension was dropped onto a slide pretreated with 2% (w/v) glycine solution and air dried. The fungal cells on the slide, either from liquid culture or from slide culture, were fixed for 4 h at room temperature or 16 h at 4°C in Histochoice Tissue Fixative (Amresco, Solon, OH). For partial digestion of the cell wall, fungal cells were subjected to a cell wall digestion enzyme solution consisting of 1.5% (w/v) Novozyme 234 in water for 1 h at 30°C. The cell membrane of the hyphal cell was permeabilized with 0.1% (w/v) saponin in Tris-buffered saline (TBS) consisting of 100 mM Tris-Cl (pH 7.5) and 0.9% (w/v) NaCl for 30 min at room temperature. The hyphae were then exposed to the triple-purified anti-Nor-1c protein polyclonal antibodies (PAb) (see Chapter 3) [10 μg /ml in modified TBS supplemented with 0.1% (w/v) saponin and 1% (w/v) bovine serum albumin (BSA)] at room temperature for 1 h. This incubation was followed by three rinses with TBS for 10 min each. The slide preparation was then incubated at room temperature with labeled secondary antibodies [a 100-fold dilution of fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO)] for 1 h. After a final wash with water, the preparation was air dried, mounted on slides with Sigma Diagnostics mounting medium (Sigma Chemical Co., St. Louis, MO), and examined under a

Nikon Labophot fluorescent microscope or a Zeiss 10 laser scanning microscope (LSM) to observe FITC fluorescence. LSM micrographs were taken with the help of Dr. J. H. Whallon (Laser Scanning Microscope Laboratory, Michigan State University, East Lansing, MI).

Semiquantitative Petri Dish Assay for *nor*-1/GUS Activity (Overlay Procedure). The semiquantitative in situ measurement of GUS activity was conducted according to Mönke and Schäfer (1993) with certain modifications. Briefly, melted agar [1.2% (w/v) in water] was kept at 45°C in a water bath and used as the overlay agar. X-glucuronide (5-bromo-4-chloro-3-indolyl glucuronide, X-gluc, Sigma Chemical Co., St. Louis, MO) was dissolved in dimethylsulfoxide (DMSO) (0.5 mg/µl), and added to 5 ml of the overlay agar at a final concentration of 0.3 mg/ml (substrate/agar) and immediately poured onto a Petri dish containing a mycelial colony which had been incubated for the appropriate periods of time. The Petri dish was gently shaken until the entire colony was covered with the overlay agar. The dish with overlay was incubated at room temperature until the blue color fully developed (approximately 3 h).

RESULTS

Distributions of Marker Enzymes and the Nor-1 Protein in Cell Fractions. Cell fractionation of mycelial cells was conducted using two methods: (1) disruption of fungal protoplasts using a Potter homogenizer followed by differential centrifugation (the **PH** method) and (2) separation of the cytosol from organelles by protoplast permeabilization followed by centrifugation (the **PP** method). The resulting cell fractions were subjected to marker enzyme assay, to Western blot analysis for the Nor-1 protein, and to protein concentration determination. The results of Western blot analysis for the Nor-1 protein are shown in Figure 4-1. Distributions of marker enzymes, the percentage of the Nor-1 protein (measured by the relative intensity of the Nor-1 protein band in the Western blot autoradiograph), and the percentage of the total protein in each fraction obtained by the PH method and the PP method are summarized in Table 4-1 and Table 4-2, respectively.

Succinate dehydrogenase (DH) was used as a mitochondrion marker, glucose-6-phosphatase (G-6-phosphatase) as a endoplasmic reticulum (ER) marker, catalase as a peroxisome marker, and acid phosphatase as a lysosome marker (Muller *et al.*, 1991). Glucose-6-phosphate dehydrogenase (G6PDH) was used as a cytosol marker (Vida *et al.*, 1993).

High percentage of each organelle marker enzyme was detected in the cytosol fraction using the PH method (Table 4-1) (succinate DH, 16.4%; G-6-phosphatase 50.6%; catalase, 39.0%; and acid phosphatase, 64.0%). These data suggest that the PH method disrupts a certain fraction of each organelle. Succinate DH (mitocondrion marker) activity was also detected in the mitochondrial fraction (32.5%) and the lysosomal fraction (20.1%). Some overlap in localization of the two markers was expected because mitochondia and lysosomes have similar sedimentation coefficients (10^4 to 10^5 s).

Analyzing the data for the organelle fraction only, the highest level of G-6-phosphatase (ER marker) was detected in the microsomal fraction (24.5%). The highest level of catalase (peroxisome marker) was detected in the lysosomal fraction (20.6%) and the mitochondrial fraction (20.5%) because the sedimentation coefficient of peroxisomes (5×10^4 s) falls within

Figure 4-1. Western blot analysis of the Nor-1 protein in cell fractions of *A. parasiticus*. Mycelial cells were grown in YES liquid medium for 60 h with constant shaking (150 rpm) at 29°C in the dark. Cell fractions were obtained from the wild-type strain SU-1 (except sample NA$^+$). The proteins in each fraction were resolved by SDS-PAGE and then transferred to a PVDF membrane. Each lane was loaded with 10 µg protein. The membrane was probed with the double-purified PAb (10 µg/ml). **A**: Cell fractions were obtained using a Potter homogenizer followed by differential centrifugation (the PH method). **Lanes:** 1 (CT), cytosol fraction; 2 (RB), free ribosomal fraction; 3 (MC), microsomal fraction; 4 (LY), lysosomal fraction; 5 (MT), mitochondrial fraction; 6 (NU) nuclear fraction; 7 (1K), 1,000 x *g* pellet from crude extract; 8 (NA$^+$), crude extract from a norsolorinic acid (NA)-accumulating strain in which *nor*-1 was disrupted; 9 (WL), crude extract from the wild-type strain SU-1. **B:** Cell fractions were obtained by protoplast permeabilization followed by centrifugation (the PP method). **Lanes:** 1 (CT), cytosol fraction; 2 (OG), total organelle fraction; 3 (NA$^+$), crude extract from an NA-accumulating strain in which *nor*-1 was disrupted.

Figure 4-1.

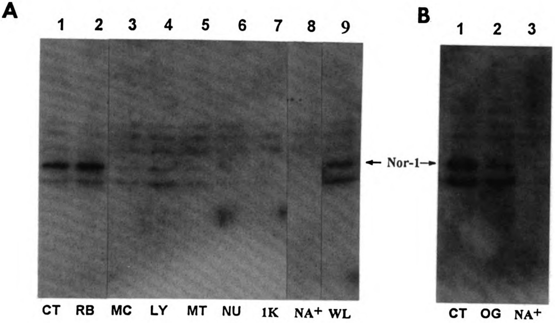


Table 4-1.

Table 4-1. Distributions of marker enzymes and the Nor-1 protein in cell fractions obtained by Potter homogenization and differential centrifugation.

Centrifugation (10^3 x g)	1	6	20	50	100	140	
Cell fraction	1,000 x g Pellet	Nucleus	Mitochondrion	Lysosome	Microsome	Ribosome	Cytosol
Succinate dehydrogenase (%)	0.7 ± 0.2	8.4 ± 1.1	32.5 ± 2.9	20.1 ± 3.4	14.3 ± 4.2	7.6 ± 2.7	16.4 ± 3.3
Glucose-6-phosphatase (%)	0.3 ± 0.1	2.2 ± 0.8	4.1 ± 2.7	9.7 ± 1.4	24.5 ± 10.5	8.6 ± 2.1	50.6 ± 5.3
Catalase (%)	0.4 ± 0.2	5.7 ± 1.2	20.5 ± 6.2	20.6 ± 2.5	8.2 ± 3.2	5.6 ± 1.2	39.0 ± 5.5
Acid phosphatase (%)	0.2 ± 0.1	5.2 ± 1.6	12.4 ± 4.6	11.1 ± 2.7	4.1 ± 2.6	2.4 ± 0.5	64.0 ± 4.8
Total protein (%)	0.3 ± 0.2	8.3 ± 1.9	18.9 ± 5.2	2.2 ± 0.4	1.4 ± 0.5	0.5 ± 0.6	68.6 ± 7.4
Nor-1 (relative) intensity	4.3 ± 0.5	6.4 ± 2.5	8.2 ± 0.8	21.5 ± 5.6	9.8 ± 1.8	100.0	82.3 ± 9.3
Nor-1 (%)	0.0 ± 0.0	0.8 ± 0.5	2.4 ± 0.7	0.6 ± 0.4	0.2 ± 0.2	0.6 ± 0.4	95.4 ± 4.7

Notes:

(1) Total protein (%): (the total protein in one fraction) ÷ (the sum of the total protein in each fraction).

(2) Nor-1 (relative) intensity: 100 × [the intensity of Nor-1 protein in one lane (from the corresponding fraction) on the Western blot autoradiograph] ÷ [the intensity of Nor-1 protein in the lane (from the corresponding fraction) with the highest intensity on the same Western blot autoradiograph] (Figure 4-1A).

(3) Nor-1 (%): {(the intensity of Nor-1 protein in one lane) × [the total protein (%) of the corresponding fraction]} ÷ { the sum of [the intensity of the Nor-1 protein in each lane × the total protein (%) of the corresponding fraction]}.

(4) Each value was the mean ± standard error (SE) from tests performed in triplicate.

Table 4-2. Distributions of marker enzymes and the Nor-1 protein in total organelle and the cytosol fractions obtained by protoplast permeabilization and centrifugation.

	Total organelles	Cytosol
Glucose-6-phosphate dehydrogenase (%)	3.0 ± 0.8	97.0 ± 2.6
Succinate dehydrogenase (%)	89.4 ± 5.4	10.6 ± 4.2
Catalase (%)	85.7 ± 4.6	14.3 ± 10.5
Total protein (%)	13.0 ± 3.7	87.0 ± 11.7
Nor-1 relative intensity	15.0 ± 3.9	100
Nor-1 (%)	2.2 ± 0.7	97.8 ± 2.3

Notes:

(1) **Total protein (%)**: (the total protein in total organelles or in the cytosol) ÷ (the sum of the total protein in total organelles and the cytosol).

(2) **Nor-1 (relative) intensity**: 100 × [the intensity of Nor-1 protein in one lane (from total organelles or the cytosol fraction) on the Western blot autoradiograph] ÷ (the intensity of the Nor-1 protein in the cytosol on the same Western blot autoradiograph) (Figure 4-1B).

(3) **Nor-1 (%)**: {[the intensity of Nor-1 protein in one lane (from total organelles or the cytosol fraction)] × [the total protein (%) of total organelles or the cytosol fraction]} ÷ { the sum of [the intensity of Nor-1 protein in each lane × the total protein (%) of the corresponding fraction]}.

(4) Each value was the mean ± standard error (SE) from tests performed in triplicate.

the range of sedimentation coefficients of lysosomes and mitochondria (10^4 to 10^5 s). The highest level of acid phosphatase (lysosome marker) was detected in the mitochondrial fraction (12.4%) and the lysosomal fraction (11.7%) because of the same reason as mentioned above. These data suggest that differential centrifugation method can successfully fractionate organelles according to their sedimentation coefficients even though a large number of organelles were destroyed by the PH method.

By the PP method, 97.0% of G6PDH (cytosol marker) was detected in the cytosol fraction while 89.4% of succinate DH (mitochondrion marker) and 85.7% of catalase (peroxisome marker) were detected in the organelle fraction (total organelles). In comparison, 83.6% of

Figure 4-2. Immunolocalization of the Nor-1 protein in hyphal cells of *A. parasiticus* SU-1 grown in YES liquid medium. Mycelial cells were incubated in YES liquid medium for 60 h with constant shaking (150 rpm) at 29°C in the dark. The triple-purified PAb was used as the primary antibodies (10 μg/ml). The secondary antibodies (FITC-conjugated anti-rabbit IgG) were diluted 100-fold. Photographs (magnification, 200 x): **A1,** fluorescence micrograph of SU-1 (the wild-type) grown for 48 h; **A2,** sample A1 under white light; **B1,** fluorescence micrograph of a *nor*-1-disrupted strain grown for 48 h; **B2,** sample B1 under white light; **C1,** fluorescence micrograph of SU-1 (the wild-type) grown for 18 h; **C2,** sample C1 under white light.

Figure 4-2.

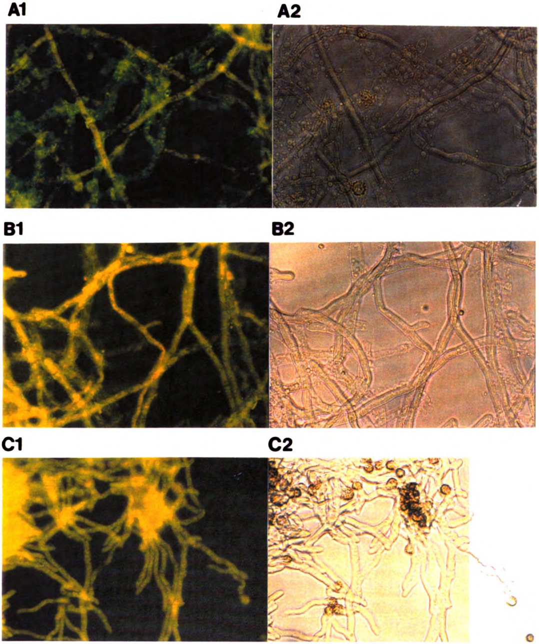


Figure 4-3. Immunolocalization of the Nor-1 protein in *A. parasiticus* SU-1 grown on YES agar. The fungus was incubated on YES agar at 29°C for 60 h in the dark. The triple-purified PAb was used as the primary antibodies (10 µg/ml). The secondary antibodies (FITC-conjugated anti-rabbit IgG) were diluted 100-fold. Photographs (magnification, 280 x): **A1,** fluorescence micrograph of SU-1 (the wild-type); **A2,** sample A1 under white light; **B1,** fluorescence micrograph of a typical hyphal cell SU-1 (the wild-type); **B2,** sample B1 under white light; **C1,** fluorescence micrograph of a *nor*-1 disrupted strain; **C2,** sample C1 under white light.

Figure 4-3.

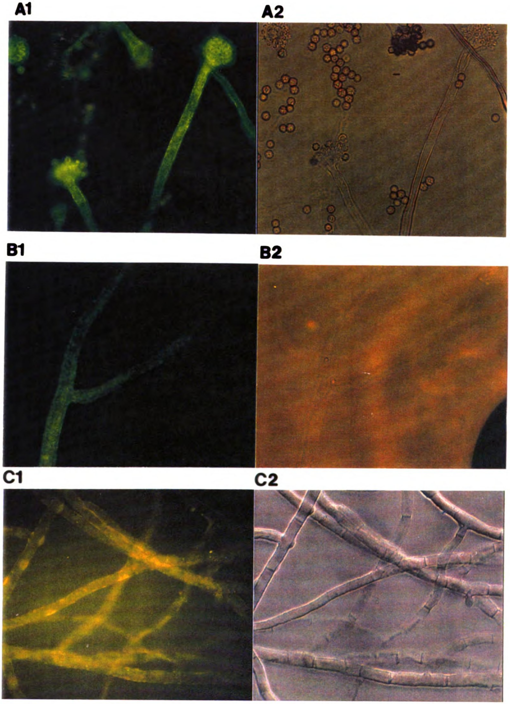
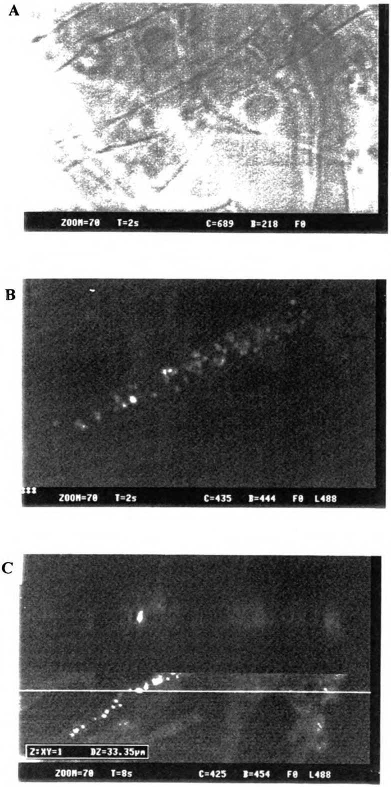


Figure 4-4. Immunolocalization of the Nor-1 protein in *A. parasiticus* SU-1 by laser scanning microscopy (LSM). Mycelial cells were grown in YES liquid medium at 29°C in the dark for 48 h with constant shaking (150 rpm). The triple-purified PAb was used as the primary antibodies (10 μg/ml). The secondary antibodies (FITC-conjugated anti-rabbit IgG) were diluted 100-fold. Photographs (magnification, 350 x): **A**, hyphal cells under white light; **B**, fluorescence micrograph of sample A; C, fluorescence micrograph (the lower part) taken in the X-Y plane and the phi-z section micrograph (the upper part) taken along the Z axis and through a straight white line selected in the lower part (see details in Discussion).

Figure 4-4.


A
ZOOM=70 T=2s C=689 B=218 F0
B
ZOOM=70 T=2s C=435 B=444 F0 L488
C
Z:XY=1 DZ=33.35µm
ZOOM=70 T=8s C=425 B=454 F0 L488

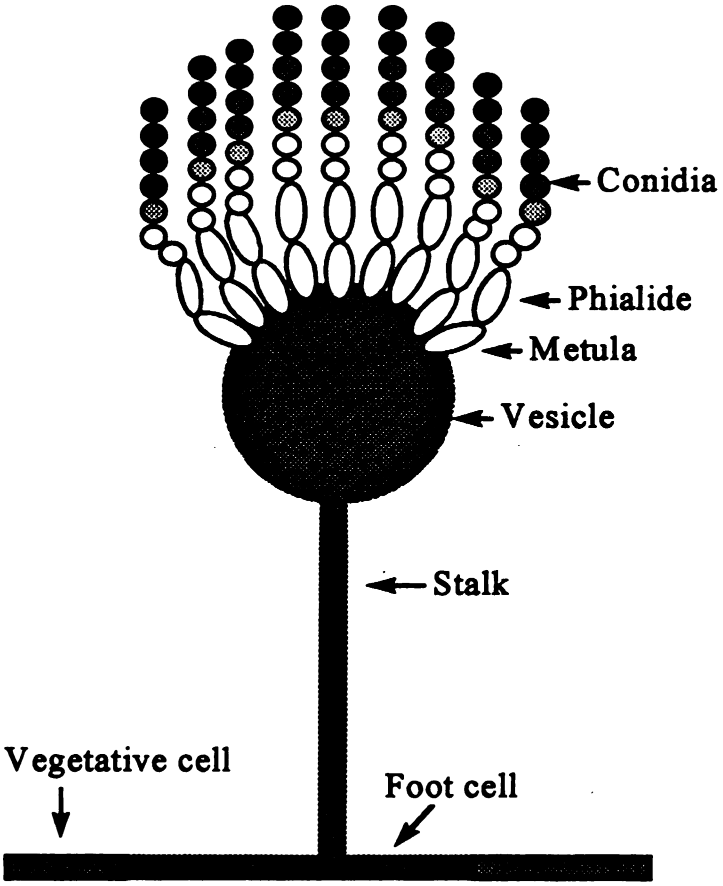


Figure 4-5. Schematic of a conidiophore of *Aspergillus parasiticus*. The process of sporulation in *A. parasiticus* starts with a substrate somic hyphal cell which is called the foot cell. The foot cell branches to give rise the conidiophore which is a long, erect hyphal cell terminating in a bulbous vesicle. As the multinucleate vesicle develops, a large number of conidiogenous cells, which are called phialides (or sterigmata), are produced over the vesicle. One or two layers of phialides (the first layer of phialides is sometimes called metula) may be produced. As phialides reach maturity, they begin to form conidia at their tips one below the other in chains (Alexopoulos and Mims, 1979; Kale *et al.*, 1994). The conidiophore does not include the foot cell and conidia. To facilitate description of the localization of Nor-1 protein accumulation and the localization of Nor-1 promoter activity, the vesicle with phialides attached is called "the conidial head"; the long and erect structure of the conidiophore is called the conidial stalk in this study. The schematic figure of a conidiophore was drawn according to previous descriptions (Alexopoulos and Mims, 1979; Kale *et al.*, 1994) and scanning electron micrographs (Tsuneda, 1983).

succinate DH and 61.0% of catalase were detected in the organelle fraction by the PH method. This suggest that fewer organelles were destroyed by the PP method than the PH method.

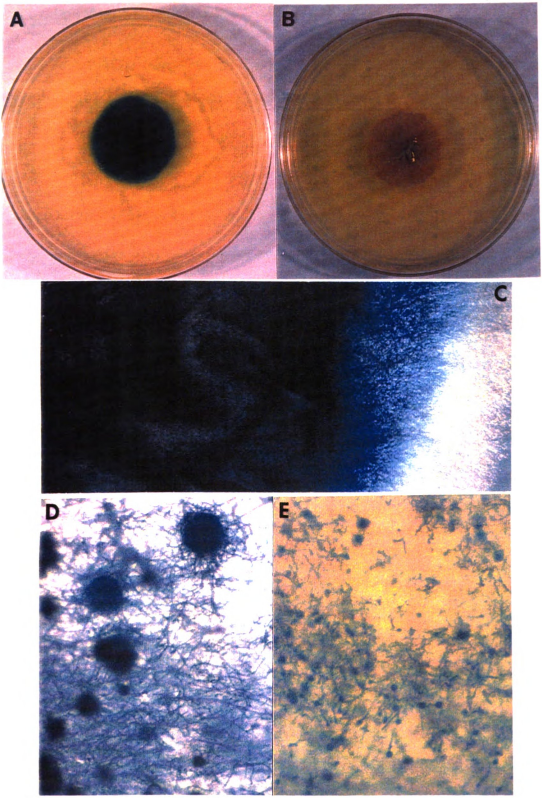
The majority of Nor-1 protein was detected in the cytosol fraction (95.4%) and a trace amount was detected in the nuclear, mitochondrial, lysosomal, microsomal, and ribosomal fractions (0.8%, 2.4%, 0.6%, 0.2%, and 0.6%, respectively) by the PH method. The majority of the Nor-1 protein was detected in the cytosol fraction (97.8%) and a trace amount was detected in the (total) organelle fraction (2.2%) by the PP method. These data suggest that the Nor-1 protein is not tightly associated with cellular organelles.

Subcellular Immunolocalization of the Nor-1 Protein. The Nor-1 protein was localized in situ in *A. parasiticus* grown in liquid medium and on solid medium using a fluorescent antibody probe. The appearance of green fluorescence (presence of FITC-labeled secondary antibody) was used as an indicator of the level of Nor-1 protein accumulation (Figure 4-2). Using this immunolocalization technique, most of the Nor-1 protein appeared to be localized in the cytosol and a significant quantity of Nor-1 protein was found to associate with "particles" of uncharacterized compositions in hyphal cells from the wild-type strain *A. parasiticus* SU-1 grown in YES liquid medium for 48 h (Figure 4-2A). In contrast, the fluorescence was not detected in two negative controls, a *nor*-1 mutant (gene disruption) of *A. parasiticus* grown in YES liquid medium for 48 h (Figure 4-2B) and the wild-type *A. parasiticus* SU-1 grown in YES liquid medium for 18 h (Chapter 2) (Figure 4-2C). More information was obtained from the fungus grown on solid media. The immature conidium showed the most intensive green fluorescence (during photography, the most intensive green color artifactually appeared yellow); while the corresponding conidial stalk showed stronger fluorescence than that observed in the vegetative hyphal cell (Figure 4-3A). In agreement with the data from liquid culture, the majority of Nor-1 protein was localized in the cytosol. However, a significant quantity was also found to associate with "particles" in hyphal cells of the wild-type *A. parasiticus* SU-1 grown on YES solid medium for 60 h (slide culture) (Figure 4-3B). The *nor*-1 disrupted mutant (negative control) grown on YES solid medium (slide culture) for 60 h did not show detectable fluorescence (Figure 4-3C). Laser scanning microscopy (LSM) was used to collect more information about the subcellular location of the Nor-1 protein. Micrographs of hyphal cells from *A. parasiticus* SU-1 grown in YES liquid medium for 48 h were generated by LSM using white light (Figure 4-4A), fluorescence (Figure 4-4B), and fluorescence in phi-z section (Figure 4-4C). The phi-z section represents the optical section taken in the vertical Z plane (the upper part of Figure 4-4C) which passes through a straight line (the white line which passes through a selected fluorescent "particle" shown in the lower part of Figure 4-4C) in the micrograph taken in the X-Y plane (the lower part of Figure 4-4C). The position and the shape of the fluorescent "particle" in the phi-z section indicated that it was located within the cell and not stuck to the outside surface. A schematic of the structure and the development of a conidiophore are shown in Figure 4-5 for reference.

In Situ Localization of *nor*-1 Promoter Activity in a Colony of *A. parasiticus*. The close correlation between *nor*-1 promoter activity and the process of sporulation in *A. parasiticus* grown on solid media was initially observed using in vitro analysis (Chapter 3). In order to confirm the in vitro data, in situ localization of *nor*-1 promoter activity was conducted in a fungal colony. An *A. parasiticus* strain, *nor*-1/GUS (the same strain as used in

Figure 4-6. In situ analysis of *nor*-1 promoter activity within a colony of *A. parasiticus* grown on solid medium. The fungus in all the colonies (except colony **B**) was a *A. parasiticus* transformant containing a *nor*-1/GUS reporter construct. The fungus in colony **B** was the wild-type SU-1 which did not contain the *nor*-1/GUS reporter construct. All colonies were incubated on YES agar in Petri dishes and then overlaid with an overlay agar containing a chromogenic GUS substrate X-glucuronide. Photographs: **A** and **B** (magnification, 0.7 x), colonies grown for 3 days (colony **A** and **B** had the same morphology before being overlaid); **C** (magnification, 5 x), part of a colony grown for 3 days; **D** (magnification, 30 x), vegetative hyphal cells, and hyphal cell masses (blue) of a colony grown for 5 days; **E** (magnification, 25 x), conidial heads and conidia of a colony grown for 4 days.

Figure 4-6.



Chapter 3) which contains a *nor*-1/GUS reporter construct integrated at the *nor*-1 locus (Chapter 3), was incubated on YES solid medium for appropriate periods of time and then overlaid with melted agar (45°C) containing a chromogenic β-glucuronidase substrate (5-bromo-4-chloro-3-indolyl glucuronide). Accumulation of a blue colored end product generated by β-glucuronidase indicates β-glucuronidase activity. The blue color was observed in all areas of the colony except at the outer margin (Figure 4-6A). No blue color was observed in the control colony of the wild-type SU-1 which did not contain the *nor*-1/GUS reporter construct (Figure 4-6B).

The following describes GUS activity in a section (Figure 4-6C with a higher magnification than Figure 4-6A) of a colony of the *nor*-1/GUS strain (grown for 3 days) proceeding from the outer margin gradually toward the center. No blue color was detectable in the peripheral region (0.1-0.4 cm in width) in which only substrate hyphae were observed. An blue color was observed in the adjacent region (0.1-0.4 cm in width) in which aerial hyphae were dominant. A more intense blue color was observed in the next adjacent region (0.8-1.0 cm in width) in which immature conidia were dominant. A less intense blue (or no blue) color was observed at the center (0.04-0.06 cm in diameter) in which only mature conidia were found. The in situ data were consistent with the in vitro data (Chapter 3) which suggested that *nor*-1 promoter activity was closely correlated to the process of sporulation in *A. parasiticus*. At certain stages of growth on solid media (e.g. after 5 days of incubation), some vegetative hyphae formed tightly interwoven round structures which showed an intense blue color (Figure 4-6D). When a colony was overlaid with the overlayer agar (containing X-glucuronide) and was observed under a dissection microscope at 15-min time intervals, the blue color appeared first in the conidial head with conidia attached, second in the conidial stalk, and last in the vegetative hyphal cell (Figure 4-6E).

DISCUSSION

Three tentative conclusions can be drawn from the data presented in this study: (1) the Nor-1 protein was localized mainly in the cytosol of the hyphal cell; (2) the Nor-1 protein was found in immature conidia and conidial heads, conidial stalks, vegetative hyphae, and mature conidia in the order from the highest level to the lowest level (undetectable); and (3) the localization of *nor*-1 promoter activity is closely correlated to the process of sporulation in *A. parasiticus* SU-1 grown on solid media.

The primary purpose of cell fractionation in this study was to check if the Nor-1 protein is enclosed in or strongly associated with certain subcellular particles or organelles. The methods used for sample preparation for cell fractionation was a major factor which influenced the reliability of subcellular localization of the Nor-1 protein in this study. One of the most widely used methods for determination of the effectiveness of cell fractionation is the bioassay for specific marker enzymes. The marker enzyme assay suggested that Potter homogenization (the PH method) destroyed a large number of organelles. However, the marker enzyme assay also suggested that differential centrifugation, a technique introduced by the Nobel laureate Albert Claude, effectively separated different subcellular particles according to their different sedimentation coefficients when used after sample preparation by the PH method.

The protoplast permeabilization (PP) method was shown to be more effective in preserving the integrity of cellular organelles. This method can remove the cytosol from the permeabilized cell and maintain organelle associations with the permeabilized cell (Vida *et al.*, 1993). The buffers used to remove cytosol contain physiological levels of magnesium and potassium ions, which are required to maintain organelle associations with the permeabilized cell. With short time and low speed centrifugation, the cytosol and permeabilized cells are effectively separated (Vida *et al.*, 1993).

For example, glucose-6-phosphate dehydrogenase (G6PDH) has been established as a useful cytosol marker in *Aspergillus* (such as *A. niger*, *A. nidulans*, *A. aspergillus*) by several researchers (Carson and Cooney, 1990; Broek *et al.*, 1995; Vida *et al.*, 1993). The high percentage (97%) of G6PDH detected in the cytosol fraction by the PP method in this study was similar to the data (98%) of Vida *et al.* (1993) and Broek *et al.* (1995). Higher percentages of succinate DH (mitochondrion marker) and of catalase (peroxisome marker) were detected in the particle fraction by the PP method than by the PH method.

Similar percentages of the Nor-1 protein were detected in the cytosol fraction by either the PH method (96%) or the PP method (98%) suggesting the Nor-1 protein is not enclosed in or strongly associated with a particle(s) or an organelle(s). If the Nor-1 protein were enclosed in or strongly associated with a particle(s) or an organelle(s), it might be predicted that a higher percentage of the Nor-1 protein would have been detected in the cytosol by the PH method than the PP method because more organelles were destroyed by the PH method than the PP method. The data did not support this hypothesis.

Even though the data suggest that the Nor-1 protein is not enclosed in or strongly associated with a particle(s) or an organelle(s), the highest concentration of Nor-1 protein was detected in the free ribosomal fraction by the PH method suggesting that a significant quantity of the Nor-1 protein is associated with free ribosomes or with particles (possible the complex consisting of proteins involved in the AFB_1 pathway) with significant mass which comigrated with free ribosomes during differential centrifugation.

The immunolocalization data supported an association of the Nor-1 protein with a particle(s) or some sort. For example, in mycelial cells grown in YES liquid medium or grown on YES solid medium, most of the Nor-1 protein appeared to be localized in the cytosol but a significant quantity appeared to be associated with particles by immunolocalization. This observation was confirmed by Laser Scanning Microscopy (LSM). Comparing the micrograph taken in the X-Y plane with the micrograph taken in the vertical Z plane, a fluorescent particle was localized inside the cell. The remaining Nor-1 protein, which was not associated with particles, could in theory spread throughout the cytosol. When the phi-z section was observed in areas where the Nor-1 protein was not attached to any particles, there should be a "smear" with weak fluorescence and the height of the "smear" along the z-axis direction should be approximately equal to the cross sectional diameter of the hyphal cell. If all of the Nor-1 protein were simply attached to the surface of the cell, the phi-z section would have shown a circle of fluorescence with no "smear". In the actual experiment, the phi-z section showed that the Nor-1 protein, when it was not associated with any particle(s) or organelle(s), was extensively distributed throughout the cytosol.

The observation that a significant quantity of the Nor-1 protein appeared to be associated with "particles" of large mass by in situ immunolocalization at first appears to be inconsistent with the data obtained by the PH method and the PP method by which only a little amount of the Nor-1 protein was localized in particles or organelles. One possible explanation which fits the experimental data is that the Nor-1 protein is associated with "particles" of small mass. These small particles are in turn loosely associated with particles or organelles of much larger mass which are detected in situ immunolocalization. Such an association could be destroyed during protoplast homogenization and protoplast permeabilization. Because of limitations of resolution and magnification, fluorescence microscopy and laser scanning microscopy cannot distinguish small organelles such as ribosomes and peroxisomes. Even if the Nor-1 protein was associated with small particles which distributed extensively in the cell, the Nor-1 protein would still seem to be localized in the cytosol. However, a larger fraction of the small particles might remain associated with the large particles when cells were fixed immediately after harvest for in situ immunolocalization. This hypothesis can be addressed more easily by electron microscopy.

The Nor-1 protein may be associated with small particles which may still reside primarily in the cytosol for an important reason. As an early intermediate in the AFB_1 pathway, norsolorinic acid could be a precursor for other secondary metabolites. A group of pigments with the basic structure of norsolorinic acid and a group of xanthones with the basic structure of sterigamatocystin were detected in the genus *Aspergillus* in the 1960's and 1970's (Steyn *et al.*, 1980). Norsolorinic acid, which does not possess a bisfuran ring, does not exhibit genotoxicity. Versicolorin A (VA) and other later intermediates (after VA) in the AFB_1 pathway, which possess a bisfuran ring, are mutagenic and genotoxic (Mori *et al.*, 1985). Therefore the earlier nontoxic or less toxic intermediates (like norsolorinic acid) in the AFB1 pathway may not be compartmentalized and may take part in other biosynthetic pathways. The more toxic intermediates may be compartmentalized in order to protect the producing cell from toxicity and to increase the efficiency of aflatoxin biosynthesis.

The localization of Nor-1 protein accumulation is closely correlated to the process of sporulation in *A. parasiticus* SU-1 grown on YES agar. Immunolocalization data showed that the conidial head, on which immature conidia were dominant, showed the highest level of the Nor-1 protein, the conidial stalk showed a higher level of the Nor-1 protein than the vegetative hyphal cell; and mature conidia, which did not attach to the conidial head, showed undetectable Nor-1 protein. These data were consistent with the in vitro data for the accumulation of the Nor-1 protein, the accumulation of aflatoxin, and the activity of the *nor*-1 promoter presented previously in this dissertation (Chapter 3).

The localization of *nor*-1 promoter activity was also closely correlated to the process of sporulation in *A. parasiticus* SU-1 grown on YES agar. The in vivo localization of *nor*-1 promoter activity in a colony was monitored by the *nor*-1/GUS reporter construct (blue color). Areas of the colony containing substrate hyphae showed no detectable blue color, areas containing aerial hyphae showed an intense blue color, areas containing immature conidia showed a more intense blue color, and areas containing mature conidia showed dark-green color which was the color of wild-type conidia (during photography the dark-green color artifactually turned to dark-brown). The pattern of appearance of blue color was consistent with the process of sporulation. Sporulation initiates with a foot cell (at the substrate level) which generates an aerial hyphal cell. This aerial hyphal cell then goes through a series of morphological steps including a conidial stalk, a conidial head with immature conidia, mature conidia attached to the conidial head, to mature conidia falling off the conidial head. Therefore the localization of the *nor*-1 promoter activity was consistent with the process of sporulation.

The correlation between the localization of *nor*-1 promoter activity and the process of sporulation was further supported by observation of the blue color development in individual conidiophores under a dissection microscope at 15-min time intervals. The conidial head with immature conidia was first to show the blue color, the conidial stalk second, and the vegetative hyphal cell last. This difference in the appearance of the *nor*-1 promoter activity in different location could be due to two possible reasons: (1) real differences of the *nor*-1 promoter activity and GUS activity in the conidial head, the conidial stalk, and the vegetative hyphal cell; or (2) differences in substrate permeability. A demonstration of the second hypothesis is difficult because substrate permeability is determined by at least two factors: the structure of cell wall and cell membrane of the fungus, and the chemical and physical nature of the substrate. However the first hypothesis is supported by in situ immunolocalization data. Further research is required to confirm this observation.

Sclerotia (surviving structures) are relatively massive structures composed entirely of vegetative cells which arise by repeated hyphal branching and intensely interwoven. At maturity, sclerotia contain a mixture of darkly pigmented and hyaline (colorless) hyphae (Deacon, 1980). Accumulation of aflatoxins in sclerotia was observed in some strains of *A. flavus* and *A. parasiticus* (Wicklow and Shotwell, 1983). In this study, relatively massive structures composed entirely of vegetative cells, which showed an intense blue color, were observed suggesting that these intensely stained blue colored masses may be developing or immature sclerotia. A wild range of environmental and internal factors influence the initiation and subsequent development of sclerotia. For example, internal morphological factors have been suggested to be involved in the initiation of sclerotia (Chet and Henis, 1975). Therefore, it is possible that aflatoxin gene expression is corregulated with two different developmental processes (sporulation and scelrotial development) in *A. parasiticus*.

In summary, the localization data suggest that the Nor-1 protein resides mainly in the cytosol of vegetative hyphae and that localization of Nor-1 protein accumulation and the localization of *nor*-1 promote activity are closely correlated to the process of sporulation in *A. parasiticus*. The studies in this chapter present preliminary data on the subcellular localization of Nor-1 protein. The in situ subcellular immunolocalization data need to be confirmed by the use of electron microscopy.

CHAPTER 5

ISOLATION AND ANALYSIS OF *FLUP*, A GENE ASSOCIATED WITH HYPHAL GROWTH, HYPHAL DEVELOPMENT AND SPORULATION IN *ASPERGILLUS PARASITICUS*

ABSTRACT: To study a gene (*flu*P) encoding a putative polyketide synthase, a disruption vector was constructed and transformed into *Aspergillus parasiticus*. Disruption of *flu*P resulted in slow growth with cotton-like hyphae, no sporulation, and reduced aflatoxin accumulation. The data suggest that *flu*P is associated with vegetative cell growth, sporulation, and in turn indirectly influences aflatoxin accumulation in *A. parasiticus*.

INTRODUCTION

Research on the molecular biology of aflatoxin biosynthesis may lead to development of novel methods to reduce or eliminate aflatoxin contamination. However research on the general biology of the toxigenic fungi encompassing areas such as the regulatory mechanisms involved in hyphal growth, hyphal differentiation, and sporulation is also useful. Blocking or interfering in any of these processes would help to eliminate or reduce aflatoxin accumulation. Studies on the genes involved in these basic cell functions may also provide useful information for the development of new fungicides, improvement of agronomic practices, development of resistant crops (by genetic engineering or classical breeding), and development of biocontrol agents (such as nontoxigenic competitive fungi) for control of aflatoxin contamination. Unfortunately, relatively little research data have been published related to this area.

Because aflatoxins are derived from a polyketide pathway (Kurtzman *et al.*, 1987), the producing fungi are predicted to have a polyketide synthase (PKS) related to aflatoxin biosynthesis. Previous studies showed that a 680-bp *ClaI*/*Hind*III DNA fragment from an aflatoxin-producing strain, *A. parasiticus* SU-1, hybridized to a 1.5-kb cDNA fragment containing the β-ketoacyl synthase functional domain of 6-methylsalicylic acid synthase (MSAS) complex from *Penicillium patulum* (Beck *et al.*, 1990). As expected, the deduced amino acid sequence from the 680-bp *ClaI*/*Hind*III DNA fragment was very similar (72% identity) to that of the β-ketoacyl synthase functional domain of MSAS of *P. patulum*. It was originally hypothesized that the gene containing the 680-bp DNA fragment from *A. parasiticus* encodes a PKS which is involved in aflatoxin biosynthesis because no published data suggested that patulin and/or its precursor 6-methylsalicylic acid is produced in *A. parasiticus*. On the other hand, it is also known that certain polyketides are closely related to cell differentiation, development, and sporulation (Summers *et al.*, 1995; Revill *et al.*, 1995; Keller and Adams, 1995). Therefore a reasonable alternative hypothesis is that the gene containing the 680-bp DNA fragment is a PKS gene involved in either aflatoxin biosynthesis, cell growth, cell differentiation, or sporulation in *A. parasiticus*. The data presented in this chapter demonstrated that disruption of the suspected PKS gene (*flu*P) resulted in slow growth with cotton-like hyphae, no sporulation, and reduced aflatoxin accumulation. This suggests that *flu*P is associated with hyphal cell growth, hyphal cell development, and sporulation, and in turn indirectly influences aflatoxin synthesis in *A. parasiticus*.

MATERIALS AND METHODS

Bacterial Strain, Fungal strains, and Incubation Conditions. Plasmids were amplified in *Escherichia coli* DH5α F'^{e}[F'/*endA1 hsdR17*(rK$^-$ mK$^+$)*supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*) U169 (*m80lacZ*ΔM15)] using standard methods (Ausubel *et al.*, 1987). *Aspergillus parasiticus* NRRL 5862 (ATCC 567755, SU-1) was used as the aflatoxin-producing wild-type strain. *A. parasiticus* NR-3, a nitrate reductase (encoded by the *nia*D gene) deficient strain derived from *A. parasiticus* SU-1 (Horng *et al.*, 1990), was used as the host stain for *flu*P disruption. Fungal strains were maintained as stock spore suspension at -80°C in 20% (v/v) glycerol. Czapek-Dox (Difco laboratories, Detroit, MI) agar (CDA) medium containing sodium nitrate as the sole nitrogen source was used for selection of *nia*D$^+$ transformants. Aflatoxin-inducing media, coconut agar medium (CAM) (Arseculeratne *et al.*, 1969), potato dextrose agar (PDA) medium (Difco Laboratories, Detroit, MI), and yeast extract-sucrose (YES) liquid medium and YES agar (Chapter 4) were used to grow fungal mycelia for genomic DNA and total RNA preparation, for aflatoxin extraction, and for observation of morphology. Reddy's medium (Reddy *et al.*, 1971), a chemically defined and aflatoxin-inducing medium, was also used to grow mycelia for RNA preparation.

Fungal Culture, Dry Weight Determination, Growth Rate Measurement, and Aflatoxin Analysis. For growth of fungi on solid medium, 5 µl of a stock spore suspension (1.5 x 10^6 spores/ml) were inoculated onto the center of agar media and incubated at 29°C in the dark. For the non-sporulating *flu*P-disrupted strain, hyphae were removed from a fluffy colony with a toothpick and transferred onto the center of agar media and incubated at 29°C in the dark. The quantity of hyphae transferred was determined by scraping the hyphae from the toothpick, resuspending in 1 ml of YES liquid medium, and spreading the hyphal suspension on CAM Petri dishes. The number of colonies which grew on each Petri dish was between 50 and 200. The differences in inoculum level did not appear to influence the growth rate of the colonies. The majority of the colonies (95%) initiated from toothpick-transfer had the same growth rate. Colonies initiated either from spore-suspension (more than 95% showed the same growth rate) or from hyphal-transfer with different growth rates (compared with the majority of the colonies prepared by the same procedure) were eliminated from the analysis. Mycelial samples were collected by using a spatula to scrape mycelia from the surface of agar. The collected mycelia were either used immediately, or frozen under liquid nitrogen and stored at -80°C until use. The dry weight of mycelia grown on solid medium was determined by the filtration-drying method of Olsson and Jennings (1991). For growth of fungi in liquid medium, a stock spore suspension was inoculated into liquid medium to a final spore concentration of 1.5x10^5 spores/ml, and grown at 29°C in the dark with constant shaking (150 rpm). Mycelia in liquid media were collected by filtration through Miracloth (Calbiochem-Novabiochem Corporation, La Jolla, CA), and were either used immediately, or frozen under liquid nitrogen and stored at -80°C until use.

Colony growth rate was measured by the time (in days) which the colony took to cover the surface of agar medium in a Petri dish or by the mycelial dry weight (mg) per Petri dish.

Aflatoxin B_1 was analyzed by using direct competitive enzyme-linked immune absorbent assay (ELISA) according to the method of Pestka (1980). Aflatoxin B_1 monoclonal antibodies used in this study were kindly provided by Dr. J. Pestka (Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI).

Temporal and Regional Collection of Samples from Solid Medium. To measure aflatoxin accumulation in wild-type *A. parasiticus* NR-3 grown on YES agar in a Petri dish, mycelial samples were collected according to a specific temporal and regional schedule as described previously (Chapter 3, Materials and Methods). Briefly, samples were collected from the central region of YES agar 2, 4, 5, and 6 days after inoculation; from the middle region 4, 5, and 6 days after inoculation; and from the peripheral region 5, and 6 days after inoculation. For collecting samples of the *flu*P-disrupted transformant grown on YES agar, the same regional schedule was used but the time points were changed to 6, 12, 15, and 18 days after inoculation, because the colony of wild-type NR-3 took 6 days to cover the YES agar in a Petri dish, while the *flu*P-disrupted transformant took 18 days to cover the same area.

Plasmid Preparation and Protoplast Transformation. Plasmid minipreparation was performed by the boiling method, and large-scale preparation was performed by the alkaline lysis procedure (Ausubel *et al.*, 1987). Transformation of protoplasts of *A. parasiticus* NR-3 with the disruption vector was conducted using minor modifications of the polyethylene glycol method (Oakley *et al.*, 1987) as previously described (Skory *et al.*, 1990).

Nucleotide Sequence Analysis and Amino Acid Sequence Comparison. Nucleotide sequence data were analyzed with the Wisconsin Genetics Computer Group Package. Comparisons of the predicted amino acid sequence to EMBL and GenBank databases libraries were conducted with TFastA and Gap and aligned with Pileup from the Wisconsin Genetics Computer Group Package.

Isolation and Analysis of Genomic DNA and Total RNA. Fungal mycelia collected from either liquid media or solid sample were used for DNA and RNA isolation and purification. Genomic DNA was isolated and purified by a published modification (Skory *et al.*, 1990) of a phenol-chloroform protocol developed for mammalian DNA isolation and purification (Ausubel *et al.*, 1995d). Total RNA was isolated and purified using the hot-phenol method as previously described (Ausubel *et al.*, 1995e). Restriction endonucleases utilized in analysis of DNA were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) or New England BioLabs (Beverly, MA) and were used according to the manufacturer's instructions. Northern (RNA) and Southern (DNA) hybridization analyses were conducted using published procedures (Ausubel *et al.*, 1995e, 1995f) with the modified hybridization buffer and conditions recommended by Stratagene Cloning Systems (La Jolla, CA). Approximately 10 μg of genomic DNA or 30 μg of total RNA per sample were separated by agarose gel electrophoresis and then transferred by capillary action to a nylon membrane (Nytran membrane, Schleicher and Schuell Inc., Keene, NH) which served as a solid support. Radio-labeled DNA probes were generated with [^{32}P]-dCTP (Du Pont Co., Boston, MA) and the Random Primed DNA labeling Kit (Boehring Mannheim Biochemical, Indianapolis, IN) according to the manufacturer's instructions. After the final wash, the membranes were exposed to X-ray film (Dodak-XAR5, Eastman Kodok Co., Rochester, NY) at -80°C. DNA probes (specified in the figure legends) were used in both Southern and Northern analysis.

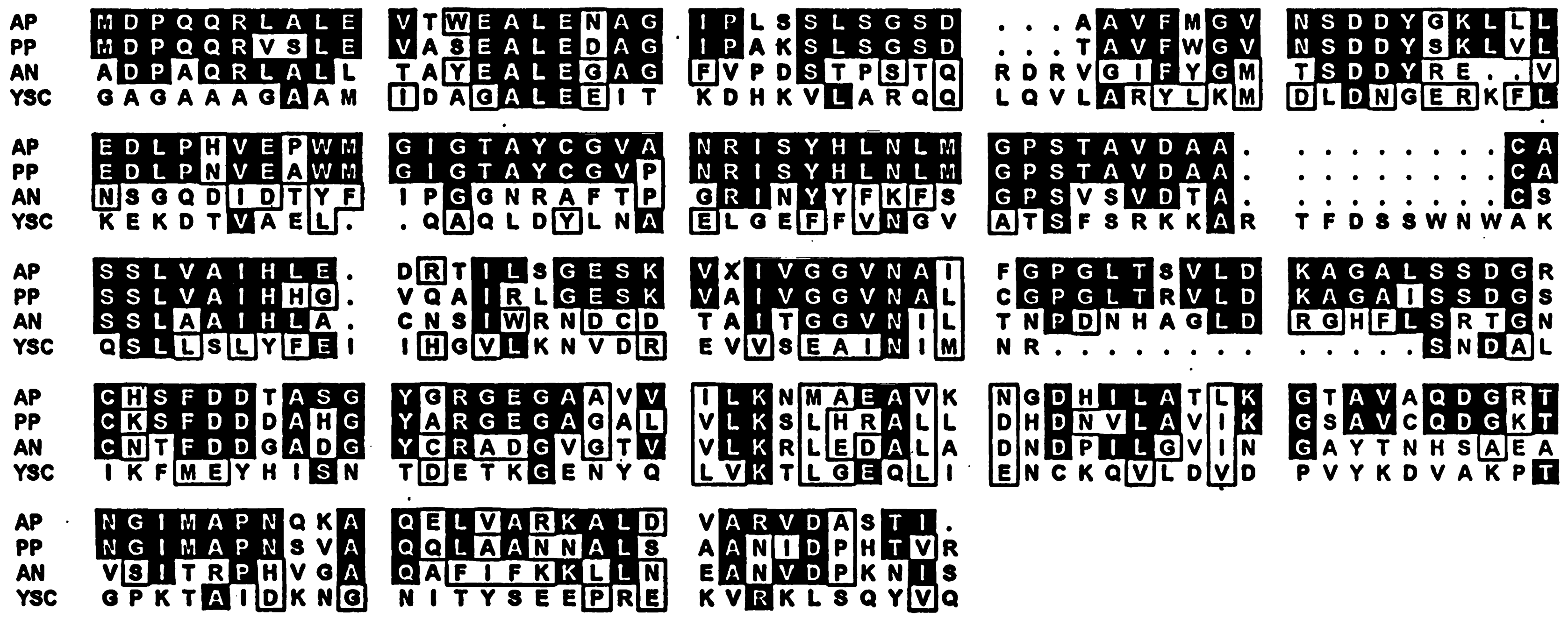
RESULTS

Analysis of the Coding Region of *flu*P. In a previous study conducted by R. Rasooly in our laboratory, a 680-bp *ClaI/Hind*III DNA fragment from *A. parasiticus* was shown to hybridize to a 1.5-kb cDNA fragment containing the β-ketoacyl synthase functional domain of 6-methylsalicylic acid synthase (MSAS) complex from *Penicillium patulum* (Beck *et al.*, 1990).

In this study, a search of EMBL and GenBank databases showed that the amino acid sequence encoded by the 680-bp *ClaI/Hind*III DNA fragment was also similar to the β-ketoacyl synthase functional domain of other 6 PKS genes (35% to 71% identity over a 216-amino-acid span). To illustrate an amino acid sequence comparison of *flu*P to two PKS genes and one fatty acid synthetase (FAS) gene is shown in Figure 5-1. Northern blot analysis was used to identify the approximate size and location of the coding region of *flu*P. Initially, the 680-bp *ClaI/Hind*III DNA fragment was used as a probe to hybridize to total RNA from *A. parasiticus* and an approximately 6-kb transcript was detected. Several genomic DNA fragments (in vector β-E2 from a phage λ genomic DNA library of *A. parasiticus*) flanking both sides of the 680-bp *ClaI/Hind*III DNA fragment were then used as probes in Northern blot analysis (Figure 5-2). Each probe (0.9-kb *SacI/Sph*I, 0.3-kb *SphI/Pst*I, 3.0-kb *Pst*I, and

Figure 5-1. Amino acid sequence comparison of *flu*P with two PKS genes and one FAS gene. AP, amino acid sequence (AAS) encoded by the 680-bp fragment of the *Aspergillus parasiticus flu*P gene; PP, AAS of the N-terminus of the *Penicllium patulum* 6-methylsalisylic acid synthase gene (PKS gene); AN, AAS of the N-terminus of the developmentally regulated *A. nidulans wA* gene (PKS-like gene); YSC, AAS of the N-terminus of the *Saccharomyces cerevisiae* fatty acid synthase 2 gene (FAS gene). The identity and similarity are 72% and 86% between AP and PP, 35% and 58% between AP and AN, and 13% and 39% between AP and YSC.

Figure 5-1.


AP MDPQQRLALE VTWEALENAG IPLSSLSGSD ...AAVFMGV NSDDYGKLLL
PP MDPQQRVSLE VASEALEDAG IPAKSLSGSD ...TAVFWGV NSDDYSKLVL
AN ADPAQRLALL TAYEALEGAG FVPDSTPSTQ RDRVGIFYGM TSDDYRE..V
YSC GAGAAAGAAM IDAGALEEIT KDHKVLARQQ LQVLARYLKM DLDNGERKFL
AP EDLPHVEPWM GIGTAYCGVA NRISYHLNLM GPSTAVDAA. ....CA
PP EDLPNVEAWM GIGTAYCGVP NRISYHLNLM GPSTAVDAA. ....CA
AN NSGQDIDTYF IPGGNRAFTP GRINYYFKFS GPSVSVDTA. ....CS
YSC KEKDTVAEL. .QAQLDYLNA ELGEFFVNGV ATSFSRKKAR TFDSSWNWAK
AP SSLVAIHLE. DRTILSGESK VXIVGGVNAI FGPGLTSVLD KAGALSSDGR
PP SSLVAIHHG. VQAIRLGESK VAIVGGVNAL CGPGLTRVLD KAGAISSDGS
AN SSLAAIHLA. CNSIWRNDCD TAITGGVNIL TNPDNHAGLD RGHFLSRTGN
YSC QSLLSLYFEI IHGVLKNVDR EVVSEAINIM NR........ .....SNDAL
AP CHSFDDTASG YGRGEGAAVV ILKNMAEAVK NGDHILATLK GTAVAQDGRT
PP CKSFDDDAHG YARGEGAGAL VLKSLHRALL DHDNVLAVIK GSAVCQDGKT
AN CNTFDDGADG YCRADGVGTV VLKRLEDALA DNDPILGVIN GAYTNHSAEA
YSC IKFMEYHISN TDETKGENYQ LVKTLGEQLI ENCKQVLDVD PVYKDVAKPT
AP NGIMAPNQKA QELVARKALD VARVDASTI.
PP NGIMAPNSVA QQLAANNALS AANIDPHTVR
AN VSITRPHVGA QAFIFKKLLN EANVDPKNIS
YSC GPKTAIDKNG NITYSEEPRE KVRKLSQYVQ

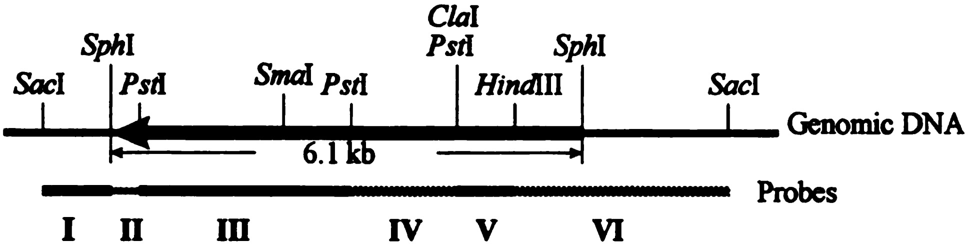


Figure 5-2. A schematic of the 9.1-kb *Sac*I genomic DNA fragment containing the *flu*P gene in *Aspergillus parasiticus*. DNA probes used in Northern analysis to define the transcribed region of *flu*P: I, 0.9-kb *Sac*I/*Sph*I; II, 0.3-kb *Sph*I/*Pst*I; III, 3.0-kb *Pst*I; IV, 1.3-kb *Pst*I; V, 0.68-kb *Cla*I/*Hind*III; VI, 2.9-kb *Hind*III/*Sac*I. The arrow represents the proposed direction of transcription of *flu*P.

1.3-kb *Pst*I fragment) except the 2.9-kb *Hind*III/*Sac*I fragment detected the same size transcript as that detected by the 680-bp *Cla*I/*Hind*III DNA fragment. Since the deduced amino acid sequence of the 680-bp *Cla*I/*Hind*III DNA fragment was similar to the β-ketoacyl synthase functional domain located in the N-terminus of MSAS in *P. patulum* (Beck *et al.*, 1990), it was predicted that *flu*P should be transcribed from right to left as shown in Figure 5-2. The 6.1-kb *Sph*I DNA fragment, which contains the 0.3-kb *Sph*I/*Pst*I, 3.0-kb *Pst*I, 1.3-kb *Pst*I, and 1.5-kb *Pst*I/*Sph*I fragments, was predicted to encompass the 5'-end plus its flanking region and most of the 3'-end of *flu*P, and therefore could be used for disruption vector construction.

Construction of a Disruption Vector pPKS. The 6.1-kb *Sph*I DNA fragment was cloned into the *Sph*I site of pUC19 resulting in plasmid pUC19-6. The disruption plasmid, pPKS, was constructed by insertion of a 6.5-kb *Pvu*II fragment from pUNH (Figure 5-3), containing a functional copy of the nitrate reductase gene, *nia*D, into the *Sma*I site of pUC19-6.

Figure 5-3. The *flu*P-disruption vector pPKS and a schematic description of the expected restriction map of genomic DNA in the *flu*P-disrupted transformant. The solid black line is the 6.1-kb *Sph*I fragment containing most of *flu*P. A 6.5-kb fragment, which was cut off from plasmid pUNH, was inserted into the middle of the *flu*P gene. The shaded line (2.7 kb) in the 6.5 kb fragment is a functional copy of the nitrate reductase gene (*nia*D). **Abbreviations:** Sp, *Sph*I; Sc, *Sac*I; B, *Bam*HI; H, *Hind*III; Sm, *Sma*I.

Figure 5-3.

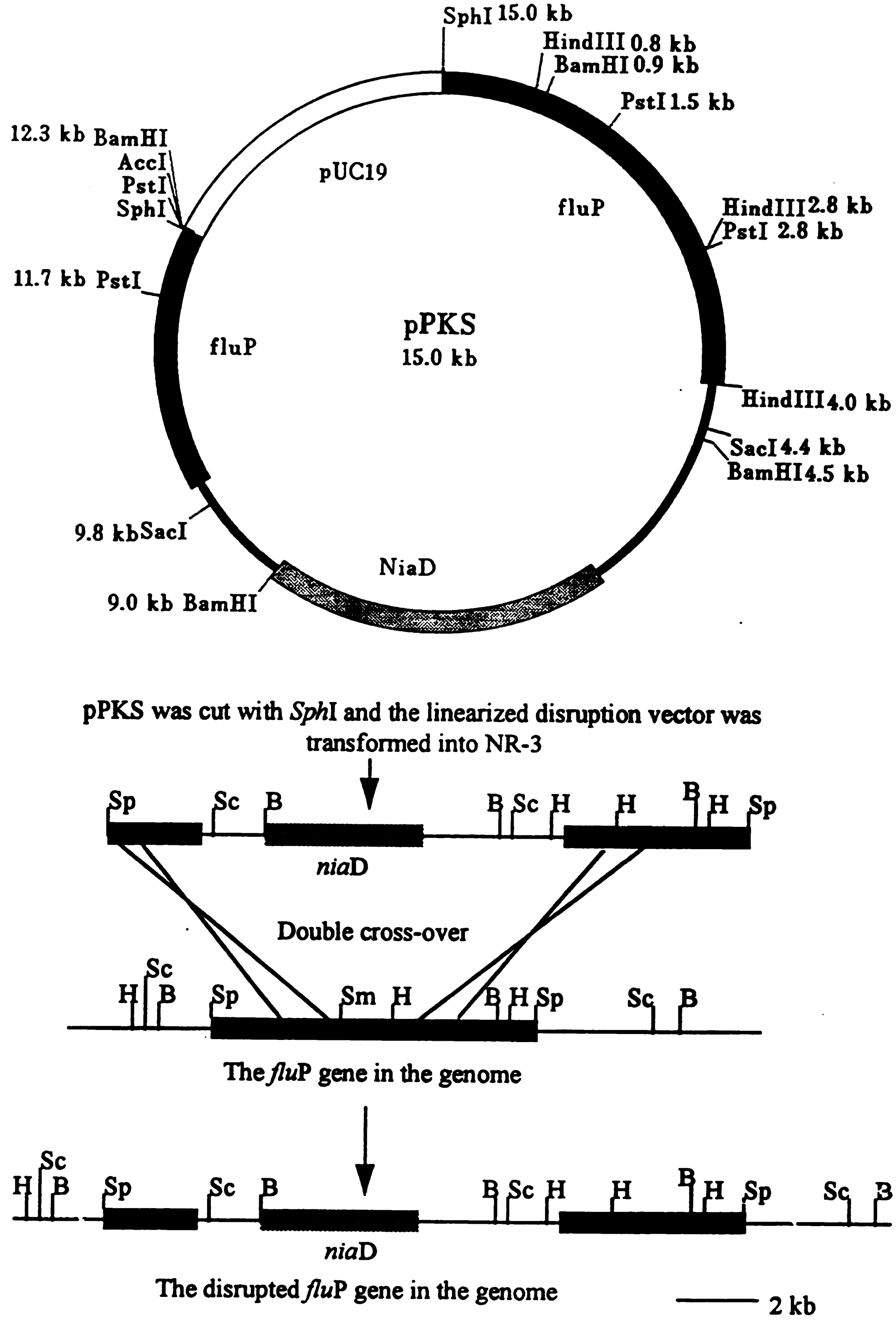
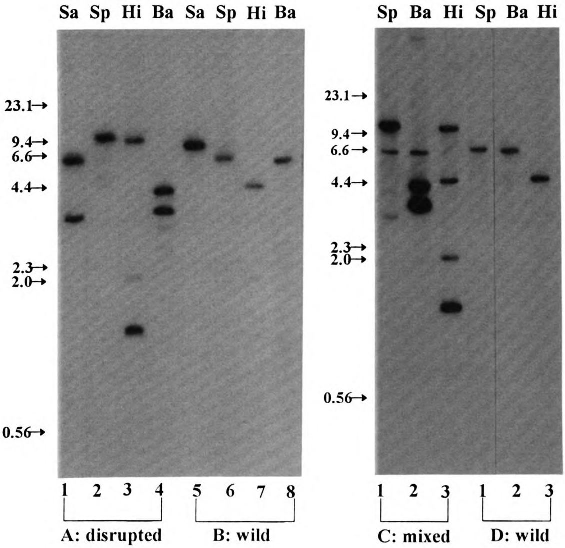

SphI 15.0 kb
HindIII 0.8 kb
BamHI 0.9 kb
PstI 1.5 kb
HindIII 2.8 kb
PstI 2.8 kb
HindIII 4.0 kb
SacI 4.4 kb
BamHI 4.5 kb
12.3 kb BamHI
AccI
PstI
SphI
11.7 kb PstI
9.8 kb SacI
9.0 kb BamHI
pUC19
fluP
fluP
NiaD
pPKS
15.0 kb
pPKS was cut with *Sph*I and the linearized disruption vector was transformed into NR-3
Sp
Sc
B
B
Sc
H
H
B
H
Sp
*nia*D
Double cross-over
H
Sc
B
Sp
Sm
H
B
H
Sp
Sc
B
The *flu*P gene in the genome
H
Sc
B
Sp
Sc
B
B
Sc
H
H
B
H
Sp
Sc
B
*nia*D
The disrupted *flu*P gene in the genome
2 kb

Figure 5-4. Southern blot analysis of genomic DNA in *A. parasiticus* NR-3 and the *flu*P-disrupted transformant. Ten µg of genomic DNA from each sample was digested with *Sac*I (Sa), *Sph*I (Sp), *Hind*III (Hi), *Bam*HI (Ba), separated by agarose gel electrophoresis, and transferred to a Nytran membrane. A 3.0-kb *Pst*I internal DNA fragment of *flu*P was used as a probe. **A: disrupted,** sample from the *flu*P-disrupted transformant (*flu*P). **B:** and **D: wild,** sample from the wild-type (*flu*P$^+$). **C: mixed,** sample from the colony with the *flu*P/*flu*P$^+$ genotype. The numbers on the left side of each panel is the molecular size standard ladder (unit: kb).

Figure 5-4.


Sa
Sp
Hi
Ba
Sa
Sp
Hi
Ba
23.1→
9.4→
6.6→
4.4→
2.3→
2.0→
0.56→
1
2
3
4
5
6
7
8
A: disrupted
B: wild
Sp
Ba
Hi
Sp
Ba
Hi
23.1→
9.4→
6.6→
4.4→
2.3→
2.0→
0.56→
1
2
3
1
2
3
C: mixed
D: wild

Transformation of *A. parasiticus* NR-3 and Disruption of *flu*P. Plasmid pPKS was linearized by cutting with the restriction enzyme *Sph*I to generate an approximately 12-kb DNA fragment. The linear disruption vector was transformed into protoplasts of *A. parasiticus* NR-3 (Figure 5-3). Nitrate-utilizing transformants (*nia*D$^+$) were selected on Czapek-Dox agar (CDA) containing sodium nitrate as the sole nitrogen source and then transferred onto coconut agar medium (CAM) to conduct a qualitative screen for aflatoxin accumulation. All colonies (more than 300) transferred to CAM produced aflatoxins. The selection of transformants containing the disrupted *flu*P gene was then conducted by Southern blot analysis.

Southern blot analysis of genomic DNA isolated from more than 200 *nia*D$^+$ transformants identified two transformants (isolates 10 and 11) in which *flu*P was disrupted (Figure 5-4). Wild-type colonies grown on CAM (Figure 5-5A) or on potato dextrose agar (PDA) (Figure 5-5B) grew with normal morphology of hyphae and spores. However colonies of disrupted transformants grew with much less aerial hyphae and had widely dispersed spores on PDA (Figure 5-5C) or with cotton-like (fluffy) hyphae and no spores on CAM (Figure 5-5D). Therefore this gene from *A. parasiticus* was tentatively named *flu*P for <u>flu</u>ffy gene. Wild-type hyphae and cotton-like hyphae of the disrupted transformant grown on CAM are shown in Figures 5-5E and 5-5F. When the hyphae of isolates 10 and 11 were transferred onto CAM (secondary isolates), about 10% of the colonies recovered the wild-type phenotype and wild-type genotype (*flu*P$^+$, nondisrupted *flu*P gene) (Figure 5-4B and 5-4D). Another 20 % of the secondary isolates grew as a mixture of normal phenotype and abnormal phenotype (cotton-like hyphae with no spores) and contained both the non-disrupted *flu*P allele and the disrupted

Figure 5-5. Morphology of *A. parasiticus* NR-3 and the *flu*P-disrupted transformants grown on coconut agar medium (CAM) or on potato dextrose agar (PDA). Pictures: **A1** (magnification, 0.7 x), a colony of NR-3 on CAM; **A2** (magnification, 1.4 x), an enlargement of part of **A1**; **B1** (magnification, 0.7 x), a colony of NR-3 on PDA; **B2** (magnification, 1.4 x), an enlargement of part of **B1**; **C1** (magnification, 0.7 x), a colony of the *flu*P-disrupted transformant on PDA; **C2** (magnification, 1.4 x), an enlargement of part of **C1; D1**, a colony of the *flu*P-disrupted transformant on CAM; **D2** (magnification, 1.4 x), an enlargement of part of **D1**; **E** (magnification, 20 x), hyphae of the wild-type (NR-3) on CAM; **F** (magnification, 20 x), hyphae of the *flu*P-disrupted transformant on CAM.

Figure 5-5.

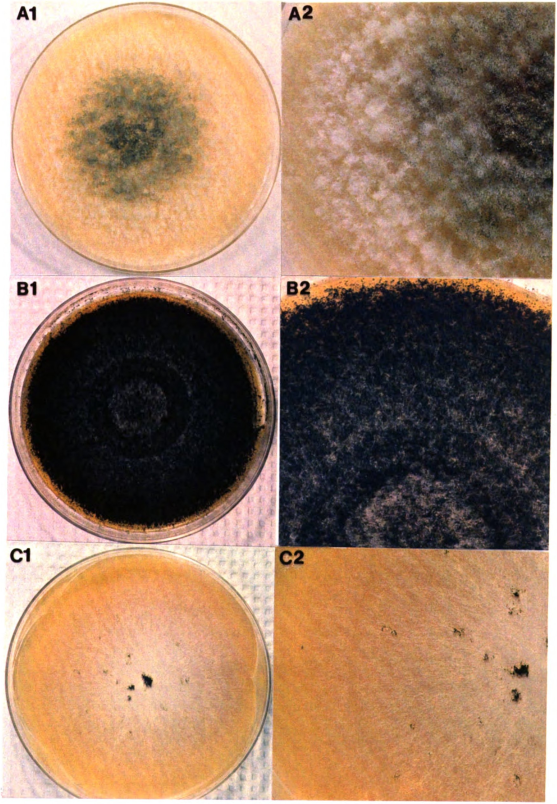


Figure 5-5 (cont'd).

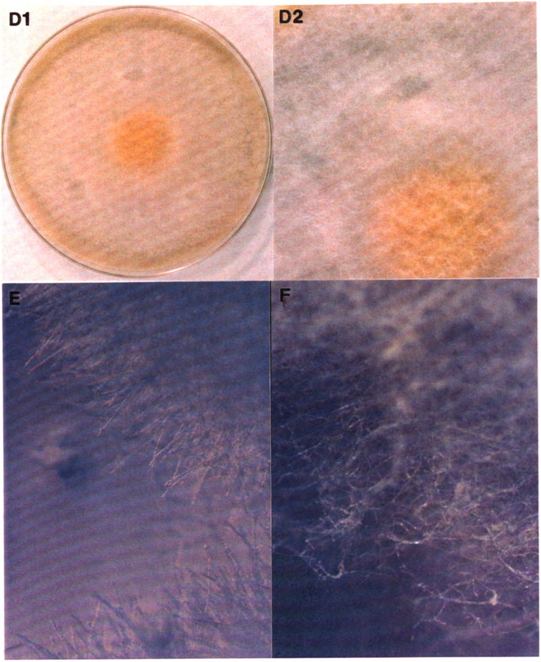


Figure 5-6. Transcript accumulations of *flu*P, the β-tubulin gene, and the *ver-1*A gene in *A. parasiticus.* Total RNA was isolated from cells grown in Reddy medium (Reddy *et al.*, 1971) for 24 h, 60 h, and 72 h, respectively. Thirty μg of total RNA in each lane was separated by agarose gel electrophoresis and transferred onto a Nytran membrane. **PKS (*fluP*):** a 1.3-kb *Pst*I internal fragment of *flu*P was used as a probe. ***ver-1*:** a 0.6-kb *Cla*I/*Eco*RI internal fragment of the *ver-1*A gene was used as a probe. **β-Tubulin**: a 2.0-kb *Hind*III/*Bam*HI internal fragment of the β-tubulin gene was used as a probe.

Figure 5-6.

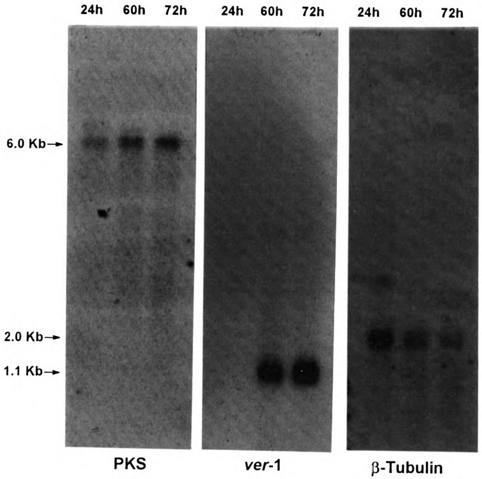
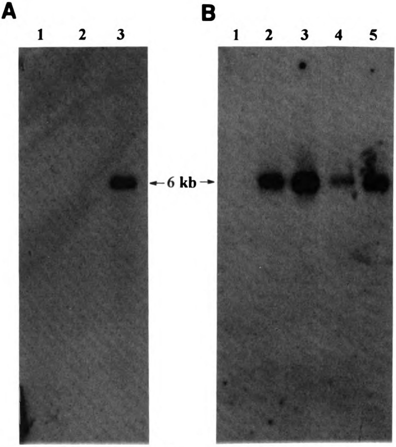

24h
60h
72h
24h
60h
72h
24h
60h
72h
6.0 Kb
2.0 Kb
1.1 Kb
PKS
ver-1
β-Tubulin

Figure 5-7. Transcript accumulations of *flu*P in *A. parasiticus* NR-3 and the *flu*P-disrupted transformant. Thirty µg of total RNA was separated by agarose gel electrophoresis, and transferred onto a Nytran membrane. A 1.3-kb *Pst*I internal fragment of *flu*P was used as a probe. Lane panel: **A: 1, 2 and 3,** genomic DNA isolated from *flu*P-disrupted isolate 11, 10, and from NR-3, respectively, grown on CAM for 72 h; **B: 1,** genomic DNA isolated from *flu*P-disrupted isolate grown on YES agar for 4 days; **2 and 3,** genomic DNA isolated from NR-3 grown on YES agar for 2 and 4 days, respectively; **4 and 5,** genomic DNA isolated NR-3 grown in YES liquid medium for 2 and 4 days, respectively.

Figure 5-7.



*flu*P allele (*flu*P$^+$/*flu*P) (Figure 5-4C). About 70 % the secondary isolates maintained only the abnormal phenotype (cotton-like hyphae with no spores) and genotype (disrupted-*flu*P allele only). The pattern of phenotype and genotype in the secondary isolates on CAM (the CAM pattern) was repeatable from one transfer to the next transfer. Interestingly, when the hyphae of isolates 10 and 11 were transferred onto PDA, the colonies grew with much less aerial hyphae and had widely dispersed spores (Figure 5-5C). This pattern is called the PDA pattern. When the spores produced on PDA were inoculated onto either CAM or PDA, all the colonies showed normal phenotype and normal genotype. When the hyphae growing on PDA were inoculated onto CAM or PDA, the CAM or PDA pattern appeared again.

***flu*P Transcript Accumulations in the Wild-Type Strain and the *flu*P-Disrupted Transformant.** Total RNA, extracted from wild-type mycelia grown in selective Reddy's liquid medium (Reddy *et al.*, 1971, an aflatoxin-inducing chemically defined medium), was subjected to Northern hybridization analysis. A 6.0-kb transcript was detected in the 24-h, 60-h, and 72-h samples using a 1.3-kb *Pst*I internal fragment of *flu*P as a probe (Figure 5-6, PKS). In contrast, a 1.1-kb transcript was only detected in the 60-h, and 72-h (not in the 24-h) samples using a 0.6-kb *Cla*I/*Eco*RI internal fragment of *ver*-1A gene (directly involved in aflatoxin biosynthesis in *A. parasiticus*) as a probe (Figure 5-6, *ver*-1). This pattern of transcript accumulation of *ver*-1A was similar to that seen in previous studies (Trail *et al.*, 1995). As a control, a 2.0-kb transcript was detected in the 24-h, 60-h and 72-h samples using a 2.0-kb *Hind*III/*Bam*HI internal fragment of the β-tubulin gene (a house keeping gene) as a probe (Figure 5-6, β-Tubulin). The transcript of the β-tubulin gene appeared in greater abundance in the 24-h sample than that in the 72-h sample, a pattern seen in previous studies

(Trail *et al.*, 1995). In contrast, the transcript of *flu*P appeared in less abundance in the 24-h sample than that in the 72-h sample. Also, the 6.0-kb transcript was not detected in the two *flu*P-disrupted transformants (figure 5-7A, lanes 1 and 2), but was detected in the wild-type cells grown on CAM for 72 h (Figure 5-7A; lane 3). Normally, sporulation can occur only on solid growth media, therefore if *flu*P is associated with sporulation the corresponding transcript on solid media is expected to appear in greater abundance than that in liquid media. Total RNA of the *flu*P-disrupted transformant was extracted from the fungus grown in YES liquid medium and YES solid medium. At two time points, the *flu*P transcript isolated from cells grown on solid media appeared in approximately 3-fold greater abundance (relative intensity on Northern blot analysis is 100) than transcript (relative intensity on Northern blot analysis is 35) isolated from cells grown in liquid media (Figure 5-7B).

Aflatoxin B$_1$ Accumulations in the Wild-Type Strain and the *flu*P-Disrupted Transformant. In order to determine if *flu*P had an effect on aflatoxin accumulation, the hyphae of the *flu*P-disrupted transformant and the wild-type (NR-3) were inoculated onto YES agar. YES agar was used to measure aflatoxin accumulation in this study for two reasons: (1) the *flu*P-disrupted transformant grew on YES agar with similar morphology to the CAM pattern (fluffy-colony with no sporulation); (2) YES is the primary medium used to study aflatoxin accumulation in this dissertation (Chapter 2, 3, and 4).

The hyphae of NR-3 took about 6 days, while the hyphae of the *flu*P-disrupted transformant took about 18 days to cover the surface of the growth medium in a Petri dish. The quantity of hyphae transferred did not appear to affect the hyphal growth rate in this experiment. Mycelial samples were collected according to the specific temporal and regional schedule as described previously (Materials and Methods). The dry weight of the mycelia

Table 5-1.

Table 5-1. Mycelial dry weights and aflatoxin B$_1$ accumulations in *A. parasiticus* NR-3 and the *flu*P-disrupted transformant grown on YES agar.

Region of sample collection	Collection time (days) *flu*P	Collection time (days) *flu*P$^+$	Dry weight (mg) *flu*P	Dry weight (mg) *flu*P$^+$	AFB$_1$ (μg/plate) *flu*P	AFB$_1$ (μg/plate) *flu*P$^+$	AFB$_1$ (μg/mg dry mass) *flu*P	AFB$_1$ (μg/mg dry mass) *flu*P$^+$
Central	6	2	0. 3 ± 0. 1	1. 7 ± 0. 3	0. 9 ± 0. 3	5. 4 ± 1. 2	2. 9 ± 0. 5	3. 1 ± 0. 9
Central	12	4	1. 1 ± 0. 3	4. 0 ± 0. 4	3. 4 ± 0. 7	23. 2 ± 4. 8	3. 1 ± 0. 7	5. 9 ± 0. 8
Central	15	5	1. 6 ± 0. 2	4. 3 ± 0. 3	5. 3 ± 1. 4	31. 8 ± 5. 6	3. 3 ± 0. 6	7. 7 ± 1. 6
Central	18	6	1. 3 ± 0. 4	4. 8 ± 0. 4	4. 4 ± 2. 3	7. 9 ± 6. 5	3. 4 ± 0. 3	7. 9 ± 1. 4
Middle	12	4	1. 5 ± 0. 5	5. 9 ± 0. 5	3. 9 ± 0. 6	25. 4 ± 5. 3	2. 6 ± 0. 7	4. 2 ± 1. 5
Middle	15	5	2. 1 ± 0. 8	9. 0 ± 0. 7	6. 1 ± 1. 3	66. 6 ± 7. 4	2. 9 ± 0. 7	7. 5 ± 1. 3
Middle	18	6	4. 2 ± 0. 9	18. 1 ± 2. 4	12. 6 ± 3. 8	126. 9 ± 7. 5	3. 0 ± 0. 8	9. 1 ± 1. 6
Peripheral	15	5	55. 5 ± 1. 4	24. 2 ± 3. 5	15. 4 ± 3. 6	154. 9 ± 12. 4	2. 8 ± 0. 8	6. 4 ± 1. 7
Peripheral	18	6	10. 9 ± 3. 6	56. 6 ± 5. 7	33. 8 ± 5,7	390. 6 ± 23. 1	3. 1 ± 0. 6	7. 0 ± 1. 3
Total	**18**	**6**	**16. 0 ± 2. 9**	**79. 5 ± 4. 7**	**50. 8 ± 7. 3**	**555. 4 ± 21. 6**	**3. 2 ± 0. 6**	**7. 1 ± 1. 1**

Notes:

(1) Colonies were grown on YES agar in Petri dishes. Mycelial samples of the wild-type NR-3 (or of the *flu*P-disrupted transformant *flu*P) were collected from the central region 2 days, 4 days, 5 days, and 6 days (or 6 days, 12 days, 15 days, and 18 days) after inoculation, collected from the middle region 4 days, 5 days, and 6 days (or 12 days, 15 days, and 18 days) after inoculation, collected from the peripheral region 5 days, and 6 days (or 15 days, and 18 days) after inoculation (see details in Materials and Methods).

(2) The accumulation of AFB1 was determined by ELISA (Materials and Methods).

(3) Each value the mean ± standard error (SE) of three independent tests.

(4) *flu*P is the *flu*P-disrupted transformant; *flu*P$^+$, the wild-type NR-3.

and the accumulation of aflatoxin B_1 (AFB_1) (from the growth medium and mycelia) of each mycelial sample were determined (Table 5-1).

The overall accumulation of AFB_1 (per Petri dish) in NR-3 was approximately 10-fold higher than that of the *flu*P-disrupted transformant, while AFB_1 accumulation per gram of mycelium of NR-3 (7.0 µg/mg) was 2-fold higher than that of the *flu*P-disrupted transformant (3.2 µg/mg). The overall growth rate of the wild-type was 3-fold faster than the *flu*P-disrupted transformant if the time to cover the Petri dish was used as the indicator, and 4-fold faster if the dry weight per Petri dish was used as the indictor suggesting that *flu*P may directly or indirectly control hyphal growth rate. The AFB_1 accumulation per gram of dry weight of mycelium was nearly the same (3.2 µg/mg) in all areas from a colony of the *flu*P-disrupted transformant which did not sporulate. However, in NR-3, AFB_1 accumulation per mg of dry mycelium (3.2 µg/mg) in the central region 2 days after inoculation (no sporulation) was almost the same as that of the *flu*P-disrupted transformant, but increased quickly in the same region when sporulation appeared [for example 4 days (5.8 µg/mg) and 5 days (7.4 µg/mg) after inoculation in the central region]. It was observed that the dry weight of mycelium and aflatoxin accumulation of the *flu*P-disrupted transformant started to decrease 15 days after incubation in the central region. These data suggested that mycelia and aflatoxin degraded after long incubation times.

DISCUSSION

Based on the data collected in this study, it is reasonable to conclude that *flu*P is associated with hyphal growth, hyphal development and sporulation, and in turn indirectly influences aflatoxin accumulation in *A. parasiticus*. The disruption of *flu*P resulted in cotton-like hyphae with no spores and reduced growth rate, and the recovery of the wild-type allele *flu*P resulted in normal hyphae with normal spores and normal growth rate supporting the proposed function of *flu*P in *A. parasiticus*.

Other evidence which supports the association of *flu*P with hyphal cell development and sporulation is derived form the comparison of *flu*P transcript level in liquid and solid media. Normally, sporulation occurs only on solid media. Therefore, if *flu*P is associated with sporulation, the corresponding transcript level on solid media may be expected to appear in greater abundance than that in liquid media. At the two time points analyzed, the transcripts from solid media did appear in greater abundance than those in liquid media. However, the fact that *flu*P is expressed in liquid media may also suggests that this gene is associated with growth in general.

The presence or absence of the *nia*D selectable marker had no observable influence on the growth rate of the *flu*P-disrupted transformant on YES or CAM. When hyphae, derived from SU-1 (*nia*D$^+$/*flu*P$^+$) and the *nia*D mutant NR-3 (*nia*D/*flu*P$^+$) of *A. parasiticus* were inoculated onto YES or CAM, there was no observable differences in growth rate between the two strains. It is likely that because CAM and YES contain many different alternative nitrogen sources, the two strains did not have to use nitrate reductase (encoded by *nia*D) for normal growth. However, when hyphae derived from SU-1 (*nia*D$^+$/*flu*P$^+$) and the *flu*P-disrupted transformant (*nia*D$^+$/*flu*P) of *A. parasiticus* were inoculated onto YES or CAM, there was a great observable difference in growth rate between the two strains. Therefore, the reduced growth rate observed for the *flu*P-disrupted transformant (*nia*D$^+$/*flu*P) on nonselective YES or CAM is the result of disruption of the *flu*P gene.

The pattern of expression of the *flu*P gene and the β-tubulin gene were different during sporulation. Microtubules are one of the 3 types of filamentous structures (cytoskeleton) found in all enkaryotic cells (the other two are microfilaments and intermediate filaments). In filamentous fungi, not only are microtubules necessary for the formation of cytoskeleton in general, but also involved in the formation of the mitotic spindle during mitosis. Microtubules consist of α-tubulin and β-tubulin. May *et al.* (1987) May and Morris (1988) suggested that β-tubulin participates in conidial development in *Aspergillus nidulans* by demonstrating that the expression of β-tubulin gene was developmentally regulated and directly correlated with the appearance of conidiating cell types. A large increase occurred in the rate of β-tubulin synthesis and there was an accumulation of the corresponding mRNA biosynthesis during sporulation in the filamentous fungus *Blastocladiella emersonii* (Da Silva and Juliani, 1988). Temperli *et al.* (1990) demonstrated that cytoplasmic microtubules arise from a nucleus-associated center (their density was highest near the nucleus) and extend into the proximal cytoplasm during mitosis by in situ immunolocalization using anti-β-tubulin monoclonal antibody. These data suggest that the increased accumulation of β-tubulin during sporulation is the result of the increased activity of mitosis in which β-tubulin is involved in the formation of the mitotic spindle.

As mentioned above, *flu*P is also thought to be associated with sporulation in *A. parasiticus*. A comparison of the transcript level between the β-tubulin gene and *flu*P may reveal more information about the function of *flu*P. For example, in liquid media, the accumulation of the transcript of the β-tubulin gene decreased from 24 to 72 h of incubation, while during the same time period the accumulation of *flu*P transcript increased. There appear to be at least two reasonable explanations for this observation. First, this experiment was conducted in liquid media and sporulation was inhibited. Therefore the requirement for a large amount of β-tubulin may not be necessary because β-tubulin acts as the building unit of mitotic spindle during sporulation (not as a signal). Second and more importantly, not only may *flu*P (may encode a signal) be associated with sporulation but it also may be associated with other biological processes in the producing fungus. Therefore even though sporulation is inhibited other biological processes still need the expression of *flu*P. These data suggest that *flu*P functions at an early stage of vegetative cell differentiation.

The *flu*P gene isolated from *A. parasiticus* is functionally different from *brl*A, *flu*G, and *flb*A in *Aspergillus nidulans*. The developmental regulatory gene *brl*A in *A. nidulans* is likely involved in controlling the switch from growth to development (Clutterbuck, 1969; Boylan *et al.*, 1987; Lee and Admas, 1994b). But DNA sequence analysis predicted that *brl*A encodes a protein similar to a number of plant phytochromes (Griffith *et al.*, 1994) which are very different from polyketide synthase. Lee and Adams (1994a) described the *flu*G gene which is required for the activation of *brl*A expression and conidiophore development. The FluG protein is present at nearly constant levels throughout the *A. nidulans* life cycle. Sequence analysis of *flu*G predicted that a 864-codon *flu*G open reading frame shares 28% identity with prokaryotic glutamine synthetase I (Lee and Adams, 1994a). The *flu*G mutant produces fluffy hyphae with no spores. The *flu*G mutant phenotype is suppressed when *flu*G mutant colonies are grown adjacent to colonies of the wild-type strain even if the two strains are separated by dialysis membrane with a 6-8 kDa cutoff (Adams *et al.*, 1992). Another fluffy gene, *flb*A was found to be required in conidiophore development prior to activation of *brl*A. A 3.0-kb mRNA corresponding to *flb*A is present at low levels throughout the *A. nidulans* asexual life cycle (Lee and Adams, 1994b). *flb*A encodes an *Aspergillus nidulans* regulator of G protein signaling (RGS) domain protein (Yu *et al.*, 1996). The phenotypic defects of the *flb*A deletion mutants could be partially remedied by growth on high osmolarity medium (e.g. 0.8 M NaCl) (Lee and Adams, 1994b). However, the *flu*P-disrupted phenotype of *A. parasiticus* could not be suppressed when being grown next to the wild-type or be remedied by growth on high osmolarity medium. Therefore *flu*P appears to be different from *brl*A, *flu*G, and *flb*A in *Aspergillus nidulans* with regard to their association with sporulation suggesting that *flu*P may be a new class of genes involved in fungal development.

The protein encoded by *flu*P in *A. parasiticus* is likely a PKS and the corresponding end product is likely a polyketide. Beck *et al.* (1990) cloned the MSAS gene together with its flanking sequences (total 7131 bp) from *Penicillium patulum*. Within this sequence the MSAS gene was identified as a 5322-bp long open reading frame. The transcript of *flu*P is about 6.0 kb in length which is similar in size to the transcript derived from the MSAS gene in *P. patulum*. A comparison of the deduced amino acid sequence of the 680-bp DNA fragment of the *flu*P gene showed a great deal of similarity with β-ketoacyl synthase function domain of MSAS gene and other PKS genes.

The disrupted-*flu*P allele is not stable. Twenty percent of the colonies derived from cotton-like hyphae "reverted" to the wild genotype and phenotype suggesting that the disrupted *flu*P allele was genetically unstable. It is possible that reversion to the wild-type *flu*P allele could occur by certain genetic mechanisms (such as a simple deletion of the inserted DNA fragment containing *nia*D). It is also possible that the transferred hyphae were contaminated with hyphae and/or spores which contained wild-type nuclei although the data would argue that this scenario is unlikely. For example, if the transferred hyphae were contaminated with hyphae and/or spores which contained wild-type nuclei, the initial colony would predominately consist of wild-type hyphae with wild-type spores because of the faster growth rate of the wild-type hyphae. Similarly, the possibility that the transferred hyphae were heterokaryons is also not consistent with the data. Heterokaryons may exist immediately after transformation, but serial mycelial transferring should reduce the possibility of a heterokaryon significantly. In the unlikely event that a heterokaryon survived serial transfer, the colony which originated from a heterokaryon would have normal hyphae and spores at the center of the colony. The normal hyphae (with spores) would continue to appear during colony growth. The cotton-like fluffy mycelium would only form a sector. The actual morphology of the colony observed suggests that neither of these possibilities is likely to be true. Therefore, the most reasonable explanation for the genetic instability of the knockout *flu*P appears to be genetic recombination within the *flu*P-disrupted allele to restore the wild-type allele.

CAM and PDA had different influences on the recovery of the wild-type *flu*P phenotype in the *flu*P-disrupted transformant. When hyphae of the *flu*P-disrupted transformant were transferred onto CAM, 10% of the resulting colonies recovered the wild-type morphology and contained only the wild-type *flu*P allele (*flu*P$^+$); 20% grew as a mixture of normal hyphae plus cotton-like hyphae and contained the wild-type *flu*P allele and the disrupted *flu*P allele (*flu*P$^+$/*flu*P); 70% grew with only the cotton-like hyphae and contained only the disrupted *flu*P allele (*flu*P). In contrast, when hyphae of the *flu*P-disrupted transformant were transferred onto potato dextrose agar (PDA), only one type of colony appeared. Such colonies grew with much less aerial hyphae and had widely dispersed *flu*P$^+$ spores. Therefore, the *flu*P allele in disrupted transformants could "revert" to the wild-type *flu*P allele on either CAM or PDA, but in different patterns.

The *flu*P gene does not appear to be directly involved in aflatoxin biosynthesis. In liquid media (Reddy's medium), the *flu*P transcript was detected in the 24-h sample while the transcript of *ver*-1A (as well as other genes in the AFB$_1$ pathway), which is directly involved in aflatoxin biosynthesis, was not detected in the 24-h sample (but detected in the 60-h sample). This difference suggests that the *flu*P gene is not directly involved in aflatoxin biosynthesis.

The decrease in AFB$_1$ accumulation of the *flu*P-disrupted transformant grown on YES agar could result from lack of sporulation and reduced growth rate (decreased cell mass). Even though the overall AFB$_1$ accumulation (per Petri dish) of the wild-type was approximately 10-fold higher than that of the *flu*P-disrupted transformant, the accumulation of AFB$_1$ per mg dry weight in the central region after 2 days incubation (which lacked sporulation) was essentially the same as the accumulation in any region of the *flu*P-disrupted transformant. In the wild-type, AFB$_1$ accumulation per gram dry weight increased quickly only when sporulation appeared. Conidiophores and developing conidia have been found to contain the highest concentration of Nor-1 protein (Chapter 4) and Ver-1A protein in a growing colony of wild type on solid medium (Liang and Linz, unpublished data). These two proteins are directly involved in AFB_1 biosynthesis. Therefore, *flu*P may directly influence vegetative cell differentiation and sporulation, which in turn influences aflatoxin biosynthesis in *A. parasiticus.*

Future studies on *flu*P in *A. parasiticus* may include: (a) a complete analysis of the nucleotide sequence of *flu*P including the deduced amino acid sequence, and comparison between *flu*P and other PKS genes involved in hyphal growth and sporulation in other fungi to narrow down the possible product of *flu*P; (b) identification of the protein product of *flu*P; and (c) a study of the mechanism of the regulation of expression of *flu*P. These studies may provide useful information for the development of new fungicides, improved agronomic practices, resistant crops (by genetic engineering or classical breeding), and biocontrol agents (such as nontoxigenic competitive fungi) for control of aflatoxin contamination.

In summary, disruption of *flu*P resulted in slow growth with cotton-like hyphae, no sporulation, and reduced aflatoxin accumulation. The data suggest that *flu*P is associated with vegetative cell growth, sporulation, and in turn indirectly reduces aflatoxin accumulation in *A. parasiticus.*

CONCLUSIONS

The data presented in this dissertation provided a further understanding of the mechanisms of regulation of aflatoxin biosynthesis and the general biology at the molecular level in the toxigenic filamentous fungus *Aspergillus parasiticus*. These information may lead to the development of new fungicides, the improvement of agronomic practices, the development of resistant crops (by genetic engineering or classical breeding), and the development of biocontrol agents (such as nontoxigenic competitive fungi) for control of aflatoxin contamination.

The data confirmed that the Nor-1c protein, derived from the *nor*-1 cDNA, is a keto-reductase which converts the AFB$_1$ pathway intermediate norsolorinic acid (NA) to averantin (AVN) in the presence of NADPH plus a factor(s) obtained from *Eschreichia loci* DH5α. The data also suggest that an unidentified cofactor(s) may also be necessary for the native Nor-1 protein function and that the *nor*-1 gene is involved in only one branch of the proposed alternative pathways for conversion of NA to averufin (AVF).

Polyclonal antibodies (PAb) raised against the Nor-1c protein were proven to be useful to monitor the accumulation of the native Nor-1 protein. A *nor*-1/GUS reporter construct was also used to monitor the activity of the *nor*-1 promoter in *A. parasiticus*. The data liquid media, and to temporal and spatial regulation on solid media; and that the activity of the *nor*-1 promoter is subjected to temporal and spatial regulation on solid media.

The data obtained by cell fractionation/Western blot analysis and in situ immunolocalization suggest that the Nor-1 protein is mainly localized in the cytosol of the fungal vegetative cell; the highest level of the Nor-1 protein is associated with the developing immature conidium, high levels are associated with the conidiophore, lower levels are associated with the vegetative hyphal cell, and none is associated with the mature conidium. Indicated by a *nor*-1/GUS reporter construct, the localization of the activity of the *nor*-1 promoter follows a temporal and spatial pattern in a fugal colony. The data also suggest that the localization of Nor-1 protein accumulation and the localization of *nor*-1 promoter activity are correlated to the process of sporulation in *A. parasiticus.*

Disruption of *flu*P, a putative polyketide synthase gene, resulted in reduced growth rate, fluffy-mycelium (cotton-like hyphae), no sporulation, and reduced aflatoxin accumulation suggesting that *flu*P is associated with vegetative cell growth, sporulation, and in turn indirectly influences aflatoxin synthesis in *A. parasiticus.*

APPENDICES

APPENDIX A

APPENDIX A

CLONING OF THREE *Eco*RI DNA FRAGMENTS FROM COSMID Nor-A AND ANALYSIS OF THEIR TRANSCRIPTS

Three different but adjacent *Eco*RI DNA fragments (in the order of 2.8-kb, 4.0-kb and 4.6-kb) from an aflatoxin gene cluster (cloned in cosmid Nor-A, Figure 1-23) in *Aspergillus parasiticus* have been tried to be cloned and mapped. Only two (the 2.8-kb *Eco*RI fragment, and the 4.0-kb *Eco*RI fragment) of them were successfully cloned and mapped. But the third one (a 4.6-kb *Eco*RI fragment) was unable to be cloned for unknown reason in at least five rounds of experiments.

In Northern blot analysis, a 7.5-kb transcript was detected using the 2.8-kb *Eco*RI DNA fragment as a probe, and a 7.5-kb transcript plus a 6.5-kb transcript were detected using the 4.0-kb *Eco*RI DNA fragment as a probe in *A. parasiticus* grown in an aflatoxin-inducing medium. The accumulations of the two transcripts had the same pattern as that of the accumulations of *nor*-1 and *ver*-1 transcripts in *A. parasiticus*. These data suggest that the two transcripts were related to or involved in aflatoxin biosynthesis.

APPENDIX B

APPENDIX B

IDENTIFICATION OF VERSICONAL CYCLASE GENE

Isolation and identification of the gene encoding versiconal cyclase (converting versiconal to versicolorin B in the AFB$_1$ pathway) have been tried for a period of time. This experiment was started with a known amino acid sequence of versiconal cyclase. A mixture of 18-mer nucleotides and a mixture of 17-mer nucleotides were prepared according to a part of the amino acid sequence of versiconal cyclase. Both the 18 mers and the 17 mers were used as probes to probe the AFB$_1$ gene cluster cloned in cosmid Nor-A. A 400-bp *Eco*RI/*Xho*I fragment (in a 4.0-kb *Eco*RI DNA fragment) in cosmid Nor-A was found to hybridize the 18-mer probes in Southern blot analysis. This 400-bp fragment was sequenced. Sequence data showed that this fragment contained a similar sequence as one of the 18-mer probes. The data suggest the gene encoding versiconal cyclase was not included in cosmid Nor-A.

APPENDIX C

APPENDIX C

A MODIFIED PROCEDURE FOR CRUDE EXTRACT PREPARATION FROM FUNGAL CELLS

Certain Proteins involved in AFB_1 biosynthesis are extremely unstable. Therefore the quality of crude extract from toxigenic fungal cells is usually one of the most important factors which influence the results of enzymatic assay or Western blot analysis.

Grinding fungal cells under liquid nitrogen followed by addition of extraction buffer containing protease inhibitors is a common protocol used by many researchers. Two of the commonly used protease inhibitors are phenol methylsulfonyl fluoride (PMSF) and aprotinin. PMSF is quickly inactivated in the aqueous solution, and aprotinin can be precipitated at low temperature. Grinding fungal cells usually cannot destroy all organelles and most proteases are enclosed in organelles. Therefore, the procedure described above cannot get all proteases inhibited. The crude extract prepared in this way is stored in the freezer (at -80°C) and later is taken out and thawed for use. The freeze-and-thaw procedure will destroy the intact organelles and proteases inside will be released. This phenomenon has been ignored in most of published literature. To get a better result of enzymatic assay or Western blot analysis, protease inhibitors can be added again after each freeze-and-thaw step as soon as possible.

LIST OF REFERENCES

LIST OF REFERENCES

Abdollahi, A., and R. L. Buchanan. 1981. Regulation of aflatoxin biosynthesis: induction of aflatoxin production by various carbohydrates. J. Food Sci. **46:** 633-635.

Abdou, R. F., S. E. Megalla, and S. G. Azab. 1984. Mutagenic effects of aflatoxin B_1 and G_1 on the Egyptian cotton leaf-worm, *Spodoptera littoralis* (Boisd.). Mycopatholo. **88:** 23-26.

Abraham, W. R., and H. A. Arfmann. 1990. 12,13-dihydroxy-fumitremorgin C from *Aspergillus fumigatus*. Phytochem. **29:**1025-1026.

Adams, T. H., W. A. Hide, L. N. Yager, and B. N. Lee. 1992. Isolation of a gene required for programmed initiation of development by *Aspergillus nidulans*. Mol. Cell. Biol. **12:** 3827-3833.

Adamson, R. H., P. Correa, and D. W. Dalgard. 1973. Occurrence of a primary liver carcinoma in a Rhesus monkey fed aflatoxin B_1. J. Natl. Cancer Inst. **50:** 549-553.

Adgigitov, F. L, L. P. Kosichenko, P. G. Popandopulo, D. A. Gubeladze, and Z. A. Djemilev. 1984. Frequency of chromosome aberrations in bone marrow of monkeys and their F1 after aflatoxin B_1 injection. Exp. Pathol. **26:** 163-169.

Alexopoulos, C. J. and Mims C. W. (Eds). 1979. Introductory mycology. pp. 292-293. John Wiley & Sons. New York, New York.

Amitani, R., G. Taylor, E. N. Elezis, C. Llewellyn-Jones, J. Mitchell, F. Kuze, P. J. Cole, and R. Wilson. 1995. Purification and characterization of factors produced by *Aspergillus fumigatus* which affect human ciliated respiratory epithelium. Infect. Immun. **63:** 3266-3271.

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

Arseculeratne, S. N., L. M. de Silva, S. Wijesundera, and C. H. Bandunatha. 1969. Coconut as a medium for the experimental production of aflatoxin. Appl. Microbiol. **18:** 88-94.

Aucamp, P. J., and C. W. Holzapfel. 1970. Polyhydroxyanthraquinones from *Aspergillus versicolor*, *Aspergillus nidulans* and *Bipolaris* spp.: their significance in relation to biogenetic theories on aflatoxin B_1. S. Afr. Chem. Inst. J. **23:** 40-56.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (Eds). 1987. *Escherichia coli*, plasmids, and bacteriophages, in "Current protocols in molecular biology". pp. 1.0.3-1.15.7. John Wiley & Sons, New York.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (Eds). 1995a. Preparation of polyclonal antisera, in "Current protocols in molecular biology". pp. 11.12.1-11.13.3. John Wiley & Sons, New York.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (Eds). 1995b. One-dimensional gel electrophoresis of protein, in "Current protocols in molecular biology". pp. 10.2.1-10.2.18. John Wiley & Sons, New York.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (Eds). 1995c. Immunoaffinity chromatography, in "Current protocols in molecular biology". pp. 10.1.1-10.11.6. John Wiley & Sons, New York.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (Eds). 1995d. Preparation of genomic DNA from mammalian tissue, in "Current protocols in molecular biology". pp. 2.2.1-1.2.2. John Wiley & Sons, New York.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (Eds). 1995e. Preparation and analysis of RNA, in "Current protocols in molecular biology". pp. 4.0.3-4.10.10. John Wiley & Sons, New York.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (Eds). 1995f. Analysis of DNA sequence by blotting and hybridization, in "Current protocols in molecular biology". pp. 6.0.3-6.12.7. John Wiley & Sons, New York.

Autrup, H., J. M. Essigmann, R. G. Croy, B. F. Trump, G. N. Wogan, and C. C. Harris. 1979. Metabolism of aflatoxin B_1 and identification of the major aflatoxin B_1-DNA adducts formed in cultured human bronchus and colon. Cancer Res. **39:** 694-698.

Baldwin, J. E., and E. Abraham. 1988. The biosynthesis of penicillins and cephalosporins. Nat. Prod. Rep. J. Curr. Dev. Bio. Org. Chem. **5:** 129-145.

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

Barredo, J. L., P. van Solingen, B. Diez, E. Alvarez, J. M. Cantoral, A. Kattevilder, E. B. Smaal, M. A. Groenen, A. E. Veenstra, and J. F. Martin. 1989. Cloning and characterization of the acyl-coenzyme A: 6-aminopenicillanic-acid-acyltransferase gene of *Penicillium chrysogenum*. Gene **83:** 291-300.

Bauer-Hofmann, R., A. Buchmann, A. S. Wright, and M. Schwarz. 1990. Mutations in the Ha-*ras* proto-oncogene in spontaneous and chemically induced liver tumours of the CF1 mouse. Carcinogenesis **11:** 1875-1877.

Bayman, P., and P. J. Cotty, 1990. Triadimenol stimulates aflatoxin production by *Aspergillus flavus in vitro*. Mycol. Res. Cambridge, Cambridge University Press. **94:** 1023-1025.

Bayman, P., and P. J. Cotty. 1991. Vegetative compatibility and genetic diversity in the *Aspergillus flavus* population of a single field. Can. J. Bot. **69:** 1007-1711.

Bayman, P., and P. J. Cotty. 1993. Genetic diversity in *Aspergillus flavus*: association wtith aflatoxin production and morphology. Can. J. Bot. **71:** 23-31.

Beck, J., S. Ripka, A. Siegner, E. Schiltz, and E. Schweizer. 1990. The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum*. Its gene structure relative to that of other polyketide synthases. Eur. J. Biochem. **192:** 487-498.

Bennett, J. W., and S. B. Christensen. 1983. New perspective on aflatoxin biosynthesis. Adv. Appl. Microbiol. **29:** 53-92.

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., P. C. Horowitz, and L. S. Lee. 1979. Production of sclerotia by aflatoxigenic and nonaflatoxigenic strains of *Aspergillus flavus* and *A. parsiticus*. Mycol. **71:** 415-422.

Bhatnagar, D., A. H. 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:** 321-349.

Bhatnagar, D., and T. E. Cleveland. 1988. Fate of the methyl group during the conversion of sterigmatocystin into O-methylsterigmatocystin and aflatoxin B_1 by cell-free preparations of *Aspergillus parasiticus*. Biochimie. **70:** 743-747.

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., S. P. McCormick, L. S. Lee, and R. A. Hill. 1987. Identification of O-methylsterigmatocystin as an aflatoxin B_1 and G_1 precursor in *Aspergillus parasiticus.* Appl. Environ. Microbiol. **53:**1028-1033.

Bhatnagar, D., T. E. Cleveland, and D. G. I. Kingston. 1991. Enzymological evidence for separate pathways for aflatoxin B_1 and B_2 biosynthesis. Biochem. **30:** 4343- 4350.

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

Bhatnagar, R. L., S. Ahmad, K. G. Mukerji, and T. A. Subramanian. 1986. Pyridine nucleotides and redox state regulation of aflatoxin biosynthesis in *Aspergillus parasiticus* NRRL 3240. J. Appl. Bacteriol. **60:** 135-141.

Bhatnager, D., K. C. Erhlich, and T. E. Cleveland. 1992. Oxidation-reduction reactions in biosynthesis of secondary metabolites, in "Handbook of applied mycology" (Bhatnagar, D., E. B. Lillehoj, and D. K. Aroa Eds). **5:** 255-286. Marcel Dekker Inc. New York.

Biollaz, M., G. Büchi, and G. Milne. 1970. The biosynthesis of aflatoxin. J. Amer. Chem. Soc. **92:** 1035-1043.

Birch, A. J., and F. W. Donovan. 1953. Studies in relation to biosynthesis. I. Some possible routes to derivatives of orcinol and phloroglucinol. Austral. J. Chem. **6:** 360-368.

Birch, A. J., and S. F. Hussain. 1969. Studies in relation to biosynthesis. 38. A preliminary study of fumagillin. J. Chem. Soc. Perkin. I. **1:** 1473-1474.

Birch, A. J., P. Fitton, E. Pride, A. J. Ryan, H. Smith, and W. B. Whalley. 1958. Studies in relation to biosynthesis. Part XVII. Sclerotiorin, citrinin, and citomycetin. J. Chem. Soc. **IV:** 4576-4581.

Birch, A. J., R. A. Massy-Westropp, and C. J. Moye. 1955. Studies in relation to biosynthesis VII. 2-Hydroxy-6-methylbenzoic acid in *Penicillium griseofulvum* Dierckx. Aust. J. Chem. **8:** 539-544.

Birkinshaw, J. H., A. Bracken, S. E. Michael, and H. Raistrick. 1943. Patulin in the common cold. Collaborative research on a derivative of *Penicillium patulum* Bainier. Lancet **245:** 625-634.

Birkinshaw, J. H., J. C. Roberts, and P. Roffey. 1966. Studies in mycological chemistry. XIX. "Product B" (averantin) [1,3,6,8-tetrahydroxy-2-(1-hydroxyhexyl)-anthraquinone], a pigment from *Aspergillus versicolor* (*Vuillemin*) Tiraboschi. J. Chem. Soc. Perkin. 1. **9:** 855-857.

Blackburn, J. M., J. D. Sutherland, and J. E. Baldwin. 1995. A heuristic approach to the analysis of enzymatic catalysis: reaction of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-α-aminobutyrate and δ-(L-α-aminoadipoyl)-L-cysteinyl-D-allylglycine catalyzed by isopenicillin N synthase isozymes. Biochem. **34:** 7548-7562.

Bloom, S. E. 1984. Sister chromatid exchange studies in the chick embryo and neonate: actions of mutagens in a developing system. Basic Life Sci. **29:** 509-533.

Blount, W. P. 1961. Turkey "X" disease. J. Brit. Turkey Fed. **9:** 52-54.

Boylan, M. T., P. M. Mirabito, C. E. Willett, C. R. Zimmerman, and W. E. Timberlake. 1987. Isolation and physical characterization of three essential conidiation genes from *Aspergillus nidulans.* Mol. Cell Biol. **7:** 3113-3118.

Brown, D. W., F. M. Hauser, R. Tommasi, S. Corlett, and J. J. Salvo. 1993. Structural elucidation of a putative conidial pigment intermediate in *Aspergillus parasiticus.* Tet. Lett. **34:** 419-422.

Brown, D. W., J. H. Yu, H. S. Kelkar, M. Fernandes, T. C. Nesbitt, N. P. Keller, T. H. Adams, and T. J. Leonard. 1996. Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans.* Proc. Natl. Acad. Sci. **93:** 1418-1422.

Brown, D. W., and J. J. Salvo. 1994. Isolation and characterization of sexual spore pigments from *Aspergillus nidulans.* Appl. Environ. Microbiol. **60:** 979-983.

Brown, R. L., P. J. Cotty, and T. E. Cleveland. 1991. Reduction in aflatoxin content of maize by atoxigenic strains of *Aspergillus flavus.* J. Food Prot. **54:** 623-626.

Brown, R. L., T. E. Cleveland, P. J. Cotty, and J. E. Mellon. 1992. Spread of *Aspergillus flavus* in cotton bolls, decay of intercarpellary membranes and production of fungal pectinases. Phytopathol. **82:** 462-467.

Buchanan, R. L., and D. F. Lewis. 1984. Regulation of aflatoxin biosynthesis: effect of glucose on activities of various glycolytic enzymes. Appl. Environ. Microbiol. **48:** 306-310.

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

Buchi, G., K. C. Luk, B. Kobbe, and J. M. Townsend. 1977. Four new mycotoxins of *Aspergillus clavatus* related to tryptoquivaline [collected from mold-damaged rice] related to trptoquivaline. J. Org. Chem. **42:** 244-246.

Buchi, G., Y. Kitaura, S. S. Yuan, H. E. Wright, J. Clardy, A. L. Demain, T. Ginsukon, N. Hunt, and G. N. Wogan. 1973. Structure of cytochalasin E, a toxic metabolite of *Aspergillus clavatus*. J. Amer. Chem. Soc. **95:** 5423-5425.

Burnham, M. K. R., A. J. Earl, J. H. Bull, D. J. Smith, and G. Turner. 1989. DNA encoding ACV synthetase. European Patent Application 8831165.0.

Busby, W. F. Jr., and G. N. Wogan. 1984. Aflatoxins, in "Chemical carcinogens" (Searle, C. E. Ed). pp. 945-1136. American Chemial Soceity. Washington D.C.

Butler, W. H., and J. S. Wigglesworth. 1966. Effects of aflatoxin B_1 on the pregnant rat. Br. J. Exp. Pathol. **47:** 242-247.

Button, M. F. 1988. Enzymes and aflatoxin biosynthesis. Microbiol. Rev. **52:** 274-295.

Caffrey, P., D. J. Bevitt, J. Staunton, and P. F. Leadlay. 1992. Identification of DEBS 1, DEBS 2 and DEBS 3, the multienzyme polypeptides of the erythromycin-producing polyketide synthase from *Saccharopolyspora erythraea*. FEBS Lett. **304:** 225-228.

Cane, D. E., and R. H. Levin. 1976. Application of carbon-13 magnetic resonance to isoprenoid biosynthesis. II. Ovalicin and the use of doubly labeled mevalonate. J. Am. Chem. Soc. **98:** 1183-1188.

Carnaghan, R. B. A. 1965. Hepatic tumors in ducks fed a low level of toxin groundnut meal. Nature **208:** 308.

Carson, D. B., and J. J. Cooney. 1990. Microbodies in fungi: a review. J. Ind. Microbiol. **6:** 1-8.

Cary, J. W., M. Wright, D. Bhatnagar, R. Lee, and F. S. Chu. 1996. Molecular characterization of an *Aspergillus parasiticus* dehydrogenase gene, *nor*A, located on the aflatoxin biosynthesis gene cluster. Appl. Environ. Microbiol. **62:** 360-366.

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

Chang, P.-K., C. D. Skory, and J. E. Linz. 1992. Cloning of a gene associated with aflatoxin B_1 biosynthesis in *Aspergillus parasiticus*. Curr. Genet. **21:** 231-233.

Chang, P.-K., J. W. Cary, D. Bhatnagar, T. E. Cleveland, J. W. Bennett, J. E. Linz, C. P. Woloshik, and G. A. Payne. 1993. Cloning of the *Aspergillus paraticicus apa-2* gene associated with the regulation of aflatoxin biosynthesis. Appl. Environ. Microbiol. **59:** 3273-3279.

Chang, P.-K., J. W. Cary, J. Yu, D. Bhatnagar, and T. E. Cleveland. 1995. The *Aspergillus parasiticus* polyketide synthase gene *pks*A, a homolog of *Aspergillus nidulans* *w*A, is required for aflatoxin B1 biosynthesis. MGG Mol. Gen. Genet. **248:** 270-277.

Chater, K. F. 1992. Genetic regulation of secondary metabolic pathways in *Streptomyces*, in "Secondary metabolites: their function and evolution. Ciba. Found. Symp. 171" (Chadwick, D. J. and J. E. Whelan Eds). pp.144-156. John Wiley & Sons, New York.

Chaves-Carballo, E., R. D. Ellefson, and M. R. Gomez. 1976. An aflatoxin in the liver of a patient with Reye-Johnson syndrome. Mayo. Clin. Proc. **51:** 48-50.

Chet, I., and Henis Y. 1975. Sclerotial morphogenesis in fungi. Annu. Rev. Phytopathol. **13:** 169-192.

Chexal, K. K., J. P. Spinger, J. Clardy, R. J. Cole, J. W. Kirksey, J. W. Dorner, H. G. Cutler, and B. J. Strawter. 1976. Austin, a novel polyisoprenoid mycotoxin from *Aspergillus ustus* [letter]. J. Am. Chem. Soc. **98:** 6748.

Chu, F. S. 1991. Mycotoxins: food contamination, mechanism, carcinogenic potential and preventive measures. Mutat. Res. **259:** 291-306.

Chung, C. H., K. H. Ling, S. S. Tung, and T. C. Tung. 1971. Study on fungi of the stored unhulled rice to Taiwan. (2) Aflatoxin B_1 like compounds from the culture of *Aspergillus genus*. J. Formosan Med. Assoc. **70:** 258-266.

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. Biochem. **21:** 125-140.

Clardy, J., J. P. Springer, G. Buchi, K. Matsuo, and R. Wightman. 1975. Letter: Tryptoquivaline and tryptoquivalone, two tremorgenic metabolites of *Aspergillus clavatus*. J. Am. Chem. Soc. **97:** 663-665.

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

Cleveland, T. E., and D. Bhatnagar. 1987. Individual reaction requirements of two enzyme activities, isolated from *Aspergillus parasiticus*, which together catalyze conversion of sterigmatocystin to aflatoxin B_1. Can. J. Microbiol. **33:** 1108-1112.

Cleveland, T. E., D. Bhatnagar, C. J. Foell, and S. P. McCormick. 1987. Conversion of a new metabolite to aflatoxin B_2 by *Aspergillus parasiticus*. Appl. Environ. Microbiol. **3:** 2804-2807.

Clevstrom, G. and H. Ljunggren. 1985. Aflatoxin formation and the dual phenomenon in *Aspergillus flavus* Link. Mycopatholo. **92:** 129-139.

Clutterbuck, A. J. 1969. A mutational analysis of conidial development in *Aspergillus nidulans.* Genet. **63:** 317-327.

Cohen, G, D. Dembiec, and J. Marcus. 1970. Measurement of catalase activity in tissue extracts. Anal. Biochem. **34:** 30-38.

Cole, R. J., and R. H. Cox (Eds). 1981. Handbook of toxic fungal metabolites. pp.111-112. Academic Press Inc. New York.

Cole, R. J., J. W. Dorner, J. P. Springer, and R. H. Cox. 1981. Indole metabolites from a strain of *Aspergillus flavus.* J. Afric. Food Chem. **29:** 293-295.

Collie, N., and W. S. Myers. 1893. The formation of orcinol and other condensation products from dehydracetic acid. J. Chem. Soc. **63:** 122-128.

Cotty, P. J. 1988. Aflatoxin and sclerotial production by *Aspergillus flavus*, influence on pH. Phytopathol. **78:**1250-1253.

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

Cotty, P. J. 1990. Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. Plant Dis. **64:** 233-235.

Cotty, P. J. 1992a. Use of native *Aspergillus flavus* strains to prevent aflatoxin contamination. United States Patent 5,171,686.

Cotty, P. J. 1992b. Soil populations of *Aspergillus flavus* group fungi in agricultural fields in Alabama, Arizona, Louisianan, and Mississippi. Phytophathol. **82:** 1064.

Cotty, P. J. 1992c. *Aspergillus flavus*, wild intruder or domesticated free loader, in "Aflatoxin elimination whorkshiop" (Robens, J. F. Ed). pp. 28. Agricultural Research Service, Beltsville.

Cotty, P. J., and P. Bayman. 1993. Competitive exclusion of a toxigenic strain of *Aspergillus flavus* by an atoxigenic strain. Phytopathol. **83:** 1283-1287.

Coulombe, R. A. Jr., and R. P. Sharma. 1985. Effect of repeated dietary exposure of aflatoxin B_1 on brain biogenic amines and metabolites in the rat. Toxicol. Appl. Pharmacol. **80:** 496-501.

Courtemanche, C., and A. Anderson. 1994. Shuttle-vector mutagenesis by aflatoxin B1 in human cells: effects of sequence context on the *sup*F mutational spectrum. Mutat. Res. **306:** 143-151.

Crosby, J., D. H. Sherman, M. J. Bibb, W. P. Revill, D. A. Hopwood, and T. J. Simpson. 1995. Polyketide synthase acyl carrier proteins from *Streptomyces*: expression in *Escherichia coli*, purification and partial characterisation. Biochim. Biophys. Acta **1251:** 32-42.

Da Silva, A. M., and M. H. Juliani. 1988. Regulation of tubulin and actin synthesis and accumulation during *Blastocladiella emersonii* development. Cell Differ. **24:** 45-54.

Dafalla, R., Y. M. Hassan, and S. E. I. Adam. 1987. Fatty hemorrhagic liver and kidney syndrome in breeding hens caused by aflatoxin B1 and heat stress in the Sudan. Vet. Hum.Toxicol. **29:** 252-254.

Davies, J. 1992. Introduction, in "Secondary metabolites: their function and evolution. Ciba. Found. Symp. 171" (Chadwick, D. J. and J. E. Whelan Eds). pp.1-2. John Wiley & Sons, New York.

De Duve, C., and H. Beaufay. 1981. A short history of tissue fractionation. J. Cell Biol. **91:** 293s-299s.

Deacon, J. W. (Ed). 1980. Sclerotia, in "Introduction to modern mycology". pp. 60-61. John Wiley & Sons. New Yowk, New Yowk.

Dimroth, P., H. Walter, and F. Lynen. 1970. Biosynthesis of 6-methylsalicylic acid. Eur. J. Biochem. **13:** 98-110.

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

Dorner, J. W. 1983. Production of cyclopiazonic acid by *Aspergillus tamarii* Kita. Appl. Environ. Microbiol. **46:**1435-1437.

Dorner, J. W., R. J. Cole, and P. D. Blankenship. 1992. Use of a biocompetitive agent to control preharvest aflatoxin in drought stressed peanuts. J. Food Prot. **55:** 888- 892.

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

Doyle, M. P., and E. H. Marth. 1978. Aflatoxin is degraded by mycelia from toxigenic and nontoxigentic strains of *Aspergilli* grown on different substrates. Mycopatholo. **63:** 145-153.

Dulaney, E. L. 1947. Some aspects of penicillin production by *Aspergillus nidulans*. Mycol. **39:** 570-582.

Dutton, M. F. 1988. Enzymes and aflatoxin biosynthesis. Microbiol. Rev. **52:** 274- 295.

Edds, G. T., K. P. C. Nair, and C. F. Simpson. 1973. Effect of aflatoxin B_1 on resistance in poultry against cecal coccidiosis and Marek's disease. Am. J. Vet. Res. **34:** 819-826.

Elis. J., and J. A. DiPaolo. 1967. Aflatoxin B_1. Induction of malformations. Arch. Pathol. **83:** 53-57.

Ellis, W. O., J. P. Smith, B. K. Simpson, and J. H. Oldham. 1991. Aflatoxin in food: occurrence, biosynthesis, effects on organisms, detection, and methods of control. Crit. Rev. Food Sci. Nutr. **30:** 403-439.

Epstein, S. M., B. Bartus, and E. Farber. 1969. Renal epithelial neoplasms induced in male Wistar rats by oral aflatoxin B_1. Cancer Res. **29:**1045-1050.

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 on relation to carcinogenesis. Drug. Metab. Rev. **13:** 581-602.

Feitelson, J. S., F. Malpartida, and D. A. Hopwood. 1985. Genetic and biochemical characterization of the red gene cluster of *Streptomyces coelicolor* A3(2). J. Gen. Microbiol. **131:** 2431-2441.

Feng, G. H., and T. J. Leonard. 1995. Characterization of the polyketide synthase gene (*pks*L1) required for aflatoxin biosynthesis in *Aspergillus parasiticus*. J. Bacteriol. **177:** 6246-6254.

Fitzell, D. L., R. Singh, D. P. Hsieh, and E. L. Motell. 1977. Nuclear magnetic resonance identification of versiconal hemiacetal acetate as an intermediate in aflatoxin biosynthesis. J. Agric. Food Chem. **25:** 1193-1197.

Foster, P. L., J. D. Groopman, and E. Eisenstadt. 1988. Induction of base substitution mutations by aflatoxin B_1 is *muc*AB dependent in *Escherichia coli*. J. Bacteriol. **170**: 3415-3420.

Franck, B. 1980. The biosynthesis of the ergochromes, in "The biosynthesis of mycotoxins: a study in secondary metabolism"(Steyn, P. S. Ed). pp. 157-191. Academic Press. New York.

Frisvad, J. C. 1988. Fungal species and their specific production of mycotoxins, in " Introduciton to food-borne fungi" (Samson, R. A., and E. S. van Reenen-Hoekstra Eds). pp. 239-249. 3rd Ed. Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

Gallagher, R. T., and B. J. Wilson. 1978. Aflatrem, the tremorgenic mycotoxin from *Aspergillus flavus*. Mycopatholo. **66:** 183-185.

Gallagher, R. T., J. Finer, J. Clardy, A. Leutwiler, F. Weibel, W. Acklin, and D. Arigoni. 1980. Paspalinine, a tremorgenic metabolite from *Claviceps paspali* Stevens and Hall. Tet. Lett. **21:** 235-238.

Gallagher, R. T., J. L. Richard, H. M. Stahr, and R. J. Cole. 1978. Cyclopiazonic acid production by aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavus*. Mycopatholo. **66:** 31-36.

Ganz, T., M. E. Selsted, D. Szklarek, S. S. Harwig, K. Daher, D. F. Bainton, and R. I. Lehrer. 1985. Defensins. Natural peptide antibiotics of human neutrophils. J. Clin. Invest. **76:** 1427-1435.

Giambrone, J. J., U. L. Diener, N. D. Davis, V. S. Panangala, and F. J. Hoerr. 1985. Effects of purified aflatoxin on broiler chickens. Poult. Sci. **64:** 852-858.

Goerttler, K., H. Lohrke, H. J. Schweizer, and B. Hesse. 1980. Effects of aflatoxin B_1 on pregnant inbred Sprague-Dawley rats and their F1 generation. A contribution to transplacental carcinogenesis. J. Natl. Cancer. Inst. **64:** 1349-1354.

Gopalan, C., P. G. Tulpule, and D. Krishnamurthi. 1972. Induction of hepatic carcinoma with aflatoxin in the Rhesus monkey. Food Cosmet Toxicol. **10:** 519-521.

Gorman, D. P., M. S. Kang, T. E. Cleveland, and R. L. Hutchinson. 1992. Field aflatoxin production by *Aspergillus flavus* and *A. parasiticus* on maize kernels. Euphytica (Netherlands) **61:** 187-191.

Gorst-Allman, C. P., P. S. Steyn, P. L. Wessels, and B. de Scott. 1978. Carbon-13 nuclear magnetic resonance assignments and biosynthesis of veriscolorin A in *Aspergillus parasiticus*. J. Chem. Soc. Perkin.Trans. I. **9:** 961-964.

Goze, A., A. Sarsin, Y. Moule, and R. Devoret. 1975. Induction and mutagenesis of prophage lambda in *Escherichia coli* K12 by metabolites of aflatoxin B_1. Mutat. Res. **28:** 1-7.

Graf, U., F. E. Wurgler, A. J. Katz, H. Frei, H. Juon, C. B. Hall, and P. G. Kale. 1984. Somatic mutation and recombination test in *Drosophila melanogaster*. Environ. Mutagen. **6:** 153-188.

Griffith, G. W.., G. I. Jenkins, E. Milner-White, and A. J. Clutterbuck. 1994. Homology at the amino acid level between plant phytochromes and a regulator of asexual sporulation in *Emericella (Aspergillus) nidulans*. Photochem. photobiol. **59:** 252-256.

Groopman, J. D., L. G. Cain, and T. W. Kensler. 1988. Aflatoxin exposure in human populations: measurements and relationship to cancer. Crit. Rev. Toxicol. **19:** 113-145.

Gupta, S. K., K. K. Maggon, and T. A. Venkitasubramanian. 1977. Regulation of aflatoxin biosynthesis. I. Comparative study of mycelial composition and glycolysis in aflatoxigen and nonaflatoxigenic strains. Microbios. **18:** 27-33.

Hamasaki, T., K. Matsui, K. Isono, and Y. Hatsuda. 1973. A new metabolite from *Aspergillus versicolor*. Agric. Biol. Chem. **37:** 1769-1770.

Harper, A. E. 1965. Glucose-6-phosphatase, in "Methods of enzymatic analysis" (Bergmeyer, H. U. Ed). pp. 788-792. Academic Press, New York.

Harris, J. L. 1986. Modified method for fungal slide culture. J. Clin. Microbiol. **24:** 460-461.

Hatsuda, Y., and S. Kuyama. 1954. Studies on metabolic products of *Aspergillus versicolor*, I. Cultivation of *Aspergillus versicolor*, isolation and purification of metabolic products. J. Agric. Chem. Soc. Jpn. **28:** 989-993.

Hayes, R. B., J. P. Van Nieuwenhuize, J. W. Raatgever, and F. J. W. Ten-Kate. 1984. Aflatoxin exposures in the industrial setting: an epidemological study of mortality [Dutch oilpress workers]. Food Chem. Toxicol. **22:** 39-43.

Heller, K., C. Schultz, R. Loser, and R. Roschenthaler. 1975. Fumitremorgin A from *Aspergillus fumigatus*. Chem. Pharm. Bull. Tokyo, **28:** 245-254.

Henderberg, A., J. W. Bennett, and L. S. Lee. 1988. Biosynthetic origin of aflatoxin G_1: confirmation of sterigmatocystin and lack of confirmation of aflatoxin B_1 as precursors. J. Gen. Microbiol. **134:** 661-667.

Herrold, K. M. 1969. Aflatoxin induced lesions in Syrian hamsters. Br. J. Cancer. **23:** 655-660.

Hetherington, A. C., and H. Raistrick. 1931. Studies in the biochemistry of microorganisms. Part XIV. On the production and chemical constitution of a new yellow coloring matter, citrinin, produced from glucose by *Penicillium ctirinum* Thom. Phil. Trans. R. Soc. London, Ser. B. **220:** 269-295.

Hill, R. A., D. M. Wilson, W. W. McMillian, N. W. Widstrom, R. J. Cole, T. H. Sanders, and P. D. Blankenship. 1985. Ecology of the *Aspergillus flavus* group and aflatoxin formation in maize and groundnut, in "Trichothecenes and other mycotoxins" (Lacey, J. Ed). pp. 79-95. John Wiley & Sons, New York.

Hohn, T. M., S. P. McCormick, and A. E. Desjardins. 1993. Evidence for a gene cluster involving trichothecene-pathway biosynthetic genes in *Fusarium sporotrichioides*. Curr. Genet. **24:** 291-295.

Holzapfel, C. W. 1980. The biosynthesis of cyclopiazonic acid and related tetramic acids, in "The biosynthesis of mycotoxins: a study in secondary metabolism" (Steyn, P. S. Ed). pp. 327-355. Academic Press, New York.

Horng, J. S., P.-K. Chang, J. J. Pestka, and J. E. Linz. 1990. Development of a homologous transformation system for *Aspergillus parasiticus* with the gene encoding nitrate reductase. Mol. Gen. Genet. **224:** 294-296.

Horsman, M. R., A. A. Khalil, D. J. Chaplin, and J. Overgaard. 1995. The ability of nicotinamide to inhibit the growth of a C3H mouse mammary carcinoma. Acta Oncol. **34:** 443-446.

Hoza, J. 1966. Secondary reactions of some new antibiotics. Fumagillin. Lek. Veda. Zahr. **8:** 156-157.

Hsieh, D. P., and R. I. Mateles. 1970. The relative contribution of acetate and glucose to aflatoxin biosynthesis. Biochem. Biophys. Acta **208:** 482-486.

Hsieh, D. P., and R. I. Mateles. 1971. Preparation of labeled aflatoxins with high specific activities. Appl. Microbiol. **22:**79-83.

Hsieh, D. P., C. C. Wan, and J. A. Billington. 1989. Conversion of versiconal acetate to versiconal and versicolorin C in extracts from *Aspergillus parasiticus*. Mycopatholo. **107:** 121-126.

Hsieh, D. P., R. Singh, R. C. Yao, and J. W. Bennett. 1978. Anthraquinones in the biosynthesis of sterigmatocystin by *Aspergillus versicolor*. Appl. Environ. Microbiol. **35:** 980-982.

Jeenah, M. S., and M. F. Dutton. 1983. The conversion of sterigmatocystin to O-methylsterigmatocystin and aflatoxin B_1 by a cell-free preparation. Biochem. Biophys. Res. Commun. **116:** 1114-1118.

Jefferson, R. A., S. M. Burgess, and D. Hirsh. 1986. β-Glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc. Natl. Acad. Sci. **83:** 8447-8451.

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

Kaden, D. A., K. M. Call, P. M. Leong, E. A. Komives, and W. G. Thilly. 1987. Killing and mutation of human lymphoblast cells by aflatoxin B_1: evidence for an inducible repair response. Cancer Res. **47:** 1993-2001.

Kale, S. P., D. Bhatnagar, and J. W. Bennett. 1994. Isolation and charaterization of morphological variants of *Aspergillsus parasiticus* deficient in secondary metabolite production. Mycol. Res. **98:** 645-652.

Keller, N. P., and T. H. Adams. 1995. Analysis of a mycotoxin gene cluster in *Aspergillus nidulans.* SAAS Bull. Biochem. Biotechnol. **8:**14-21.

Keller, N. P., H. C. Dischinger Jr., D. Bhatnagar, T. E. Cleveland, and A. H. J. Ullah. 1993. Purification of a 40-kilodalton methyltransferase active in the aflatoxin biosynthetic pathway. Appl. Environ. Microbiol. **59:** 479-484.

Keller, N. P., N. J. Kantz, and T. H. Adams. 1994. *Aspergillus nidulans ver*A is required for production of the mycotoxin sterigmatocystin. Appl. Environ. Microbiol. **60:** 1444-1450.

Keller, N. P., S. Segner, D. Bhatnagar, and T. H. Adams. 1995. stcS, a putative P-450 monooxygenase, is required for the conversion of versicolorin A to sterigmatocystin in *Aspergillus nidulans.* Appl. Environ. Microbiol. **10:** 3628-3632.

Khan, S. N. and T. A. Venkitasubramanian. 1986. Regulation of aflatoxin biosynthesis: effect of adenine nucleotides, cyclic AMP and N-6, O-2'-dibutyryl cyclic AMP on the incorporation of (1-^{14}C)-acetate into aflatoxins by *Aspergillus parasiticus* NRRL-3240. J. Environ. Sci. Health. B. **211:** 67-85.

Kimura, N., and T. Tsuge. 1993. Gene cluster involved in melanin biosynthesis of the filamentous fungus *Alternaria alternata.* J. Bacteriol. **175:** 4427-4435.

King, R. C., and W. D. Stansfield. 1985. "A dictioany of gnetics". pp. 76. Oxford Univrsity Press, New York.

Kirby, G. W., and D. J. Robins. 1980. The biosynthesis of gliotoxin and related epipolythiodioxopiperazines in *Trichoderma viride,* in "The biosynthesis of mycotoxins: a study in secondary metabolism" (Steyn, P. S. Ed). pp. 301-326. Academic Press, New York.

Kirby, G. W., G. L. Patrick, and D. J. Robins. 1978. Cyclo-(L-phenylalanyl-L-seryl) as an intermediate in the biosynthesis of gliotoxin. J. Chem. Soc. Perkin Trans. I. **1:** 1336-1338.

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

Kodama, M., F. Inoue, and M. Akao. 1990. Enzymatic and non-enzymatic formation of free radicals from aflatoxin B1. Free Radic. Res. Commun. **10:** 137-142.

Kreuzaler, F., H. Ragg, W. Heller, R. Tesch, I. Witt, D. Hammer, and K. Hahlbrock. 1979. Flavanone synthase from *Petroselinum hortense*. Molecular weight, subunit composition, size of messenger RNA, and absence of pantetheinyl residue. Eur. J. Biochem. **99:** 89-96.

Krishnamachari, K. A., R. V. Bhat, V. Nagarajan, and T. B. Tilak. 1975. Hepatitis due to aflatoxicosis. An outbreak in Western India. Lancet **1:** 1061-1063.

Krogh, P., B. Hald, and E. J. Pedersen. 1973. Occurrence of ochratoxin A and citrinin in cereals associated with mycotoxic porcine nephropathy. Acta Pathol. Microbiol. Scand. B. Microbiol. Immunol. **81:** 689-695.

Kuo, H. L., and W. P. Yun. 1988. Biosynthesis of territrems by *Aspergillus terreus.* Appl. Environ. Microbiol. **54:** 585-587.

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

Laakso, J. A., M. R. TePaske, P. F. Dowd, J. B. Gloer, and D. T. Wicklow. 1992. Sulpinine, secopenitrem B and alfatrem B antiinsectan metabolites. U. S. Dep. Agric. Pat. **5,130,236:** 1.

Labuza, T. P. 1983. Regulation of mycotoxins in food. Ames: International Association of Milk, Food, and Environmental Sanitarians. J. Food Prot. **46:** 260-265.

Lamb, M. J., and L. J. Lilly. 1971. Induction of recessive lethals in *Drosophila melanogaster* by aflatoxin B1. Mutat. Res. **11:** 430-433.

Lauren, D. J., A. Wishkovsky, J. M. Groff, R. P. Hedrick, and D. E. Hinton. 1989. Toxicity and pharmacokinetics of the antibiotic fumagillin in yearling rainbow trout (*Salmo gairdneri*). Toxicol. Appl. Pharmacol. **98:** 444-453.

Lebron, C.I., R. A. Molins, H. W. Walker, A. A. Kraft, and H. M. Stahr. 1989. Inhibition of growth and aflatoxin production of *Aspergilli* in medium containing phosphates. J. Food Prot. **52:** 4-6.

Lee, B. N., and T. H. Adams. 1994a. The *Aspergillus nidulans fluG* gene is required for production of an extracellular developmental signal and is related to prokaryotic glutamine synthetase I. Gene. Dev. **8:** 641-651.

Lee, B. N., and T. H. Adams. 1994b. Overexpression of *flbA*, an early regulator of *Aspergillus nidulans* asexual sporulation, leads to activation of *brlA* and premature initiation of development. Mol. Microbiol. **14**: 323-334.

Lee, B. N., and T. H. Adams. 1996. *FluG* and *flbA* function interdependently to initiate conidiophore development in *Aspergillus nidulans* through *brlA* beta activation. EMBO J. **15:** 299-309.

Lee, L. S., J. W. Bennett, A. F. Cucullu, and J. B. Stanley. 1975. Two distinct O-methyltransferases in aflatoxin biosynthesis. J. Agric. Food Chem. **23:** 1132-1134.

Lee, L. S., J. W. Bennett, L. A. Goldblatt, and R. E. Lundin. 1971. Norsolorinic acid from a mutant strain of *Aspergillus parasiticus*. J. Amer. Oil Chem. Soc. **48:** 93-94.

Lee, S. S., F. C. Peng, C. M. Chiou, and K. H. Ling. 1992. NMR assignments of territrems A, B, and C and the structure of MB2, the major metabolite of territrem B by rat liver microsomal fraction. J. Nat. Prod. **55:** 251-255.

Lee, Y. J., and W. M. Hagler Jr. 1991. Aflatoxin and cyclopiazonic acid production by *Aspergillus flavus* isolated from contaminated maize. J. Food Sci. **56:** 871-872.

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

Lin, M. T., D. P. Hsieh, R. C. Yao, and J. A. Donkersloot. 1973. Conversion of averufin into aflatoxins by *Aspergillus parasiticus*. Biochem. **12:** 5167-5171.

Ling, K. H. 1976. Study on mycotoxins contaminated in food in Taiwan. (2) Tremor inducing compounds from *Aspergillus terreus*. Proc. Natl. Aci. Council, Republic of China **9:** 121-129.

Ling, K. H. 1994. Territrems, tremorgenic mycotoxins isolated from *Aspergillus terreus* J. Toxicol. Toxin Rev. **13:** 243-252.

Ling, K. H., H. H. Liou, C. M. Yang, and C. K. Yang. 1984. Isolation, chemical structure, acute toxicity, and some physicochemical properties of territrem C from *Aspergillus terreus*. Appl. Environ. Microbiol. **7:** 98-100.

Lowery, C., T. C. Orton, and R. C. Garner. 1983. Metabolic activation and bacterial mutagenicity of aflatoxin B_1 in two different test systems. Toxicol. **29:** 131-141.

Lück, H. 1965. Catalase, in "Methods of enzymatic analysis" (Bergmeyer, H. U. Ed). pp. 885-894. Academic Press, New York.

Luckner, M. 1990. Secondary products derived from L-phenylalanine and L-tyrosine (phenylpropanoinds), in "Secondary metabolism in microorganisms, plants, and animals" (Luckner, M. Ed). pp. 361-417. 3rd Ed. Springer-Verlag. New York

Luk, K. C., B. Kobbe, and J. M. Townsend. 1977. Production of cyclopiazonic acid by *Aspergillus flavus* Link. Appl. Environ. Microbiol. **33:** 211-212.

MacCabe, A. P., H. van Liempt, H. Palissa, S. E. Unkles, M. B. R. Riach, E. Pfeifer, H. von Dohren, and J. R. Kinghorn. 1991. δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase from *Aspergillus nidulans.* Molecular characterization of the *acv*A gene encoding the first enzyme of the penicillin biosynthetic pathway. J. Bio. Chem. **266:** 12646-12654.

MacCabe, A. P., M. B. R. Riach, S. E. Unkles, and J. R. Kinghorn. 1990. The *Aspergillus nidulans npe*A locus consists of three contiguous genes required for penicillin biosynthesis. EMBO J. **9:** 279-287.

Mace, K., F. J. Gonzalez, I. R. McConnell, R. C. Garner, O. Avanti, C. C. Harris, and A. M. Pfeifer. 1994. Activation of promutagens in a human bronchial epithelial cell line stably expressing human cytochrome P-450 1A2. Mol. Carcinog. **11:** 65-73.

Macgregor, J. T., D. H. Gould, A. D. Mitchell, and G. P. Sterling. 1979. Mutagenicity tests of diflubenzuron in the micronucleus test in mice, the L5178Y mouse lymphoma forward mutation assay, and the Ames *Salmonella* reverse mutation test. Mutat. Res. **66:** 45-53.

Maebayashi, Y., K. Miyaki, and M. Yamazaki. 1972. Application of ^{13}C-NMR to the biosynthetic investigations. I. Biosynthesis of ochratoxin A. Chem. Pharm. Bull. (Tokyo) **20:** 2172-2175.

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

Manitto, P. 1981. The isoprenoids, in "Biosynthesis of natural products" (Manitto, P. Ed). pp. 213-313. John Wiley & Sons, New York.

Martin, J. F., and S. Gutierrez. 1995. Genes for β-lactam antibiotic biosynthesis. Antonie van leeuwenhoek **67:** 181-200.

Mashaly, R. L, S. L. Habib, S. A. el Deeb, M. H. Salem, and M. M. Safwat. 1988. Isolation, purification and characterization of enzyme(s) responsible for conversion of sterigmatocystin to aflatoxin B_1. Z. Lebensm. Unters. Forsch. **186:** 118-124.

Matsushima, K., Y. Ando, T. Hamasaki, and K. Yabe. 1994. Purification and characterization of two versiconal hemiacetal acetate reductases involved in aflatoxin biosynthesis. Appl. Environ. Microbiol. **60:** 2561-2567.

May, G. S., M. L. Tsang, H. Smith, S. Fidel, and N. R. Morris. 1987. *Aspergillus nidulans* β-tubulin genes are unusually divergent. Gene. **55:** 231-243.

May, G. S., N. R. Morris. 1988. Developmental regulation of a conidiation specific beta-tubulin in *Aspergillus nidulans.* Dev. Biol. **128:** 406-414.

Mayorga, M. E., and W. E. Timberlake. 1992. The developmentally regulated *Aspergillus nidulans wA* gene encodes a polypeptide homologous to polyketide and fatty acid synthases. Mol. Gen. Genet. **235:** 205-212.

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. Microbiol. **53:** 14-16.

McGuire, S. M., S. W. Brobst, T. L. Graybill, K. Pal, and C. A. Townsend. 1989. Partitioning of tetrahydro- and dihydro-bisfuran formation in aflatoxin biosynthesis defined by cell-free and direct incorporation experiments. J. Am. Chem. Soc. **111:** 8308-8309.

McLean , M., P. Berjak, M. P. Watt, and M. F. Dutton. 1992. The effects of aflatoxin B_1 on immature germinating maize (Zea mays) embroyos. Mycopathol. **119:** 181-190.

Mönke, E., and W. Schäfer. 1993. Transient and stable gene expression in the fungal maize pathogen *Cochliobolus heterostrophus* after thransformation with the β-glucuronidase (GUS) gene. Mol. Gene. Genet. **241**: 73-80.

Montenegro, E., J. L. Barredo, S. Gutierrez, B. Diez, E. Alvarez, and J. F. Martin. 1990. Cloning, characterization of the acyl-CoA: 6-amino penicillanic acid acyltransferase gene of *Aspergillus nidulans* and linkage to the isopenicillin N synthase gene. Mol. Gen. Genet. **221:** 322-330.

Mori, H., J. Kitamura, S. Sugie, K. Kawai, and T. Hamasaki. 1985. Genotoxicity of fungal metabolites related to aflatoxin B_1 biosynthesis. Mutat. Res. **143:** 121-125.

Moss, M. O. 1977. *Aspergillus* mycotoxins, in "Genetics and physiology of *Aspergillus*" (Simth, J. E., and J. A. Pateman Eds). pp. 499-524. Academic Press, London.

Moss, M. O. 1994. Biosynthesis of *Aspergillus* toxins-non-aflatoxins. in "The genus *Aspergillus*" (Powell, K. A., A. Renwick, and J. F. Peberdy Eds). pp. 29-50. Plenum Press, New York.

Muller, W. H., T. P. van der Krift, A. J. J. Krouwer, H. A. B. Wosten, L. H. M. Voort, E. B. van der Smaal, and A. J. Verkleij. 1991. Localization of the pathway of the penicillin biosynthesis in *Penicillium chrysogenum.* MBO J. Eur. Mol. Biol. Organ. **10:** 489-495.

Ngindu, A., B. K. Johnson, P. R. Kenya, J. A. Ngira, D. M. Ocheng, H. Nandwa, T. N. Omondi, A. J. Jansen, W. Ngare, J. N. Kaviti, D. Gatei, and T. A. Siongok. 1982. Outbreak of acute hepatitis caused by aflatoxin poisoning in Kenya. Lancet. **1:** 1346-1348.

Niehaus, W. G. Jr, and R. P. Dilts Jr. 1984. Purification and characterization of glucose-6-phosphate dehydrogenase from *Aspergillus parasiticus.* Arch. Biochem. Biophys. **228:** 113-119.

Nishimura, Y., R. Murata, and M. Hiraoka. 1996. Combined effects of an angiogenesis inhibitor (TNP-470) and hyperthermia. Br. J. Cancer. **73:** 270-427.

Nose, H., F. Imazeki, M. Ohto, and M. Omata. 1993. p53 gene mutations and 17p allelic deletions in hepatocellular carcinoma from Japan. Caner **72:** 355-360.

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 *pyr*G (orotidine-5'-phosphate decarboxylase) gene of *Aspergillus nidulans.* Gene **61:** 385-399.

Olsson, S., and D. H. Jennings. 1991. A glass fiber filter technique for studying nutrient uptake by fungi: the technique used on colonies grown on nutrient gradients of carbon and phosphorus. Exp. Mycol. **15:** 292-301.

Ottinger, M. A., and J. A. Doerr. 1980. The early influence of aflatoxin upon sexual maturation in the male Japanese quail. Poult. Sci. **59:** 1750-1754.

Pachler, K. G., P. S. Steyn, R. Vleggaar, and P. L. Wessels. 1976. Carbon-13 nuclear magnetic resonance assignments and biosynthesis of aflatoxin B_1 and sterigmatocystin. J. Chem. Soc. Perkin. I. **11:** 1182-1189.

Papa, K. E. 1986. Heterokaryon incompatibility in *Aspergillus flavus.* Mycol. **78:** 98-101.

Park, D. L., and B. Liang. 1993. Perspectives on aflatoxin control for human food and animal feed. Trends Food Sci. Technol. **4:** 334-342.

Payne, G. A., G. J. Nystrom, D. Bhatnagar, T. E. Cleveland, and C. P. Woloshuk. 1993. Clonig of the *afl*-2 gene involved in aflatoxin biosynthesis from *Aspergillus falvus.* Appl. Environ. Mirobiol. **59:**156-162.

Peng, F. C., K. H. Ling, Y. Wang, and G. H. Lee. 1985. Isolation, chemical structure, acute toxicity, and some physicochemical properties of territrem B from *Aspergillus terreus.* Appl. Environ. Microbiol. **49:** 721-723.

Pestka, J. J, P. K. Gaur, and F. S. Chu. 1980. Quantitation of aflatoxin B_1 and aflatoxin B_1 antibody by an enzyme-linked immunosorbent microassay. Appl. Environ. Microbiol. **40:** 1027-1031.

Puisieux, A., S. Lim, J. Groopman, and M. Ozturk. 1991. Selective targeting of p53 gene mutational hotspots in human cancers by etiologically defined carcinogens. Cancer Res. **51:** 6185-6189.

Raina, V., H. L. Gurtoo, M. Berrigan, and B. Paigen. 1980. Genetic dependence of hepatic microsome-mediated depression of aflatoxin B_1 activation to mutagens in Ames *Salmonella typhimurium* TA-98 system. Biochem. Biophys. Res. Commun. **94:** 737-743.

Rainey, P. B., C. L. Brodey, and K. Johnstone. 1993. Identification of a gene cluster encoding three high-molecular-weight proteins, which is required for synthesis of tolasin by the mushroom pathogen *Pseudomonas tolaasii.* Mol. Microbiol. **8:** 643-652.

Ramon, D., L. Carramolino, C. Patino, F. Sanchez, and M. A. Penalva. 1987. Cloning and characterization of the isopenicillin N synthetase gene mediating the formation of the beta-lactam ring in *Aspergillus nidulans.* Gene **57:** 171-181.

Reddy, J. K., D. J. Svoboda, and M. S. Rao. 1976. Induction of liver tumors by aflatoxin B_1 in the tree shrew (*Tupaia glis*), a nonhuman primate. Cancer Res. **36:** 151-160.

Reddy, T. V., L. Viswanathan, and T. A. Venkitasubramanian. 1971. High aflatoxin production on a chemically defined medium. [*Aspergillus parasiticus*]. Appl. Microbiol. **22:** 393-396.

Refolo, L. M., C. B. Bennett, and M. Z. Humayun. 1987. Mechanisms of frameshift mutagenesis by aflatoxin B_1-2,3-dichloride. J. Mol. Biol. **193:** 609-636.

Revill, W. P., M. J. Bibb, and D. A. Hopwood. 1995. Purification of a malonyltransferase from *Streptomyces coelicolor* A3(2) and analysis of its genetic determinant. J. Bacteriol. **177:** 3946-3952.

Richard, J. L., A. C. Pier, S. J. Cysewski, and C. K. Graham. 1973. Effect of aflatoxin and aspergillosis on turkey poults. Avian Dis. **17:** 111-121.

Richard, J. L., and R. T. Gallagher. 1979. Multiple toxin production by an isolate of *Aspergillus flavus*. Mycopatholo. **67**: 161-163.

Roach, P. L., C. J. Schofield, J. E. Baldwin, I. J. Clifton, and J. Hajdu. 1995. Crystallization and preliminary X-ray diffraction studies on recombinant isopenicillin N synthase from *Aspergillus nidulans*. Protein Sci. **4**:1007-1009.

Robens, J. F., and J. L. Richard. 1992. Aflatoxins in animal and human health. Rev. Environ. Contam. Toxicol. **127**: 69-94.

Rodig, O. R., L. C. Ellis, and I. T. Glover. 1966. The biosynthesis of citrinin in *Penicillium citrinum*. I. Production and degradation of citrinin. Biochem. **5**: 2451-2458.

Rose, M. E., and R. A. W. Johnstone. 1982. Structure elucidation, in "Mass sepctrometry for chemists and biochemists". pp. 185-246. Cambridge University Press, Cambridge, Great Britain.

Rothweiler, W., and C. Tamm. 1966. Isolation and structure of phomin. Experientia **22**: 750-752.

Russell, T. E. 1979. Field conditions which influence aflatoxin *Aspergillus flavus* production in cottonseed. Prepr. Pap. Oilseed Process Clin. New Orleans, Oilseed Processing Clinic **28**: 44-45.

Samson, R. A., and J. C. Frisvad. 1991. Current taxonomic concepts in *Penicillium* and *Aspergillus*. Dev. Food Sci. **26**: 405-439.

Samson, S. M., R. Belagaje, D. T. Blankenship, J. L. Chapman, D. Perry, P. L. Skatrud, R. M. Van Frank, E. P. Abraham, J. E. Baldwin, and S. W. Queener. 1985. Isolation, sequence determination and expression in *Escherichia coli* of the isopenicillin N synthetase gene from *Cephalosporium acremonium*. Nature **318**: 191-194.

Schoppner, A., and H. Kindl. 1984. Purification and properties of a stilbene synthase from induced cell suspension cultures of peanut. J. Biol. Chem. **259**: 6806-6811.

Scott, D. B. 1965. Toxigenic fungi isolated from cereal and legume products. Mycopathol. Mycol. Appl. **25**: 213-222.

Seary, J. W., N. D. Davis, and U. L. Diener. 1969. Biosynthesis of ochratoxin A. Appl. Microbiol. **18**: 622-627.

Shantha, T., E. R. Rati, and T. N. B. Shankar. 1990. Behaviour of *Aspergillus flavus* in presence of *Aspergillus niger* during biosynthesis of aflatoxin B_1. Antonie Van Leeuwenhoek **58**: 121-127.

Shearer, J. F., L. E. Sweets, N. K. Baker, and L. H. Tiffany. 1992. A study of *Aspergillus flavus/parasiticus* in Iowa crop fields: 1988-1990. Plant. Dis. **76:** 19-22.

Shih, C. N., and E. H. Marth. 1974. Aflatoxin formation, lipid synthesis, and glucose metabolism by *Aspergillus parasiticus* during incubation with and without agitation. Biochim. Biophys. Acta **338:** 286-296.

Sieber, S. M., P. Correa, D. W. Dalgard, and R. H. Adamson. 1979. Induction of osteogenic sarcomas and tumors of the hepatobiliary system in nonhuman primates with aflatoxin B_1. Cancer Res. **39:** 4545-4554.

Silva, J. C., R. E. Minto, C. E. Barry, K. A. Holland, and C. A. Townsend. 1996. Isolation and characterization of the versicolorin B synthase gene from *Aspergillus parasiticus.* Expansion of the aflatoxin B_1 biosynthetic gene cluster. J. Biol. Chem. **271:** 13600-13608.

Simpson, T. J. 1987. The biosynthesis of polyketides. Nat. Prod. Rep. **4:** 339-376.

Simpson, T. J., D. J. Stenzel, A. J. Bartlett, E. O'Brien, and J. S. E. Holker. 1982. Studies on fungal metabolites. 3. ^{13}C-carbon isotopes NMR (nuclear magnetic resonance) spectral and structural studies on austin and new related meroterpenoids from *Aspergillus ustus, Aspergillus variecolor,* and *Penicillium diversum* Fungi. J. Chem. Soc. Perkin. Trans. I. **11:** 2687-2692.

Singer, T. P., P. Bernath, and C. J. Lusty. 1965. Succinate, in "Methods of enzymatic analysis" (Bergmeyer, H. U. Ed). pp. 340-345. Academic Press, New York.

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

Sinnhuber, R. O., J. H. Wales, J. L. Ayres, R. H. Engebrecht, and D. L. Amend. 1968. Dietary factors and hepatoma in rainbow trout (*Salmo gairdneri*). I. Aflatoxins in vegetable protein feedstuffs. J. Natl. Cancer. Inst. **41:** 711-718.

Skory, C. D., J. S. Horng, J. J. Pestka, and J. E. Linz. 1990. Transformation of *Aspergillus parasiticus* with a homologous gene (*pyr*G) involved in pyrimidine biosynthesis. Appl. Environl. Microbiol. **56:** 3315-3320.

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

Skory, C. D., P.-K. Chang, J. Cary, and J. E. Linz. 1992. Isolation and characterization of a gene from *Aspergillus parasiticus* associated with the conversion of versicolorin A to sterigmatocystin in aflatoxin biosynthesis. Appl. Environ. Microbiol. **58**: 3527-3537.

Soman, N. R., and G. N. Wogan. 1993. Activation of the c-ki-*ras* oncogene in aflatoxin-B_1-induced hepatocellular carcinoma and adenoma in the rat: detection by denaturing gradient gel electrophoresis. Pro. Natl. Acad. Sci. **90:** 2045-2049.

Springer, J. P., J. W. Dorner, R. J. Cole, and R. H. Cox. 1979. Terretonin, a toxic compound from *Aspergillus terreus*. J. Org. Chem. **44:** 4852-4854.

Stark, A. 1980. Mutagenicity and carcinogenicity of mycotoxins: DNA binding as a possible mode of action. Annu. Rev. Microbiol. **34:** 235-262.

Steenkamp, D. J., J. C. Schabort, and N. P. Ferreira. 1973. β-cyclopiazonate oxidocyclase from *Penicillium cyclopium,* III, preliminary studies on the mehanism of action. Biochim. Biophys. Acta **309:** 440-456.

Steyn, P. S., and R. Vleggaar. 1975. Dihydrofuro[3',2',4,5] furo [3,2-*b*]xanthenones: the structures of austocystins G, H, and I. J. S. Afr. Chem. Inst. **28:** 375-377.

Steyn, P. S., and R. Vleggaar. 1985. Tremorgenic mycotoxins. Fortschr. Chem. Org. Naturst. **48:** 1-80.

Steyn, P. S., C. W. Holzapfel, and N. P. Ferreira. 1970. The biosynthesis of the ochratoxins, metabolites of *Aspergillus ochraceus*. Phytochem. **9:** 1977-1983.

Steyn, P. S., R. Vleggaar, and P. L. Wessels. 1980. The biosynthesis of aflatoxin and its congeners, in "The biosynthesis of mycotoxins: a study in secondary metabolism"(Steyn, P. S. Ed). pp. 105-155. Academic Press, New York.

Steyn, P. S., R. Vleggaar, N. P. Ferreira, G. W. Kirby, and M. J. Varley. 1975a. Steric course of proton removal during the cyclistation of beta-cyclopiazonic acid in *Penicillium cyclopium*. Chem.Commun. **12:** 465-466.

Steyn, P. S., R. Vleggaar, P. L. Wessels, and B. de Scott. 1975b. Biosynthesis of aflatoxin B_1 from [2-^{13}C] and [1,2-^{13}C]-acetate [*Aspergillus flavus, Aspergillus parasiticus, fungi*]. J. Chem. Soc. Chem. Comm. **6:**193-195.

Suhadolnik, R. J., and R. G. Chenoweth. 1958. Biosynthesis of gliotoxin. I. Incorporation of phenylalanine-1-and-2-^{14}C. J. Am. Chem. Soc. **80:** 4391-4392.

Summers, R. G., A. Ali, B. Shen, W. A. Wessel, and C. R. Hutchinson. 1995. Malonyl-coenzyme A: acyl carrier protein acyltransferase of *Streptomyces glaucescens*: a possible link between fatty acid and polyketide biosynthesis. Biochem. **34:** 9389-9402.

Tada, S., K. Gomi, K. Kitamoto, K. Takahashi, G. Tamura, and S. Hara. 1991. Construction of a fusion gene comprising the Taka-amylase A promoter and the *Escherichia coli* β-glucuronidas gene and analysis of its expressin in *Asperglius oryzae.* Mol. Gen. Genet. **229:** 301-306.

Tamm, C. H. 1980. The biosynthesis of the cytochalasans, in "The biosynthesis of mycotoxins: a study in secondary metabolism" (Seytn, P.S. Ed). pp. 269-299. Academic Press, New York.

Temperli, E., U. P. Roos, and H. R. Hohl. 1990. Actin and tubulin cytoskeletons in germlings of the oomycete fungus *Phytophthora infestans.* Eur. J. Cell Biol. **53:** 75-88.

TePaske, M. R., J. B. Gloer, D. T. Wicklow, and P. F. Dowd. 1992. Aflavarin and beta-aflatrem: new anti-insectan metabolites from the sclerotia of *Aspergillus flavus.* J. Nat. Prod. **55:** 1080-1086.

Thomas, R. 1965. Biosynthetic pathways involving ring cleavage, in "Biogenesis of antibiotic substances" (Vanek, Z., and Z. Hostalek Eds). pp. 115-168. Academic Press, New York.

Tilak, T. B. 1975. Induction of cholangiocarcinoma following treatment of a Rhesus monkey with aflatoxin. Food Cosmet Toxicol. **13:** 247-249.

Timberlake, W. E., and E. C. Barnard. 1981. Organization of a gene cluster expressed specifically in the asexual spores of *Aspergillus nidulans* Fungi. Cell **26:** 29-37.

Townsend, C. A., K. A. Plancan, K. Pal, and S. W. Brobst. 1988. Hydroxyversicolorone: isolation and characterisation of a potential intemediate in aflatoxin biosynthesis. J. Org. Chem. **53:** 2472-2477.

Townsend, C. A., S. B. Christensen, and K. Trantwein. 1984. Hexanoate as a starter unit in polyketide biosynthesis. J. Amer. Chem. Soc. **106:** 3868-3869.

Townsend, C. A., S. B. Christensen. and S. G. Davis. 1982. Bisfuran formation in aflatoxin biosynthesis: the role of versiconal acetate. J. Am. Chem. Soc. **104:** 6154-6155.

Trail, F., N. Mahanti, M. Rarick, R. Mehigh, S. H. Liang, R. Zhou, and J. E. Linz. 1995. Physical and transcriptional map of an aflatoxin gene cluster in *Aspergillus parasiticus* and functional disruption of a gene involved early in the aflatoxin pathway. Appl. Environ. Microbiol. **61:** 2665-2673.

Trail, F., P.-K. Chang, J. Cary and J. E. Linz. 1994. Structural and functional analysis of the *nor*-1 gene involved in the biosynthesis of aflatoxins by *Aspergillus parasiticus.* Appl. Environ. Microbiol. **60:** 4078-4085.

Trucksess, M. W., P. B. Mislivec, K. Young, V. R. Bruce, and S. W. Page. 1987. Cyclopiazonic acid production by cultures of *Aspergillus* and *Penicillium* species isolated from dried beans, corn meal, macaroni, and pecans. J. Assoc. Off. Anal. Chem. **70:** 123-126.

Tsuneda, A. (Ed). 1983. Fungal morphology and ecology. pp. 196. Tokyo Press Co., Ltd. Tokyo, Japan.

Turner, W. B., and D. C. Aldridge (Eds). 1983. Fungal metabolites II. pp. 1-631. Academic Press, London.

Ueda, K., K. Miyake, S. Horinouchi, and T. Beppu. 1993. A gene cluster involved in aerial mycelium formation in *Streptomyces griseus* encodes proteins similar to the response regulators of two-component regulatory systems and membrane translocators. J. Bacteriol. **175:** 2006-2016.

Umeda, M., T. Tsutsui, and M. Saito. 1977. Mutagenicity and inducibility of DNA single-strand breaks and chromosome aberrations by various mycotoxins. Gann **68:** 619-625.

Van den Broek, P., T. Goosen, B. Wennekes, and H. van den Broek. 1995. Isolation and characterization of the glucose-6-phosphate dehydrogenase encoding gene (*gsd*A) from *Aspergillus niger*. Mol. Gen. Genet. **247**: 229-239.

Van Liempt, H., H. von Doehren, and H. Kleinkauf. 1989. δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Aspergillus nidulans*. The first enzyme in penicillin biosynthesis is a multifunctional peptide synthetase. J. Biol. Chem. **264:** 3680-3684.

Vederas, J. C. 1987. The use of stable isotopes in biosynthetic studies. Nat. Prod. Rep. **4:** 277-337.

Verrett, M. J., J. Winbush, E. F. Reynaldo, and W. F. Scott. 1973. Collaborative study of the chicken embryo bioassay for aflatoxin B_1. Ass. Offic. Anal. Chem. J. **56:** 901-904.

Vida, T. A., G. Huyer, and S. D. Emr. 1993. Yeast vacuolar proenzymes are sorted in the late Golgi complex and transported to the vacuole via a prevacuolar endosome-like compartment. J. Cell. Biol. **121:** 1245-1256.

Webster, J., and L. Lomas. 1964. Does *Thichoderma viride* produce gliotoxin and viridin? Thans. Brit. Mycol. Soc. **47:** 535-540.

Wei, R. D., F. M. Strong, and E. B. Smalley. 1971. Incorporation of chlorine-36 into ochratoxin A. Appl. Microbiol. **22:** 276-277.

Weiss U., and J. M. Edwards. (Eds). 1980. The biosynthesis of aromatic compounds. John Wiley & Sons, New York.

Whiteman, P. A., E. P. Abraham, J. E. Baldwin, M. D. Fleming, C. J. Schofield, J. D. Sutherland, and A. C. Willis. 1990. Acyl coenzyme A: 6-aminopenicillanic acid acyltransferase from *Penicillium chrysogenum* and *Aspergillus nidulans.* FEBS Lett. **262:** 342-344.

Wicklow, D. T. 1990. Environmental factors and other stresses important in the production of aflatoxin in corn. USDA, Agricultural Research Service. **83:** 53-61.

Wicklow, D. T., and O. L. Shotwell. 1983. Intrafungal distribution of aflatoxins among conidia and sclerotia of *Aspergillus flavus* and *Aspergillus parasiticus.* Can. J. Microbiol. **29:** 1-5.

Wicklow, D. T., and R. J. Cole. 1982. Tremorgenic indole metabolites and aflatoxins in sclerotia of *Aspergillus flavus*, an evolutionary perspective. Can. J. Bot. **60:** 525-528.

Wicnienski, N. A. 1979. Preliminary studies of the biosynthesis of austin. Govt. Reports Announcements & Index (GRA&I), Issue 23.

Wilkinson, S., and J. F. Spilsbury. 1965. Gliotoxin from *Aspergillus chevalieri* (Mangin) Thom et Church. Nature **206:** 619.

Willetts, H. J., and S. Bullock. 1992. Developmental biology of sclerotia. Mycol. Res. Cambridge **96:** 801-816.

Wilson, B. J., and C. H. Wilson. 1964. Toxin from *Aspergillus flavus*: production on food materials of a substance causing tremors in mice. Science **144:** 177-178.

Wiseman, D. W., and R. L. Buchanan. 1987. Determination of glucose level needed to induce aflatoxin production in *Aspergillus parasiticus.* Can. J. Microbiol. **33:** 828-830.

Wogan, G. N., and P. M. Newberne. 1967. Dose-response charateristics of aflatoxin B_1 carcinogenesis in the rat. Cancer Res. **27:** 2370-2376.

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. Microbiol. **55:** 86-90.

Woloshuk, C. P., K. R. Foutz, J. F. Brewer, D. Bhatnagar, T. E. Cleveland, and G. A. Payne. 1994. Molecular characterization of *afl*R, a regulatory locus for aflatoxin biosynthesis. Appl. Environ. Microbiol. **60:** 2408-2414.

Wood, M. L., J. R. Smith, and R. C. Garner. 1987. Aflatoxin B_1 activation to a plasmid mutagen by a chemical model of cytochrome P-450. Mutat Res. 176:11-20.

Woodward, R. B., and G. Singh. 1949. The structure of patulin. J. Am. Chem. Soc. **71:** 758-759.

Yabe, K., and T. Hamasaki. 1993. Stereochemistry during aflatoxin biosynthesis: cyclase reaction in the conversion of versiconal to versicolorin B and reacemization of versiconal hemiacetal acetate. Appl. Environ. Microbiol. **59:** 2493-2500.

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

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

Yabe, K., Y. Matsuyama, Y. Ando, H. Nakajima, and T. Hamasaki. 1993. Stereochemistry during aflatoxin biosynthesis: conversion of norsolorinic acid to averufin. Appl. Environ. Microbiol. **59:** 2486-2492.

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

Yamazaki, M. 1980. The biosynthesis of neurotropic mycotoxins, in "The biosynthesis of mycotoxins: a study in secondary metabolism" (Steyn, P. S. Ed). pp. 193-222. Academic Press, New York.

Yamazaki, M., E. Okuyama, and Y. Maebayashi. 1979. Isolation of some new tryptoquivaline-related metabolites from *Aspergillus fumigatus*. Chem. Pharm. Bull. Tokyo, **27:** 1611-1617.

Yamazaki, M., H. Fujimoto, and T. Kawasaki. 1980. Chemistry of tremorogenic metabolites. I. Fumitremorgin A from *Aspergillus fumigatus*. Chem. Pharm. Bull. (Tokyo) **28:** 245-254.

Yamazaki, M., S. Suzuki, and K. Miyaki. 1971. Tremorgenic toxins from *Aspergillus fumigatus* Fres. Chem. Pharm. Bull. Tokyo. **19:** 1739-1740.

Yan, R. Q., J. J. Su, D. R. Huang, Y. C. Gan, C. Yang, and G. H. Huang. 1996. Human hepatitis B virus and hepatocellular carcinoma. II. Experimental induction of hepatocellular carcinoma in tree shrews exposed to hepatitis B virus and aflatoxin B_1. J. Cancer. Res. Clin. Oncol. **122:** 289-295.

Yao, R. C., and D. P. H. Hsieh. 1974. Step of dichlorvos inhibition in the pathway of aflatoxin biosynthesis. Appl. Microbiol. **28:** 52-57.

Yoneyama, M., R. P. Sharma, and Y. Y. Elsner. 1987. Effects of mycotoxins in cultured kidney cells: cytotoxicity of aflatoxin B_1 in Madin-Darby and primary fetal bovine kidney cells. Ecotoxical. Environ. Safety **13:** 174-184.

Yu, J. H., J. Wieser, and T. H. Adams. 1996. The *Aspergillus flb*A RGS domain protein antagonizes G protein signaling to block proliferation and allow development. EMBO J. **15:** 5184-5190.

Yu, J. H., and T. J. Leonard. 1995. Sterigmatocystin biosynthesis in *Aspergillus nidulans* requires a novel type I polyketide synthase. J. Bacteriol. **177:** 4792-4800.

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

Zamir, L. O. 1980. The biosynthesis of patulin and penicillic acid, in "The biosynthesis of mycotoxins: a study in secondary metabolism"(Steyn, P. S. Ed). pp. 223-268. Academic Press, New York.