THE FUNCTION AND EXPRESSION OF THE *VER-1* GENE AND LOCALIZATION OF THE VER-1 PROTEIN INVOLVED IN AFLATOXIN B_1 BIOSYNTHESIS IN *ASPERGILLUS PARASITICUS*

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ABSTRACT

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The ultimate goal of the present research is the elimination of preharvest aflatoxin contamination. One approach to accomplish this goal is to develop an understanding of the molecular mechanisms regulating aflatoxin biosynthesis in toxigenic aspergilli. This study focused on the *ver-1* gene associated with the aflatoxin biosynthetic pathway. This gene was cloned and its nucleotide sequence was determined as part of a previous study on aflatoxin B_1 (AFB_1) biosynthesis in *Aspergillus parasiticus* NRRL 5862. Two copies of the *ver-1* gene, *ver-1A* and *ver-1B*, were tentatively identified in this fungal strain by Southern hybridization analysis. Genetic complementation and nucleotide sequence data suggested that the *ver-1* gene is involved in the conversion of versicolorin A (VA) to sterigmatocystin (ST).

To clearly establish the function of the *ver-1* gene, I proposed to test three hypotheses: (1) the *ver-1* gene encodes a protein which has enzymatic activity associated with the conversion of VA to ST; (2) the pattern of Ver-1 protein accumulation parallels AFB_1 synthesis; and (3) the Ver-1 protein is closely associated with other proteins involved in AFB_1 biosynthesis allowing the pathway to function in an efficient way. To address these hypotheses, studies were designed to achieve the following specific aims : (1) identify *ver-1* gene function by nucleotide sequence and gene disruption analyses; (2) identify the

accumulation pattern of the Ver-1 protein in fungal cells grown in liquid or solid media; and (3) identify the intracellular location of the Ver-1 protein.

In specific aim 1, the methods consisted of gene disruption and nucleotide sequence analysis. The methods used in specific aim 2 included generation of polyclonal antibodies against the Ver-1 protein, batch fermentation analysis, nutritional shift assay, and analysis of *ver-1* promoter activity using the GUS reporter strain. In specific aim 3, the subcellular localization of the Ver-1 protein was performed by differential centrifugation and immunofluorescence microscopy. The major findings of this research are : (1) *ver-1A* but not *ver-1B* is directly involved in aflatoxin biosynthesis; (2) the timing of Ver-1 protein accumulation is positively correlated to aflatoxin accumulation; (3) expression of the *ver-1* gene in a fungal colony is subject to temporal and spatial regulation; (4) although the *ver-1* gene is expressed in both vegetative hyphae and conidiophores, there is significantly more Ver-1 protein in the structures involved in asexual reproduction; and (5) the Ver-1 protein appears to be located in or tightly bound to a membrane-bound organelle in fungal cells.

To my parents, brother, and lovely wife, Lie-Ken

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INTRODUCTION AND RESEARCH RATIONALE

Aflatoxin contamination of agricultural commodities and dietary staples such as corn, peanuts, and cottonseed has caused worldwide economic and food safety problems (Jelinek *et al.*, 1989). Its impact on the agricultural economy and potential impact on human health has drawn scientists' efforts in an attempt to prevent aflatoxin contamination. The long term goal of this research is the elimination of preharvest aflatoxin contamination from the food chain. An elucidation of the molecular mechanisms which regulate aflatoxin biosynthesis in toxigenic aspergilli may be the best approach to achieve the ultimate goal. For this purpose, it is very important to study aflatoxin biosynthesis at the molecular level and to identify the control points in the aflatoxin biosynthetic pathway. The information derived from these analyses will help in development of strategies to inhibit aflatoxin production in the field.

In order to effectively understand the regulation of expression of genes involved in aflatoxin biosynthesis, it is necessary to clone several of these genes and to clearly establish their function. Two pathway genes, ***nor-1*** and ***ver-1***, have been cloned in our laboratory by genetic complementation of ***Aspergillus parasiticus*** mutants blocked at unique steps in aflatoxin B_1 (AFB_1) biosynthesis (Chang *et al.*, 1992; Skory *et al.*, 1992). The present research is mainly focused on the structural and functional characterization of the two copies of the *ver-1* gene (*ver-1A* and *ver-1B*) and of the Ver-1 protein in *A. parasiticus*. The objective of this study is to understand aflatoxin biosynthesis at the genetic and cellular levels

using the *ver-1* gene as the model system. To achieve this aim, an initial effort was made to establish the function of two *ver-1* gene copies by recombinational inactivation and genetic complementation experiments. The function of the *ver-1* gene was further examined by studying the relationship between Ver-1 protein accumulation and aflatoxin biosynthesis using batch fermentation analysis and nutritional shift assay (liquid media systems). After the *ver-1* gene was confirmed to be directly involved in AFB_1 biosynthesis, the regulation of its expression was studied during growth on solid media. Finally, the location of the Ver-1 protein in fungal cells was analyzed by subcellular fractionation and immunofluorescence microscopy.

This research represents the first analysis to study the regulation of aflatoxin gene expression and the location of aflatoxin-associated enzymes in the fungal cells using solid growth media. The resulting data will help in understanding the function of aflatoxin biosynthesis in fungi and may provide a potential approach to develop an efficient way to prevent aflatoxin contamination.

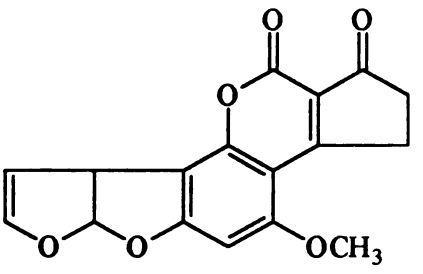
CHAPTER 1

LITERATURE REVIEW

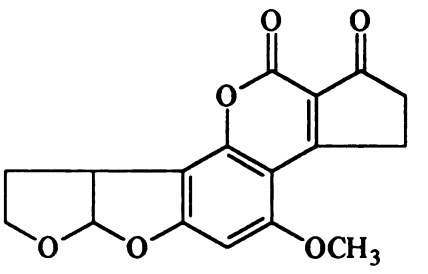
I. Natural occurrence of aflatoxins

Aflatoxins are biologically active mycotoxins produced by certain strains of the imperfect fungi ***Aspergillus parasiticus***, ***A. flavus***, and ***A. nomius*** (Bennett, 1979; Cotty *et al.*, 1994). *A. nomius*, however, is less important to the contamination of foods and feeds by aflatoxins. The major aflatoxins of concern include aflatoxin B_1, B_2, G_1, and G_2 (Figure 1). When resolved by thin-layer chromatography (TLC), these aflatoxins separate into individual fluorescent compounds in the order given above. Aflatoxins are freely soluble in moderately polar solvents such as chloroform, methanol, and dimethylsulphoxide, and also have some water solubility (McLean and Dutton, 1995). These mycotoxins are usually found together in contaminated foods and feeds. Aflatoxin B_1 (AFB_1), however, is the most abundant and toxic. Aflatoxin M_1 is the major metabolic product of AFB_1 in animals and is usually found in the milk and urine of diary cattle and other mammalian species that have consumed aflatoxin-contaminated foods or feeds (Allcroft *et al.*, 1966).

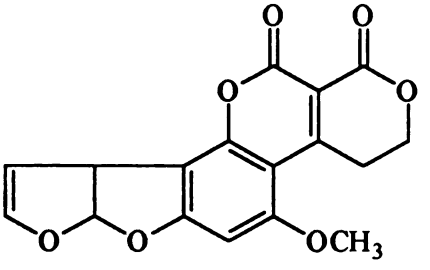
Although both species can produce aflatoxins, *A. parasiticus* and *A. flavus* have different abilities in toxin production. First, *A. parasiticus* produces the aflatoxin B and G groups but *A. flavus* only produces the aflatoxin B group. Second, most *A. parasiticus* isolates (> 90 %) produce aflatoxin whereas up to 35% of the *A. flavus* isolates may not



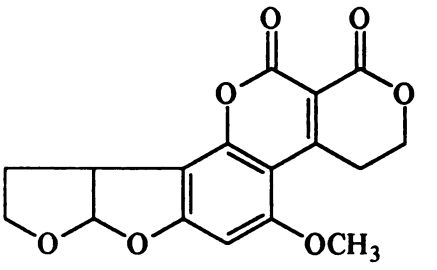
AFLATOXIN B_1



AFLATOXIN B_2



AFLATOXIN G_1



AFLATOXIN G_2

Figure 1. Chemical structures of naturally occurring aflatoxins.

produce aflatoxin (Bennett and Papa, 1988; Trail *et al.*, 1995). The contamination of tree nuts, peanuts, and other oilseeds such as corn and cottonseed by aflatoxins occurs under certain environmental conditions when these crops are infected with toxigenic strains of the fungus (Jelinek *et al.*, 1989). Although the fungus may be killed or removed during processing, aflatoxins often remain in the final product and thus contribute to the main point of entry of aflatoxins into the food chain (Smith and Moss, 1985). For this reason, the elimination of aflatoxin contamination at the preharvest stage is very important. An alternative route of aflatoxin contamination is the postharvest infection of food or feed with toxigenic fungi and the subsequent formation of toxin at some stage during processing, transport, and storage.

A variety of factors which contribute to the contamination of aflatoxins in the food chain include biological and environmental factors (Pestka and Casale, 1990). The biological factors consist of substrate availability for toxigenic fungi, competing microflora (Ellis *et al.*, 1991), and susceptibility of the crops. The environmental factors include temperature, moisture, insect/bird damage, and mechanical injury of the crops. Warm temperatures and drought conditions favor fungal growth and aflatoxin production in the field. Climatic patterns thus could determine which regions are more prone to aflatoxin contamination. For example, corn in the southeastern regions of the United States is frequently contaminated with aflatoxins. Other areas of the country are occasionally susceptible. For example, due to severe drought, widespread aflatoxin contamination in corn occurred in the midwestern regions of the United States in 1983 and 1988 (Chu, 1991). In the postharvest stage, aflatoxin contamination may result from warm temperatures and high humidity during storage.

II. The aflatoxin problem

Worldwide, aflatoxins are considered a public health and economic problem because of their potent toxic effects on humans and animals (Chu, 1991; Eaton and Gallagher, 1994) and the huge cost incurred by farmers or producers due to the loss of crops, animals and the need for more careful agronomic practices (Shane, 1994).

A. Aflatoxins and public health

The threat of aflatoxins to human health was initially realized after they were directly linked to acute hepatotoxicity in poultry (Turkey X disease) in 1960 (Blount, 1961) and later their association with fatal human aflatoxicoses in India (Krishnamachari *et al.*, 1975) and West Africa (Ngindu *et al.*, 1982). In the outbreak of aflatoxicosis in northwest India, 108 persons died among 397 persons affected due to consumption of contaminated corn with aflatoxin at levels of 0.25 to 15 mg/kg. The daily AFB_1 intake was about 55 μg/kg body weight. The symptoms of affected individuals in this outbreak included vomiting, high fever, rapid progressive jaundice, edema of the limbs, and swollen livers. Histopathological examination showed extensive periportal fibrosis and bile duct proliferation of the liver and gastrointestinal hemorrhages. In studies on animals, it was found that no species is resistant to the acute toxic effects of aflatoxins (Newberne and Butler, 1969). The first signs of aflatoxicosis in animals are the lack of appetite and the loss of weight. Liver centrilobular necrosis, fatty degeneration, and bile duct proliferation are the most common pathological findings. For most of the animals tested, the LD_{50} for a single dose of AFB_1 is in the range of 0.5 to 10 mg/kg of body weight. Although interspecific variation has been recognized for acute effects, many factors, such as age, sex, nutritional status of diet, and mode of application, affect the degree of toxicoses. In general, aflatoxin is more toxic to young

animals and males than females (Cullen and Newberne, 1994). Besides the liver, many other organs such as pulmonary (Wieder *et al.*, 1968), gastrointestinal (Bulter, 1964; Deger, 1976), renal (Epstein *et al.*, 1969), nervous (Egbunike and Ikeguonu, 1984), reproductive (Ottinger and Doerr, 1980), and immune systems (Kadian *et al.*, 1988; Pestka and Bondy, 1990) are more or less severely affected with high doses of AFB_1.

In well-developed countries, aflatoxin contamination in foods rarely reaches the level that causes acute aflatoxicosis in humans. Hence, studies of aflatoxin toxicity on humans have been focusing on its carcinogenic potential. The carcinogenic properties of aflatoxins have been studied extensively, and much information has been produced concerning various aspects of their mechanisms of action and their putative importance as risk factors for primary hepatocellular carcinoma (PHC) in humans (Busby and Wogan, 1984). Primary liver tumors have been induced by experimental administration of AFB_1 to animals of many species, including fish (rainbow trout, salmon, and guppy), birds (duck), rodents (5 strains of rats, the B6C3F1 mouse, tree shrew, and hamster), a carnivore (ferret), and subhuman primates (rhesus, cynomolgus, and African green monkeys) (Wogan, 1991). Each of these species has been shown to be susceptible to induction of PHC by AFB_1 administration. However, in relating this information to putative effects in humans, it is important to note that wide species differences exist with respect to the carcinogenic potency of aflatoxins. The estimated potency of AFB1 in inducing liver tumors in animals showed that rats are highly susceptible, mice are highly resistant, and primates are of intermediate susceptibility (Eaton and Gallagher, 1994). In utilizing information derived from these experimental systems for assessing cancer risks for humans resulting from aflatoxin exposures, it is of particular importance to compare the animal data with estimates of human susceptibility derived from epidemiological

observations. The consolidated data have been collected from studies in Africa and Asia (Hsieh, 1989; van Rensburg *et al.*, 1985; Yu *et al.*, 1898), where aflatoxin intake was measured in populations in which PHC incidence was variable, as determined from cancer registry information. In general, aflatoxin intake values increase in parallel with cancer incidence. Although the causative role for aflatoxins in human liver cancer has not been universally accepted because of the presence of endemic hepatitis B virus in high risk populations (Stoloff, 1989), the International Agency for Research on Cancer has determined that the combined experimental and epidemiological evidence was sufficient to designate aflatoxins as human carcinogens (IARC, 1993).

Studies of the metabolism of AFB_1 have revealed that the compound is activated to its toxic form predominantly by liver-specific, cytochrome P450 IIIA4 monooxygenases in mammals (Coros *et al.*, 1990). Metabolic activation of AFB_1 to AFB_1-8,9-epoxide (Figure 2) is believed to result in its toxicity, mutagenicity, and carcinogenicity (Campbell and Hayes, 1976; Gurtoo and Dave, 1975). The aflatoxins listed in order of terms of acute and chronic toxicity are $AFB_1 > AFG_1 > AFB_2 > AFG_2$, suggesting that epoxidation of the 8,9-double bond and also the presence of the cyclopentenone ring of the B compounds (when compared with the six-membered lactone ring of the G compounds) may play a major role in the harmful effects (McLean and Dutton, 1995). The AFB_1-8,9-epoxide and its hydration product, the dihydrodiol form of AFB_1 (Figure 2) are highly reactive and can covalently bind to cellular macromolecules. The epoxide specifically binds to the N^7 position of guanine of DNA and RNA (Cory and Wogan, 1981), while the dihydrodiol links to proteins by the formation of a Schiff base structure (Hsieh, 1987). The major DNA adduct formed *in vivo* and *in vitro* after depurination is 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy AFB_1 (AFB_1-guanine), which can

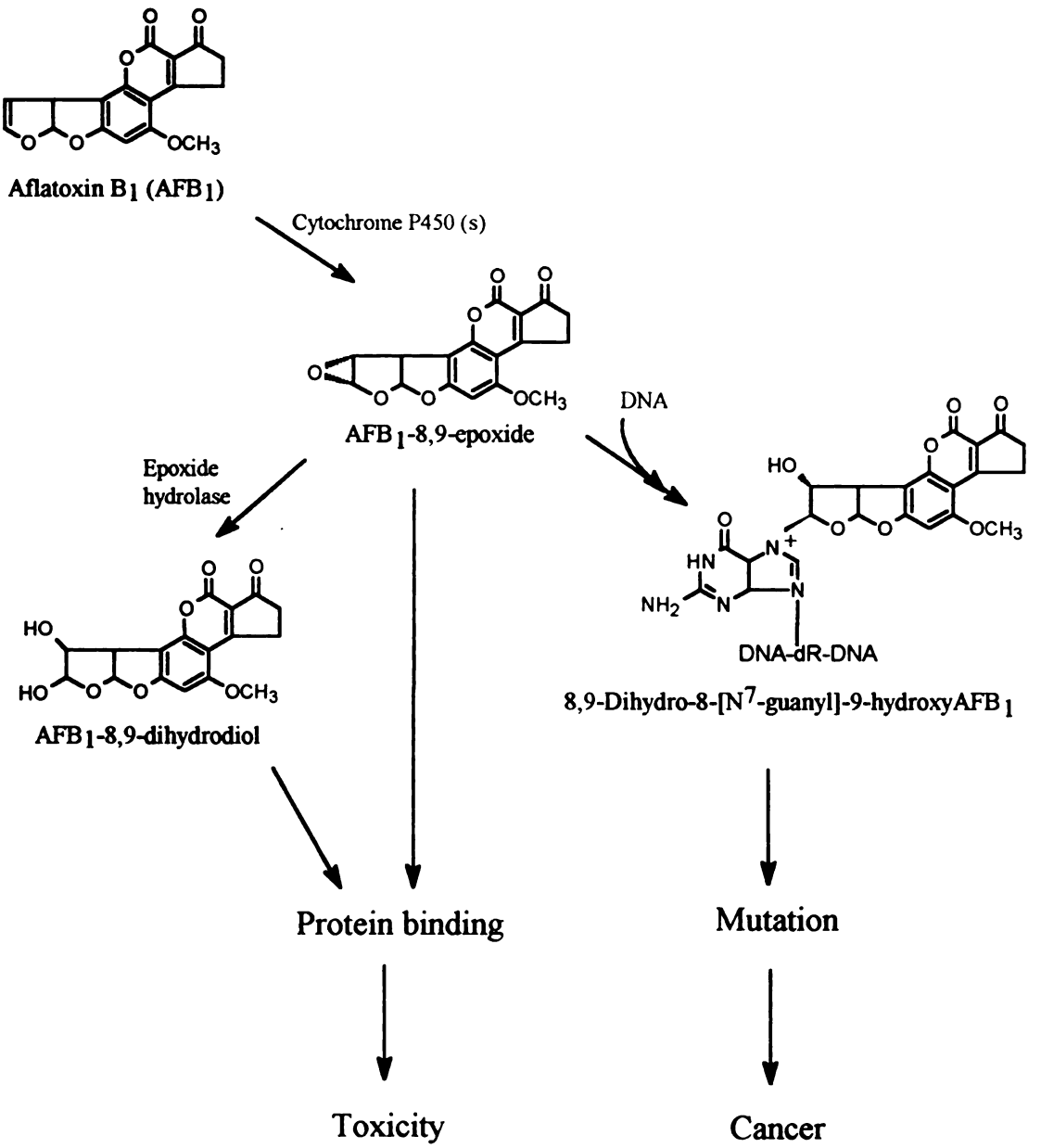


Figure 2. Metabolic activation of aflatoxin B_1 and its harmful effects. (Eaton and Gallagher, 1994)

thus be used as a biomarker in urine for aflatoxin exposure (Groopman, 1994). Depurination at guanine residues could lead to a GC → TA transversion during replication. Evidence has been collected which suggests that AFB_1 induces a mutation of the *p*53 tumor-suppressor gene in codon 249 (Hsu *et al.*, 1991; Bressac *et al.*, 1991) and may result in the development of human hepatocellular carcinoma. It has also been reported that the activated form of AFB_1 induces virus expression and tumor formation associated with *ras* (McMahon *et al.*, 1980) and *myc* (Larson *et al.*, 1980) oncogenes.

B. Aflatoxins and economic losses

While considerable research has been directed to the prevalence, chemical characterization, and biochemical action of aflatoxins, less attention has been paid toward calculating the economic impact of aflatoxins on society. This is because hidden or indirect factors may result in the significant financial losses which are difficult to evaluate. Instead of considering aflatoxins only, people generally take all mycotoxins into account when evaluating economic losses. Nevertheless, it is believed that aflatoxin-associated losses play a major part because of the widespread contamination of aflatoxins in the world and their extremely toxic effects. It was estimated that billions of dollars are lost annually in direct and indirect costs which result from the fact that approximately 25% of the world's crops are affected by mycotoxins (CAST, 1989). This huge loss encompassed a broad category of crop and animal industries, and extends through the food chain from producers to the consumer (Shane, 1994).

Because of the threat of aflatoxins to public health, the U.S. Food and Drug Administration has set action levels of 20 ppb for aflatoxins in human food, 0.5 ppb for AFM_1 in milk and dairy products, and 20 to 300 ppb in most animal feeds (CAST, 1989). In some

European countries the action level is even more restrictive. The cost to the agronomic sector is thus increased due to efforts to meet these guidelines.

The preharvest mycotoxin contamination of corn was recognized in the mid-1970s (Lisker and Lillehoj, 1991). Since then, significant costs have been associated with mainly two approaches to reduce preharvest aflatoxin contamination, namely the development of resistant cultivars and the improvement of farming practices. For example, significant costs arise from implementation of breeding programs which are utilized to select cultivars resistant to insect damage, drought, and fungal infection. Financial losses also may arise from the reduced yield of the resistant crops. The costs associated with improved farming practices include irrigation to prevent desiccation, the use of insecticides or fungicides to prevent infection, the use of additional fertilizers to reduce environmental stress, and the use of modified harvesting methods to avoid crop damage. Costs can also result from improvements in postharvest handling of grains and other ingredients. These include modified transport and mechanization to reduce damage, intensified drying of grain to achieve desirable moisture levels, improved storage control to prevent fungal contamination, and proper quality control procedures to monitor toxin levels. If, at last, the contamination level is too high, it is necessary to reduce the toxin content by physical or chemical treatment or the contaminated grain must be either destroyed or downgraded and hence reduce the revenue for producers.

Aflatoxin contamination is also responsible for financial losses in domestic animal production, including ruminant, monogastric, and aquatic species (Nelson and Christensen, 1978). The major economic impact consists of aflatoxin-induced death (Smith and Hamilton, 1970), a depression in growth rate and feed conversion efficiency (Dalvi, 1986), and an increase in plant condemnation (Shane, 1991). In the poultry industry, ducklings are the most

susceptible species to aflatoxicosis. Turkey poults are more resistant than ducklings but are ten times more sensitive than four-week-old chickens.

In summary, aflatoxin contamination causes huge financial losses to food processors, and producers of commodities and domestic animals. The costs ultimately are borne by consumers or the national economy.

III. Control of aflatoxins

Because of the public health and economic problems caused by aflatoxins, it is very important and urgent to find ways to efficiently control their occurrence. Ideally, any method should be technically and economically feasible if it is to be applied practically. Some other factors should also be considered including safety, retention of nutritional elements, and the lack of harmful effects to the environment. To date numerous methods have been tested although they still do not meet all of the control criteria. In general, the control of aflatoxins can be divided into three principal categories : prevention, decontamination or detoxification, and reduction of aflatoxin bioavailability.

A. Prevention

The prevention of aflatoxin formation in agricultural products and other foodstuffs can occur at the pre- and post-harvest level by regulating the environmental factors influencing fungal growth and toxin production. Many environmental conditions have been identified which can promote aflatoxin formation in growing crops including : insect infestation, drought conditions, mechanical damage, nutritional deficiencies, and unseasonable temperatures and rainfall (Smith and Moss, 1985a). Conventional on-farm preventative techniques such as methods of cultivating to improve plant vigor, the use of insecticides and

fungicides to reduce insect and fungal infestation, irrigation to avoid drought conditions, and the use of resistant varieties (Darrah and Barry, 1991; Scott and Zummo, 1988) have been utilized to help overcome these environmental stress although these are often too costly or are ineffective. Post-harvest (during processing, storage, and shipment) control of aflatoxin contamination prevents or delays toxigenic mold growth through manipulation of moisture levels, temperature, aeration, and mold spore density (Darrah and Barry, 1991).

Biocontrol using nontoxigenic strains of *A. parasiticus* and *A. flavus* is another potential approach for the preharvest prevention of aflatoxin contamination. It has been demonstrated that this strategy can significantly reduce aflatoxin contamination in peanuts and cottonseed (Cotty, 1990; Dorner *et al.*, 1992; Ehrlich, 1987). Biocompetitive control, however, has to be evaluated for its feasibility regarding the stability of the non-aflatoxin producing strains and the environmental impact. The potential for naturally occurring nontoxigenic strains to produce aflatoxins may be a major concern (Rarick *et al.*, 1994) of this technology. Additional studies are needed to address the microbial ecological changes after releasing the biocontrol agent in the field.

B. Decontamination or detoxification

Once a product is contaminated with aflatoxins, there are only two options if it is to be used for human or animal consumption : to remove aflatoxins or to degrade them into non-toxic compounds. Practical methods are being investigated for removal and detoxification of aflatoxins from foods and feeds.

1. Removal of aflatoxins

(a) Physical separation. In large particle size agricultural products such as the peanut, Brazil nut or almonds, aflatoxin contamination, when it occurs, is normally confined in any

batch to a small number of contaminated seeds or kernels (Smith and Moss, 1985a). It has been shown that the level of aflatoxin in peanuts can be correlated with the proportion of loose-shelled or discolored kernels. When these are discarded the remaining kernels are relatively free of aflatoxins. Off-colored kernels can be separated either by hand or by passing through color sorters (Cole, 1898). Density segregation by flotation in water or a salt solution is another way to separate toxic kernels from sound, nontoxic kernels (Hagler, 1991; Huff, 1980; Huff and Hagler, 1985; Kirksey *et al.*, 1989). Physical separation, however, causes some loss of raw materials.

(b) Chemical separation. Numerous solvent extraction systems have been developed to remove aflatoxins from contaminated materials with minimal effects on protein content or nutritional quality (Rayner, 1977). These systems include 95% ethanol, 90% aqueous acetone, 80% isopropanol, hexane-methanol, and hexane-acetone-water mixtures. Although solvent extraction can be highly successful in removing aflatoxins, the cost of the additional processing and the need for special solvent-removing equipment, etc., have made these processes of questionable economic value. Besides, residues of solvent in food and feed would cause additional safety problems.

2. Degradation or detoxification

(a) Physical methods. Of these methods, irradiation and thermal inactivation are considered here. Aflatoxins are sensitive to ultraviolet light. However, the degradation of aflatoxin in contaminated products is dependent on the nature of the solvent, the toxin concentration and the length of exposure to UV light (Shantha, 1986). Aflatoxins are resistant to thermal inactivation and are not destroyed completely by boiling water, autoclaving, or a variety of food and feed processing procedures (Phillips, 1994). With dry

heat such as roasting, temperatures approaching the melting point (250°C) of aflatoxin must be used to effectively degrade the toxin. Increasing the moisture content and/or time of heating increases the rate of aflatoxin degradation (Mann, 1967). However, the adverse effects of heat treatment on the appearance and nutritional value of the product makes the practical application of these methods highly doubtful.

(b) Chemical methods. Several chemicals have been tested for the destruction of aflatoxins including acids, aldehydes, oxidizing agents and gases. The treatment of grain with ammonia appears to be a valuable approach to the detoxification of aflatoxins. Ammonia used as an anhydrous gas at elevated temperatures and pressures can cause a 95-98% reduction in total aflatoxin concentration in peanuts, cottonseed meal and corn (Brekke *et al.*, 1977; Gardner *et al.*, 1971; Park *et al.*, 1984). This method is legal and is being used on a commercial scale in certain states of the US (Alabama, Arizona, California, Georgia, North Carolina and Texas) for animal feeds. Ammoniation is also used routinely in Mexico, France, South Africa, Senegal, Brazil, and India (Piva *et al.*, 1995; Phillips *et al.*, 1994). The drawback of ammonia treatment is the need to build special plants because ammonia corrodes metal and becomes explosive in the air. This would result in a substantial increase in costs which cannot be afforded by most farmers. Besides, ammonia treatment may lead to an undesirable brown color in the feed and the diminished content of specific amino acids such as cystine, methionine and lysine (Piva *et al.*, 1995).

Oxidizing agents such as sodium bisulfite have been shown to degrade aflatoxins in naturally contaminated grain (Doyle and Marth, 1978). When compared with ammonia treatment, bisulfite treatment is less efficient in detoxification of aflatoxins. Bisulfite treatment, however, is much less costly than ammonia treatment. In addition, sodium bisulfite

is commonly added to food and drinks where it acts as antioxidant, enzyme inhibitor, and bacteriostatic agent. It therefore may be competitive with the ammoniation process.

Calcium hydroxide is another chemical which has been shown to be able to reduce aflatoxin levels (Codifer *et al.*, 1976). The advantages of using sodium hydroxide are its low cost and easy application because it is the cheapest alkali and can be readily mixed with the feed to be detoxified. However, the low efficiency (less than 45 %) in the destruction of AFB_1 is a big concern (Piva *et al.*, 1995).

(c)Biological methods. Many microorganisms including bacteria, actinomycetes, yeast, molds, and algae show varying abilities to degrade aflatoxin. The most active organism so far discovered is *Flavobacterium aurantiacum* (NRRL B-184) which in aqueous solution can take up and metabolize aflatoxin B_1, G_1 and M_1 (Ciegler *et al.*, 1966). As yet no commercial application has been developed because the safe and practical use of *Flavobacterium aurantiacum* in feed or food has not been established.

C. Reduction of aflatoxin bioavailability

1. Selective chemisorption

Numerous studies have demonstrated that the use of clays in contaminated feeds can reduce aflatoxin absorption in the intestine of animals. Tests *in vitro* showed that absorbents such as aluminas, silicas, and aluminosilicates are capable of binding aflatoxin in solution (Phillips *et al.*, 1988). It was observed that hydrated sodium calcium aluminosilicates (HSCAS) were the most efficient in binding aflatoxins. HSCAS are currently used as anticaking agents for animal feeds and were found to prevent aflatoxicosis in domestic animals (Davidson *et al.*, 1987; Kubena *et al.*, 1990) and to decrease the level of AFM_1 in the milk of dairy cattle (Harvey *et al.*, 1991).

2. Chemoprotection against aflatoxin toxicity

Methods have been suggested for the protection of animals and humans against the effects of aflatoxins by prior treatment with chemicals or drugs that induce protective detoxifying liver enzymes (Kensler *et al.*, 1991). A dithiolethione compound, oltipraz, has been demonstrated to be a potent inhibitor of AFB_1-induced hepatocarcinogenesis in rats. It is believed that this drug induces phase II enzymes, including the glutathione S-transferase, and thus could enhance the detoxification of AFB_1 (Ansher *et al.*, 1986; Bueding *et al.*, 1982).

In summary, since no practical method is currently available for preharvest prevention of aflatoxin contamination of foods and feeds, all potential methods for postharvest aflatoxin detoxification must be considered as important at this point in time. Nevertheless, the prevention of aflatoxin contamination before harvest is the best long-term approach because the technology would eliminate or reduce the need for handling aflatoxin-contaminated commodities by growers or processors.

IV. Biosynthesis of aflatoxins

A. Chemical and biochemical aspects

Because of the extreme toxicity of aflatoxins, elucidation of the biosynthetic pathway has become a very popular topic since their discovery in 1960. To date aflatoxin biosynthesis is the best characterized biosynthetic pathway of fungal secondary metabolism. Studies using blocked mutants, metabolic inhibitors, and radiolabeled precursors in bioconversion experiments have lead to a relatively clear picture of the biochemical pathway utilized for synthesis of AFB_1 (Bennett *et al.*, 1980; Bhatnagar *et al.*, 1987; Hsieh *et al.*, 1973, 1976; Lee

et al., 1976; McCormick *et al.*, 1987; Shroeder *et al.*, 1974; Steyn *et al.*, 1980). Data on the biosynthetic intermediates and enzymes in the AFB_1 pathway have been collected (Bhatnagar *et al.*, 1992; Dutton, 1988) and the putative biosynthetic scheme is shown in Figure 3. AFB_1 is a polyketide-derived secondary metabolite which is synthesized from acetate and malonate in a process analogous to fatty acid synthesis. As described by Bennett and Christensen (1983), the precursors in biosynthetic pathway include one acetyl CoA and 9 malonyl CoA. The early steps of AFB_1 biosynthesis are the same as those in fatty acid biosynthesis with condensation of acetyl CoA and 2 Malonyl CoA molecules in the presence of NADPH to form a hexanoate starter unit (Towsend *et al.*, 1984). Condensation of this starter unit with 7 malonyl CoA molecules then proceeds to form a C_{20} polyketide chain without further ketoreduction. Cyclization and oxidation then occur to form the first stable anthraquinone intermediate, norsolorinic acid (NA), which is then converted to (sequentially) averantin (AVN), averufanin (AVNN), averufin (AVF), versiconal hemiacetal acetate (VHA), versiconal (VHOH), versicolorin B (VB), versicolorin A (VA), sterigmatocystin (ST), *O*-methylsterigmatocystin (OMST), and the final product AFB_1.

Mutants blocked in aflatoxin biosynthesis played an important role in elucidating this pathway. In this regard, the pathway blocked mutants of *A. parasiticus* were more useful than those of *A. flavus*. This is because mutants isolated from *A. parasiticus* accumulate pigmented pathway intermediates. Most of the mutants of *A. flavus*, on the other hand, do not accumulate pigments. For example, the pigments NA, AVN, AVF, and VA were identified from *A. parasiticus* non-aflatoxin producing mutants. These four pigments were then isolated from fungal mycelia and their chemical structures identified. Radiolabelled pigments were used in feeding studies to demonstrate that they could be converted to AFB_1

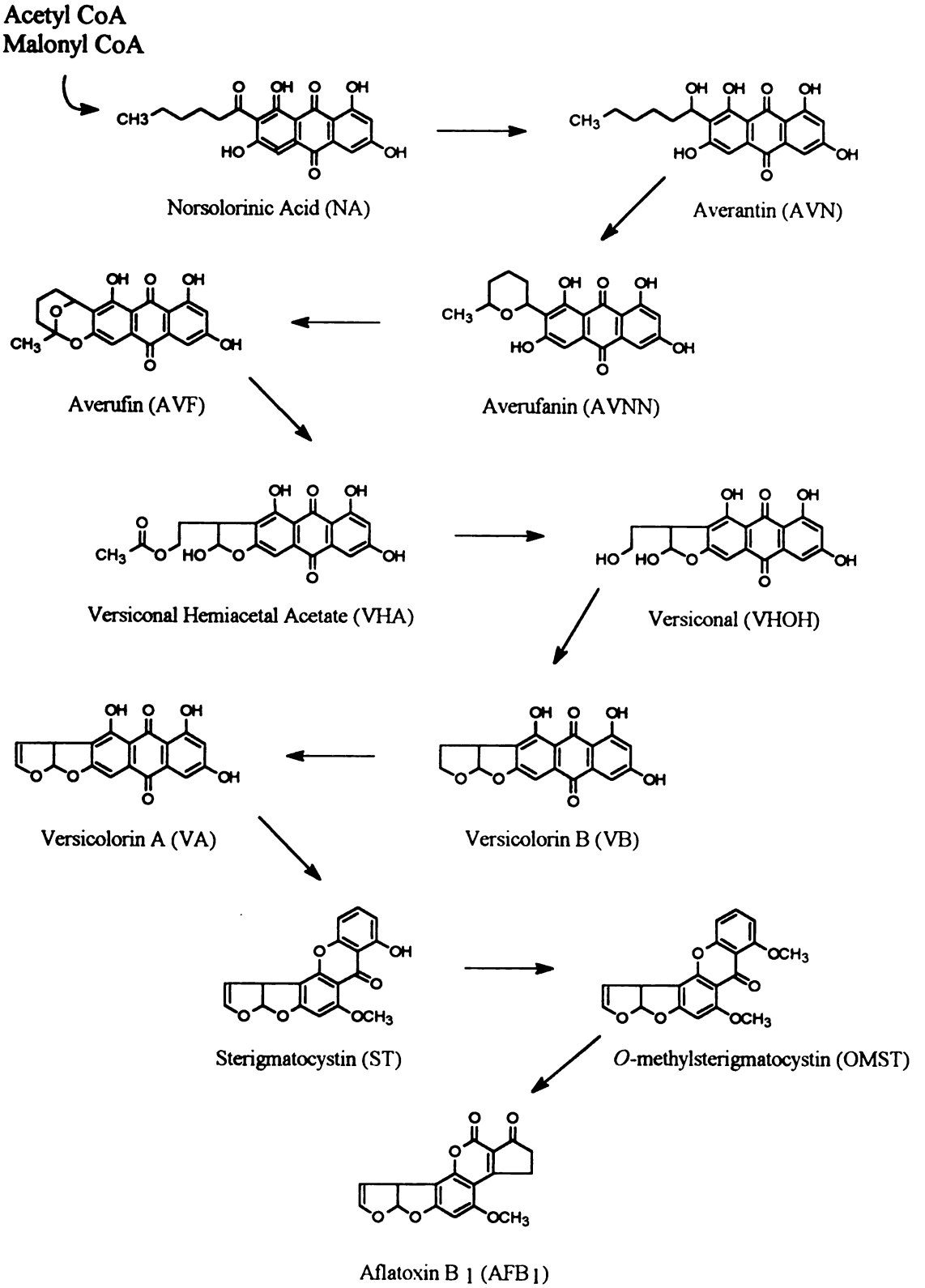


Figure 3. The proposed biosynthetic pathway for aflatoxin B1

by toxigenic fungi. The sequential order of these intermediates was determined according to their efficiencies of conversion to AFB_1 and their relative chemical structures. AVNN, a metabolite of *A. parasiticus* was then demonstrated to be incorporated into AFB_1 and was placed between AVN and AVF based on a logical organic conversion mechanism (McCormick *et al.*, 1987). VHA was identified because it accumulated after inhibition of an esterase activity by the insecticide dichlorvos (Yao and Hsieh, 1974). The intermediates after VA are not pigmented. ST and OMST isolated from *A. versicolor* and *A. parasiticus*, respectively, were placed downstream from VA by a bioconversion assay (Hsieh *et al.*, 1973; Bhatnagar *et al.*, 1987).

It is estimated that at least 17 enzymatic activities are associated with this complex pathway (Bhatnagar *et al.*, 1992; Dutton, 1988). Several pathway enzymes have been purified to homogeneity including two NA reductases (NA→AVN; Bhatnagar and Cleveland, 1990; Chuturgoon *et al.*, 1990), two VHA reductases (VHA→versiconol acetate[VOAc]; Matsushima *et al.*, 1994), one versiconal cyclase (VHOH→VB; Lin and Anderson, 1992; Townsend *et al.*, 1991), and two *O*-methyltransferases (ST→OMST; Bhatnagar *et al.*, 1988; Keller *et al.*, 1992). Those enzymes which have been identified or partially purified are the esterase (VHA→VHOH; Yabe and Hamaski, 1993), the desaturase (VB→VA; Yabe *et al.*, 1991), and the oxidoreductase (OMST→AFB1; Bhatnagar *et al.*, 1989). Nevertheless, many other enzymes involved in the biosynthetic pathway have not been identified.

B. Genetic and molecular aspects

1. Classical genetic analysis

Conventional genetic analysis of aflatoxin biosynthesis was hampered because neither *A. flavus* nor *A. parasiticus* has a sexual reproductive stage. However, genetic linkage of

genes for aflatoxin biosynthesis in both fungi was studied using the parasexual cycle (Bennett, 1979; Papa, 1973). The genetic markers in genetic linkage analysis included auxotrophic mutants, spore color mutants, and aflatoxin pathway mutants. The genetics of *A. flavus*, however, is better understood than that of *A. parasiticus*. Over 30 genes including 11 aflatoxin genes have been mapped to 8 linkage groups (Bennett and Papa, 1988). Of the 11 aflatoxin loci mapped, 1 is on linkage group II, 9 are on linkage group VII, and 1 is on linkage group VIII. The aflatoxin loci are nonallelic and recessive in diploids, with the exception of the mutant containing the *afl-1* allele.

The generation of physical mapping data of the aflatoxin genes was enhanced by the development of the electrophoretic karyotype analysis. Using pulsed-field gel electrophoresis, the genomes of *A. flavus* and *A. parasiticus* have been separated into 6 to 8 chromosomes with sizes ranging from 3 to 7 Mb (Keller et al., 1992). In an attempt to assign the linkage groups identified from genetic linkage studies to these separated chromosomes, Foutz *et al.* (1995) have cloned seven previously mapped auxotrophic genes which hybridized to 7 individual chromosomes. These specific probes could help in future studies to determine the karyotypic map of aflatoxin genes.

2. Molecular genetics

A logical approach to prevent aflatoxin contamination is to block their production in the field at the preharvest stage. The development of a thorough understanding of aflatoxin biosynthesis at the molecular level may aid in this approach. Cloning of genes associated with aflatoxin biosynthesis is the first step to effectively understand the regulation of gene expression. The cloned genes can be used as targets for gene disruption to generate genetically stable nontoxigenic strains of *Aspergillus spp.* which can be utilized in the field

as biocontrol agents. On the other hand, cloning of aflatoxin pathway genes and subsequent identification of regulatory genes will provide molecular probes for investigating the specific molecular regulation of aflatoxin biosynthesis in fungal culture and in host plant tissue in which aflatoxin contamination occurs. This could lead to the identification of candidate compounds which are able to block aflatoxin biosynthesis in fungi. It may be possible to directly use these compounds in the field or to construct resistant plants which naturally produce these compounds.

Three different strategies have been used to isolate genes involved in aflatoxin biosynthesis. Differential screening of a cDNA library, a relatively nonspecific approach, has been used to identify genes in *A. parasiticus* (Feng *et al.*, 1992) and *A. flavus* (Woloshuk and Payne, 1994) that may be associated with aflatoxin biosynthesis. This method is based on the principle that aflatoxin associated genes are transcribed under the conditions supporting aflatoxin production. These genes, on the contrary, are not transcribed or are transcribed in much lower amounts under non-aflatoxin supporting conditions. Therefore toxin specific cDNA clones theoretically could be isolated by hybridization to RNA extracted from aflatoxin-producing cultures but not to RNA from non-aflatoxin-producing cultures. The major disadvantage of this approach is that many nonspecific cDNA clones may be obtained and cause difficulties in the selection and study of their exact functions.

A second approach is reverse genetics. In this method, it is necessary to identify and purify pathway proteins which can be used to generate antibodies for use as immunoscreening probes. Alternatively, protein sequence data should allow the design of oligonucleotide probes to isolate the specific genes. Using a reverse genetics approach, a 1.5-kb genomic DNA clone (pF9-1) from *A. flavus* NRRL 3357 was identified with an oligonucleotide based

on the amino acid sequence of the N-terminus of the purified methyltransferase which converts ST to OMST (Keller *et al.*, 1992). This *omtA* gene also has been cloned from *A. parasiticus* by using antibodies (raised to the purified methyltransferase) to screen a cDNA expression library (Yu *et al.*, 1993). Recently, an *A. parasiticus* dehydrogenase gene, *norA*, was cloned by the same strategy using a monoclonal antibody raised against a purified norsolorinic acid reductase involved in the conversion of NA to AVN.

A third approach involves genetic complementation of fungal mutants deficient in aflatoxin biosynthesis followed by rescuing genes associated with aflatoxin production. Genetic transformation systems have been developed for *A. parasiticus* (Horng *et al.*, 1990; Skory *et al.*, 1990) and *A. flavus* (Woloshuk *et al.*, 1989). Using these transformation systems, the *nor-1* gene, associated with the conversion of NA to AVN (Chang *et al.*, 1992), the *ver-1* gene, associated with the conversion of VA to ST (Skory *et al.*, 1992), and the *fas-1A* gene, encoding a putative fatty acid synthetase involved in polyketide backbone synthesis (Mahanti *et al.*, 1996), were cloned by genetic complementation of *A. parasiticus* mutants blocked at unique steps in AFB_1 synthesis. Complementation was performed by transformation of a cosmid DNA library constructed using genomic DNA from a wild-type aflatoxin-producing strain *A. parasiticus* NRRL 5862 (SU-1). A regulatory gene, *aflR*, was first isolated from *A. flavus* by genetic complementation (Payne *et al.*, 1993). The homologue of *A. flavus aflR* was later identified in *A. parasiticus* based on its ability to upregulate aflatoxin biosynthesis when it was transformed into a wild-type toxigenic strain (Chang *et al.*, 1993).

The isolation of these pathway genes made it possible to elucidate a detailed physical map of the genes involved in aflatoxin biosynthesis. In the process of cloning the *nor-1* and

ver-1 genes from *A. parasiticus*, it was found that these two genes are located within a 35-kb genomic DNA fragment contained in cosmid NorA and on a single chromosome. The aflatoxin gene cluster was further confirmed by mapping the position of *fas-1A*, *aflR*, and *omtA* in the genomic DNA of both *A. flavus* and *A. parasiticus*. Transcriptional mapping studies have shown that this cluster may extend up to 60-kb (Trail *et al.*, 1995; Yu *et al.*, 1995). Taking advantage of transcripts identified in this cluster, it was possible to elucidate the function of originally non-identified genes by gene disruption analysis. For example, a *pksA* gene encoding a putative polyketide synthase was identified adjacent to *nor-1* by gene disruption analysis (Chang *et al.*, 1995; Trail *et al.*, 1995). The order of aflatoxin genes in the clusters is similar in the two aflatoxin-producing *Aspergillus*. The spacing between these genes, however, is different (Yu *et al.*, 1995).

V. Regulation of aflatoxin biosynthesis

As a secondary metabolite, aflatoxins are produced when the fungus reaches the end of active growth phase during batch fermentation. Because the precursor (i.e. acetate) of aflatoxin biosynthesis is the product of the primary metabolism, the factors which regulate primary metabolism could influence aflatoxin biosynthesis. Carbohydrate metabolism has been shown to affect the production of aflatoxins. Several simple carbon sources, especially glucose, sucrose, and maltose, have been shown to support and stimulate aflatoxin production (Luchese and Harrigan, 1993). Glucose can be catabolized by aspergilli either by way of the Embden-Meyerhoff or the hexose monophosphate pathways simultaneously (Zaika and Buchanan, 1987). Aerobic conditions favor utilization of the hexose monophosphate pathway and anaerobic conditions favor the Embden-Meyerhoff pathway. It has been reported that

the amount of aflatoxin production depends on the pathway by which glucose is catabolized by the fungi (Shih and Marth, 1974). The study showed that less aflatoxins were produced in extensively aerated cultures of *A. parasiticus* and the efficiency of [1-^{14}C]glucose incorporation was less than that in stationary cultures. Shih and Marth (1974) concluded that in less aerobic conditions oxidation of acetate (via the citric acid cycle) would be decreased and more acetate would be available for synthesis of aflatoxins. It has also been proposed that a low NADPH/NADP ratio favors aflatoxin production (Niehaus and Dilts, 1984). The increased aflatoxin production observed in a less aerobic environment could therefore result from the decreased formation of NADPH via the hexose monophosphate pathway. An alcohol dehydrogenase gene, *adh1*, has been cloned from *A. flavus* (Woloshuk and Payne, 1994). The transcription of this gene is induced during aflatoxin production. Although the exact role of *adh1* in aflatoxin biosynthesis in not known, it is hypothesized that alcoholic fermentation may be important for the use of glucose through the glycolytic pathway under less aerobic conditions and may be associated with aflatoxin biosynthesis.

Instead of relying solely on the regulation of primary metabolism, aflatoxin biosynthesis could also be regulated at the level of secondary metabolism. Skory *et al.* (1993) have reported that the regulated expression of the aflatoxin pathway genes, *nor-1* and *ver-1*, is in part at the level of transcription. The accumulation of the RNA transcripts from other aflatoxin associated genes was shown to follow a similar pattern as *nor-1* and *ver-1* (Trail *et al.*, 1995). This suggested that aflatoxin genes may be regulated by a common regulatory factor or some specific factors activated at the same time for aflatoxin synthesis. The genetic evidence suggests that *aflR* is a specific regulatory gene in aflatoxin biosynthesis. Metabolite feeding studies demonstrated that *aflR* is required for the conversion of several different

pathway intermediates to aflatoxins (Payne *et al.*, 1993). Moreover, the *aflR* gene is capable of inducing transcription of several aflatoxin pathway genes and expression of several aflatoxin pathway enzymatic activities simultaneously in cell extracts (Chang *et al.*, 1995; Payne *et al.*, 1993). Analysis of the predicted amino acid sequence of *aflR* showed that there is a cysteine-rich binuclear zinc cluster DNA-binding motif, Cys-Xaa2-Cys-Xaa6-Cys-Xaa6-Cys-Xaa2-Cys-Xaa6-Cys, which has been found in several fungal transcriptional regulatory proteins (Woloshuk *et al.*, 1994).

In addition to *aflR*, a putative regulatory locus, *afl-1*, was identified in *A. flavus* (Bennett and Papa, 1988). Recently, it was demonstrated that a heterozygous diploid strain with both mutant (*afl-1*) and wild-type (*afl-1*$^+$) alleles lost its ability to produce aflatoxin and to transcribe the *nor-1*, *ver-1*, and *omtA* genes. However, expression of the *aflR* gene was not suppressed (Woloshuk *et al.*, 1995) which suggests that *aflR* is not the only regulator of AFB_1 biosynthesis. The real function of *afl-1* in regulation of aflatoxin biosynthesis will not be clear until the *afl-1* gene can be isolated in the future study.

Since several aflatoxin pathway genes have been cloned, it is now possible to elucidate the *cis*-acting DNA elements and *trans*-acting proteins that regulate aflatoxin synthesis. One approach to accomplish this task is to fuse the aflatoxin gene promoter to a reporter gene such as the GUS gene (*uidA* gene; encodes β-glucuronidase). Fungal strains containing these reporter constructs could be used to identify the *cis*-acting elements (Trail *et al.*, 1994; Wu and Linz, 1994). After identification of the *cis*-acting elements, promoter fragments can be used in mobility shift assays to identify and thereafter purify the specific *trans*-acting proteins which regulate aflatoxin biosynthesis.

CHAPTER 2

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE *VER-1* GENE

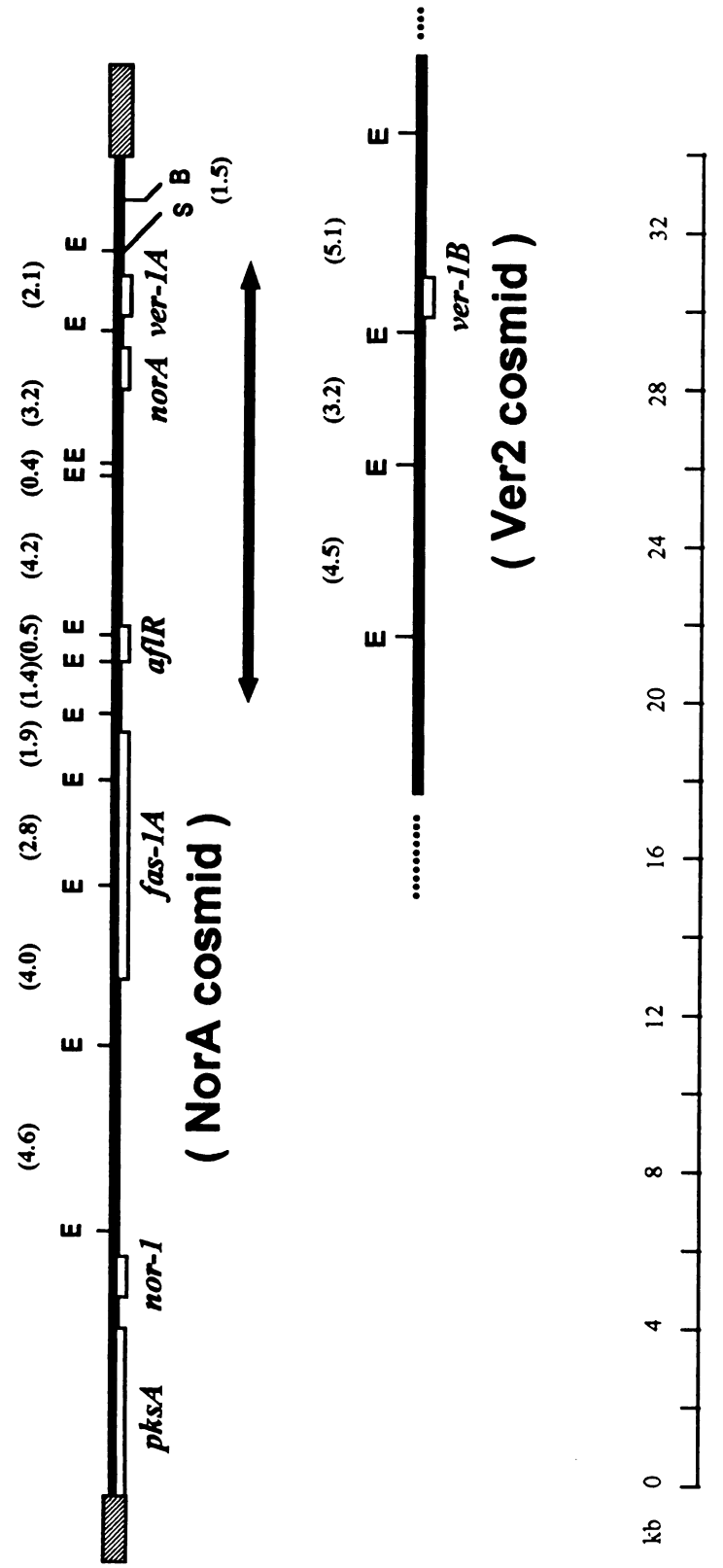
I. INTRODUCTION

In a previous study (Skory *et al.*, 1992), the *ver-1* gene was cloned based on its ability to complement the versicolorin A (VA) accumulating strain, *A. parasiticus* CS10 (*ver-1*, *wh-1*, *pyrG*; derived from ATCC 36537; Lee *et al.*, 1975), to produce aflatoxins. The nucleotide sequence of a genomic DNA fragment and cDNA fragment covering the entire *ver-1* coding region has been determined (Skory *et al.*, 1992). The predicted amino acid sequence, deduced from the *ver-1* nucleotide sequence, was compared with the EMBL and GenBank data bases. The search revealed striking similarity with *Streptomyces* ketoreductases involved in polyketide biosynthesis. This observation resulted in the prediction that the *ver-1* gene encodes a protein that has enzymatic activity associated with AFB_1 biosynthesis.

Southern hybridization analysis using the cloned *ver-1* gene as a probe indicated that there are two copies of the *ver-1* gene in *A. parasiticus* SU-1 (Skory, 1992). By in situ colony hybridization of the genomic DNA library constructed by Skory *et al* (1992), two cosmid vectors, **NorA** and **Ver2**, were found to contain the *ver-1* gene. The location of the *ver-1* gene on the two cosmid vectors is shown in Figure 4. The gene copy located on a 2.1-kb *Eco*RI fragment of the cosmid NorA was named *ver-1A*. The other *ver-1* gene copy

Figure 4. Schematic representation of the location of *ver-1A* and *ver-1B* on cosmids NorA and Ver2, respectively. The number in parentheses is the size of the restriction fragment in kilobase pairs. The arrow indicates the duplicated region. B, *Bam*HI; E, *Eco*RI; S, *Sal*I.

Figure 4.


pksA
nor-1
fas-1A
aflR
norA
ver-1A
ver-1B
S B
(1.5)
(2.1)
(3.2)
(0.4)
(4.2)
(0.5)
(1.4)
(1.9)
(2.8)
(4.0)
(4.6)
(5.1)
(3.2)
(4.5)
E
(NorA cosmid)
(Ver2 cosmid)
kb 0 4 8 12 16 20 24 28 32

located on a 5.1-kb *Eco*RI fragment of the cosmid Ver2 was designated *ver-1B*. The flanking regions (3.2-kb *Eco*RI fragments; see Figure 4) of *ver-1A* and *ver-1B* genes were found to hybridize to each other in Southern hybridization analysis. This indicated that there is a duplicated region around the *ver-1* gene in the chromosomal DNA.

Based on the complementation analysis and nucleotide sequence data, it was hypothesized that the cloned *ver-1* gene encodes a protein involved in the conversion of the AFB_1 pathway intermediate VA to sterigmatocystin (ST). To address this hypothesis, the following experiments were designed and accomplished in this study. First, restriction fragment length polymorphism (RFLP) analysis of *ver-1A*, *ver-1B*, and the cloned *ver-1* gene confirmed that the gene previously cloned is *ver-1A*. Second, a duplicated chromosomal region (approximately 12-kb) was identified upstream from *ver-1A* and *ver-1B* by Southern hybridization analysis. Third, the nucleotide sequence of *ver-1B* was determined. A translational stop codon, found in the *ver-1B* coding region, indicated that it encodes a truncated polypeptide with no function. Fourth, recombinational inactivation and genetic complementation experiments confirmed that *ver-1A* is the only functional copy of *ver-1* in *A. parasiticus* SU-1 and that its gene product is directly involved in the conversion of VA to ST.

II. MATERIALS AND METHODS

A. Strains and plasmids

Escherichia coli DH5α F' ^{e} [F'/*endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1*Δ(*lacZYA-argF*)$_{u169}$(m80*lacZΔM15*)](Gibco BRL, Life Technologies, Inc. Gaithersburg, MD) was used to propagate plasmid DNA. *A. parasiticus* NRRL5862 (SU-1;

ATCC 56775; Bennett, 1979), a wild-type aflatoxin-producing strain, was used as the control strain. The nitrate reductase (*niaD*) deficient strain, NR-1, derived from *A. parasiticus* SU-1, was used as the recipient strain for *ver-1* gene disruption analysis. VA, used as a standard in thin-layer chromatography (TLC) assays, was purified from *A. parasiticus* ATCC 36537 (*ver-1*, *wh-1*) according to the procedure of Lee *et al.* (1975).

Plasmid pDV-VA (Figure 5A) was constructed for *ver-1* gene disruption. A 4.4-kb *Pst*I genomic DNA fragment containing *ver-1A* was inserted into pBluescriptII SK(-) to generate pVer-AP. This plasmid was cut near the middle of the *ver-1A* coding region (*Stu*I) and blunt-end ligated to a 6.2-kb *Pvu*II fragment containing the functional *niaD* gene. For *ver-1* complementation experiments, plasmid pVer-Ben (Figure 5B) was constructed. A 4.1-kb *Xba*I/*Xho*I DNA fragment containing *ver-1A* was subcloned into pBluescriptII SK (-) to generate pVer-AX. Then, a 7.0-kb *Xba*I fragment containing the *ben*^{r} gene (confers resistance to benomyl) from pYT1 (Wu *et al.*, 1996) was inserted into pVer-AX to generate pVer-Ben. pSL82 (Horng *et al.*, 1990), a plasmid containing a complete copy of *niaD*, and pYT1, containing *ben*^{r}, were used as positive controls in recombinational inactivation and genetic complementation experiments, respectively.

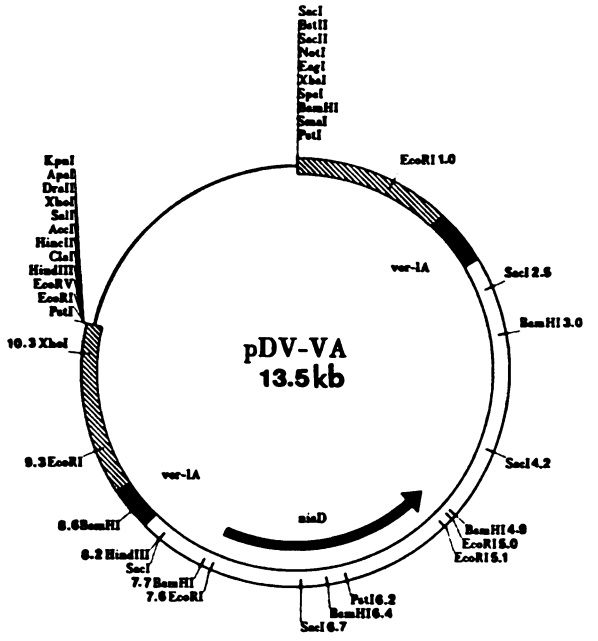
B. Bacterial cell transformation and plasmid purification

The preparation and transformation of competent cells were conducted by a calcium chloride method (Ausubel *et al.*, 1993). Recombinant cells were screened on MacConkey agar (Difco) or selected by standard colony hybridization techniques (Maniatis *et al.*, 1989). Minipreparation of plasmid DNA using alkaline lysis and large scale preparation of plasmid DNA by CsCl/ethidium bromide equilibrium centrifugation were conducted using standard methods described by Ausubel *et al.*(1993).

Figure 5. Plasmids used for functional analysis of the *ver-1* gene in recombinational inactivation and complementation experiments. (A) The plasmid pDV-VA used for recombinational inactivation of the *ver-1* gene. The white block region is a 6.2-kb *Pvu*II fragment containing the *niaD* gene. The black blocks represent the coding region of *ver-1A* split by the *niaD* containing fragment. The hashed regions are the 5' and 3' flanking regions of *ver-1A*. The single black line represents pBluescriptII SK(-). (B) The plasmid pVer-Ben used for complementation of *A. parasiticus* VAD-102. The white block is a 7.0-kb *Xba*I fragment containing the *ben*^{r} gene. The black block is the coding region of *ver-1A*. The hashed blocks are the 5' and 3' flanking regions of *ver-1A*. The single black line represents pBluescriptII SK(-).

Figure 5.

A.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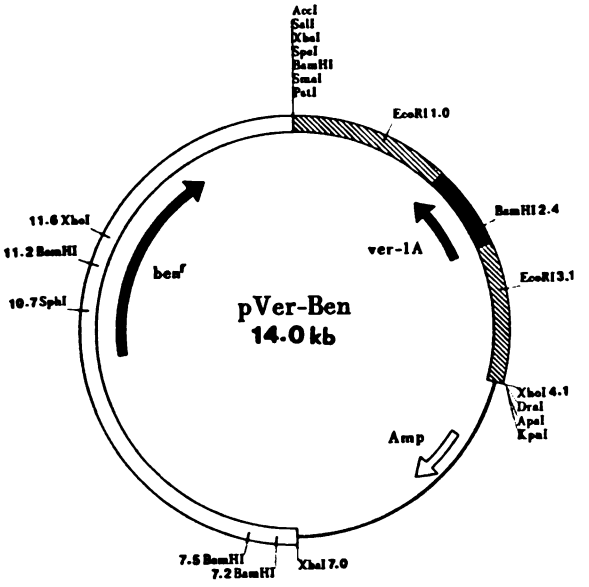
BstII
SacII
NotI
EagI
XbaI
SpeI
BamHI
SmaI
PstI
EcoRI 1.0
SacI 2.5
BamHI 3.0
SacI 4.2
BamHI 4.9
EcoRI 5.0
EcoRI 5.1
PstI 6.2
BamHI 6.4
SacI 6.7
7.6 EcoRI
7.7 BamHI
SacI
6.2 HindIII
6.6 BamHI
9.3 EcoRI
10.3 XbaI
KpnI
ApaI
DraII
XbaI
SalI
AccI
HincII
ClaI
HindIII
EcoRV
EcoRI
PstI
ver-1A
ver-1A
minD
pDV-VA
13.5kb

B.


AccI
SalI
XbaI
SpeI
BamHI
SmaI
PstI
EcoRI 1.0
BamHI 2.4
EcoRI 3.1
XbaI 4.1
DraI
ApaI
KpnI
XbaI 7.0
7.2 BamHI
7.5 BamHI
10.7 SphI
11.2 BamHI
11.6 XbaI
ver-1A
Amp
pVer-Ben
14.0 kb

C. Transformation of fungal protoplasts

Fungal protoplasts were transformed using a polyethylene glycol procedure (Oakley *et al.*, 1987) with minor modifications as described by Skory *et al.* (1990). Approximately 10^8 conidia of *A. parasiticus* NR-1 were incubated in yeast extract-sucrose liquid medium (YES; 2% yeast extract, 6% sucrose, pH 5.8) for 15 hr at 29°C with shaking (150 rpm) in the dark. The mycelia were harvested and digested with Novozyme 234 (Novo Industries, Danbury, Conn.) to generate protoplasts. To 100 µl protoplast suspension, 5 - 10 µg DNA in 10 µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and 50 µl PEG solution (25% polyethylene glycol 3350, 50mM $CaCl_2$, 10mM Tris-HCl, pH 7.5) were added. The mixture was incubated on ice for 20 min. 1 ml PEG solution was added and the mixture was further incubated at room temperature for 30 min. Finally, the protoplasts were spread onto selective agar media. Cells transformed with plasmid pSL82 and pDV-VA were screened for their ability to utilize nitrate on Czapek-Dox agar (CZ agar, Difco), a defined medium containing 20% sucrose as the osmotic stabilizer. When transformed with pYT1 or pVer-Ben, benomyl resistant transformants were selected on CZ containing benomyl (2 µg/ml). Transformant colonies were then transferred to coconut agar medium (CAM) for screening aflatoxin production by visualization of blue fluorescence under UV light (Davis *et al.*, 1987).

D. Preparation and analysis of genomic DNA from fungal cells

YES broth was used to grow fungal mycelia for preparation of genomic DNA. 100 ml of YES in a 250 ml Erlenmeyer flask was inoculated with $2x10^6$ spores of individual fungal isolates and incubated on a rotary shaker at 150 rpm at 30°C in the dark. Cultures were grown for 48 h and a phenol-chloroform protocol previously described (Skory *et al.*, 1990) was used to isolate genomic DNA from mycelia. Restriction enzymes were purchased from

Boehringer Mannheim Biochemicals (Indianapolis, IN) and used according to the manufacturer's instructions. Enzyme digestion, agarose gel electrophoresis, and Southern hybridization analyses were performed according to standard procedures (Ausubel *et al.*, 1993). Radiolabeled DNA probes were generated with a Random-Primed DNA Labeling kit (Boehringer Mannheim Biochemicals) by incorporation of [α-^{32}P]dCTP (DuPont).

E. Analysis of versicolorin A and aflatoxin production

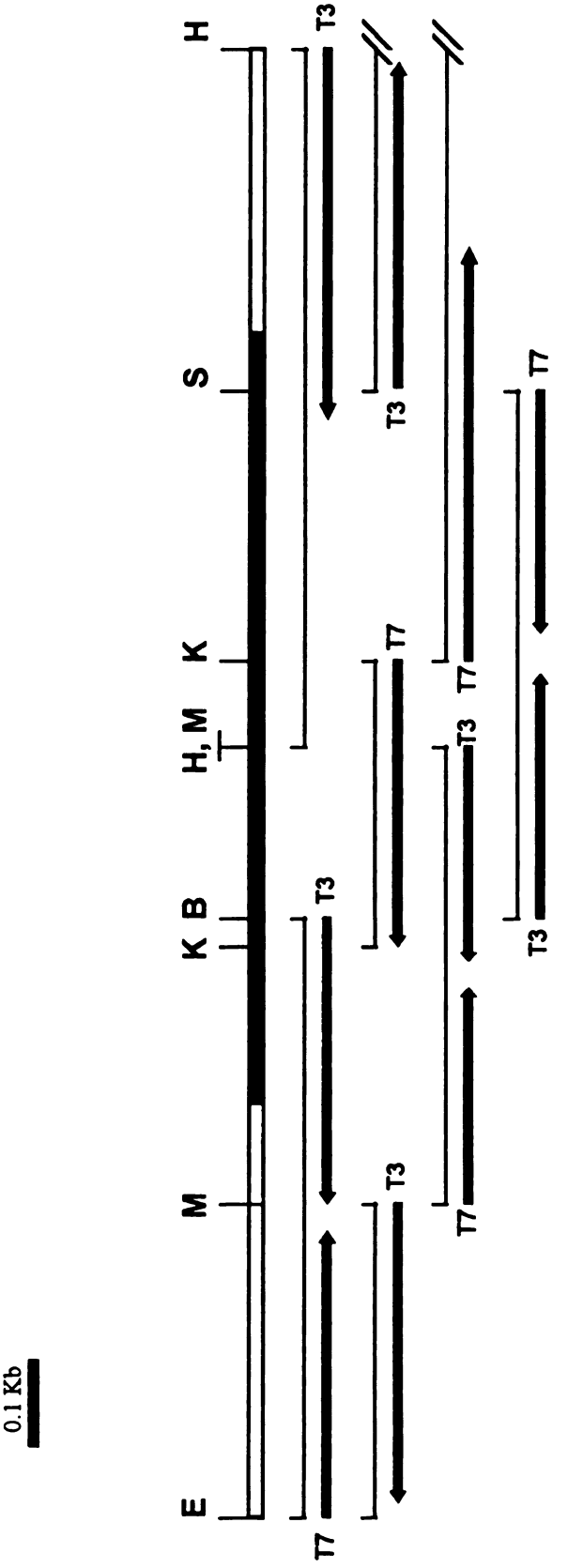
To qualitatively determine the metabolites which accumulated in transformant colonies, TLC analysis was performed on activated silica TLC plates (10 by 10 cm) using chloroform-acetone (95:5) as a solvent system. A mixture of aflatoxin B_1, B_2, G_1 and G_2 (Sigma) and semipurified VA were resolved on the same plate as reference standards. To identify the production of VA in *ver-1* gene disrupted transformants, TLC analysis was performed using benzene-acetic acid (95:5) as a solvent system. The yellow pigment that comigrated with the VA standard was scraped from the plates and dissolved in methanol or ethanol and the absorbance spectrum from 200 to 600 nm was determined. The spectrum was compared to previously published spectrum data for pure VA (Hamasaki *et al.*, 1967).

F. Nucleotide sequence analysis

A 1.7-kb *Eco*RI/*Hin*dII DNA fragment containing the *ver-1B* gene was sequenced using eight overlapping subclones which were inserted into pBluescriptII SK(-) (Figure 6). DNA sequence analysis was performed on both strands with T3 and T7 primers by the dideoxy-chain termination method (Sanger *et al.*, 1977) with an automated nucleotide sequencer (ABI robotic catalyst and 373A DNA sequencer) at the Plant Research Laboratory at Michigan State University. Nucleotide sequence data were analyzed with the Wisconsin Genetics Computer Group (GCG) package. A comparison between the predicted amino acid

Figure 6. Strategy for nucleotide sequence analysis of the 1.7-kb *Eco*RI-*Hind*II fragment which contains the *ver-1B* gene. Several subclones were cloned into the plasmid pBluescriptII SK(-) and T3 and T7 primers were used for double-stranded sequencing. The arrows indicate the direction and extent of sequencing. The black region represents the open reading frame. B, *Bam*HI; E, *Eco*RI; H, *Hind*II; K, *Kpn*I; M, *Msc*I; S, *Sma*I.

Figure 6.



sequences of *ver-1A*, *ver-1B*, the *Streptomyces coelicolor actIII* gene, and the *Magnaporthe grisea ThnR* gene was made with Gap and aligned with Pileup software (GCG).

III. RESULTS

A. Restriction fragment length polymorphism (RFLP) analysis

During the isolation of the *ver-1* gene, several DNA fragments containing the *ver-1* gene were obtained by marker rescue from an *A. parasiticus* CS-10 aflatoxin-producing transformant (Skory *et al.*, 1992). In the current study, several DNA fragments were subcloned from cosmid NorA and Ver2. The DNA fragments subcloned from cosmid NorA included a 2.1-kb *Eco*RI fragment (containing *ver-1A*), a 3.2-kb *Eco*RI fragment (5' flanking region of the *ver-1A*), and a 2.5-kb *Sal*I fragment (3' flanking region of the *ver-1A*). The DNA fragments subcloned from cosmid Ver2 included a 5.1-kb *Eco*RI fragment (containing *ver-1B*), and a 3.2-kb *Eco*RI fragment (5' flanking region of the *ver-1B*) (see Figure 4 for relative location of these DNA fragments). Restriction enzyme mapping was conducted on these subcloned DNA fragments and the restriction patterns were compared with restriction maps of the DNA fragments containing the cloned *ver-1* gene. The results indicated that the cloned *ver-1* gene is *ver-1A*.

B. Identification of a duplicated chromosomal region containing the *ver-1* gene

The 5' flanking region of *ver-1A* (3.2-kb *Eco*RI fragment) hybridized to a similar sized DNA fragment upstream from *ver-1B* indicating that there is a duplicated region flanking the *ver-1* gene in the chromosomal DNA. To determine the extent of this duplication, Southern hybridization analysis of *A. parasiticus* genomic DNA was performed using DNA fragments adjacent to *ver-1A* (isolated from cosmid NorA) as probes (see schematic in Figure 4). At

least two DNA fragments hybridized with the 1.4-kb, 3.2-kb, and 2.1-kb *Eco*RI probes in genomic DNA digests but only one fragment was detected with the 1.9-kb *Eco*RI and 1.5-kb *Bam*HI/*Sal*I probes (Figure 7). Since only one of the restriction enzymes utilized in the analysis cut within any of the fragments used as probes (*Sma*I cut within the 2.1-kb *Eco*RI fragment), the data suggested that the region of duplication extended approximately 12-kb upstream from the *ver-1A* and *ver-1B* genes.

C. Nucleotide sequence analysis of *ver-1B*

Nucleotide sequence analysis of a 1.7-kb *Eco*RI/*Hin*dII DNA fragment containing *ver-1B* was conducted on both DNA strands. The alignment of the nucleotide and predicted amino acid sequences of *ver-1A* and *ver-1B* is shown in Figure 8. The data demonstrated that these two genes share 93% identity in nucleotide sequence. The deduced amino acid sequences of the products of *ver-1A* and *ver-1B* share 97% similarity and 95% identity. A translational stop codon was identified in the coding region of *ver-1B* (see asterisk in Figure 8) indicating that *ver-1B* may encode a truncated and nonfunctional polypeptide.

D. Functional analysis of the *ver-1* genes via recombinational inactivation and complementation

To confirm gene function, plasmid pDV-VA (Figure 5A) was designed to disrupt either copy of the *ver-1* gene by homologous recombination (gene replacement). pDV-VA was linearized with *Xba*I and *Xho*I and transformed into an AFB_1 producing strain *A. parasiticus* NR-1(*afl*$^+$, *niaD*$^-$), in order to disrupt the *ver-1* gene by gene replacement through double cross-over recombination at homologous sites flanking the *ver-1* gene (Figure 9). Because of the similarity between the flanking regions of *ver-1A* and *ver-1B*, this disruption vector could in theory be used to disrupt both *ver-1* genes. In two separate experiments, a

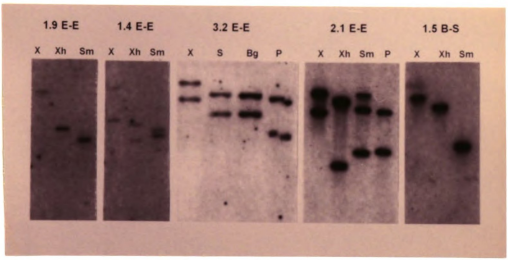


Figure 7. Identification of a duplicated chromosomal region containing the *ver-1* genes by Southern hybridization analysis. The genomic DNA from *A. parasiticus* SU-1 was digested with several restriction endonucleases. Digested DNA was electrophoresed in a 0.8% agarose gel, transferred to Nytran membranes and probed with DNA fragments subcloned from cosmid NorA. The location of probes indicated above the blots is shown in Figure 4. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; X, *Xba*I; Xh, *Xho*I.

Figure 8. An alignment of the nucleotide sequences and the deduced amino acid sequences of *ver-1A* and *ver-1B*. A stop codon within the coding region of *ver-1B* gene is indicated by an asterisk.

Figure 8.

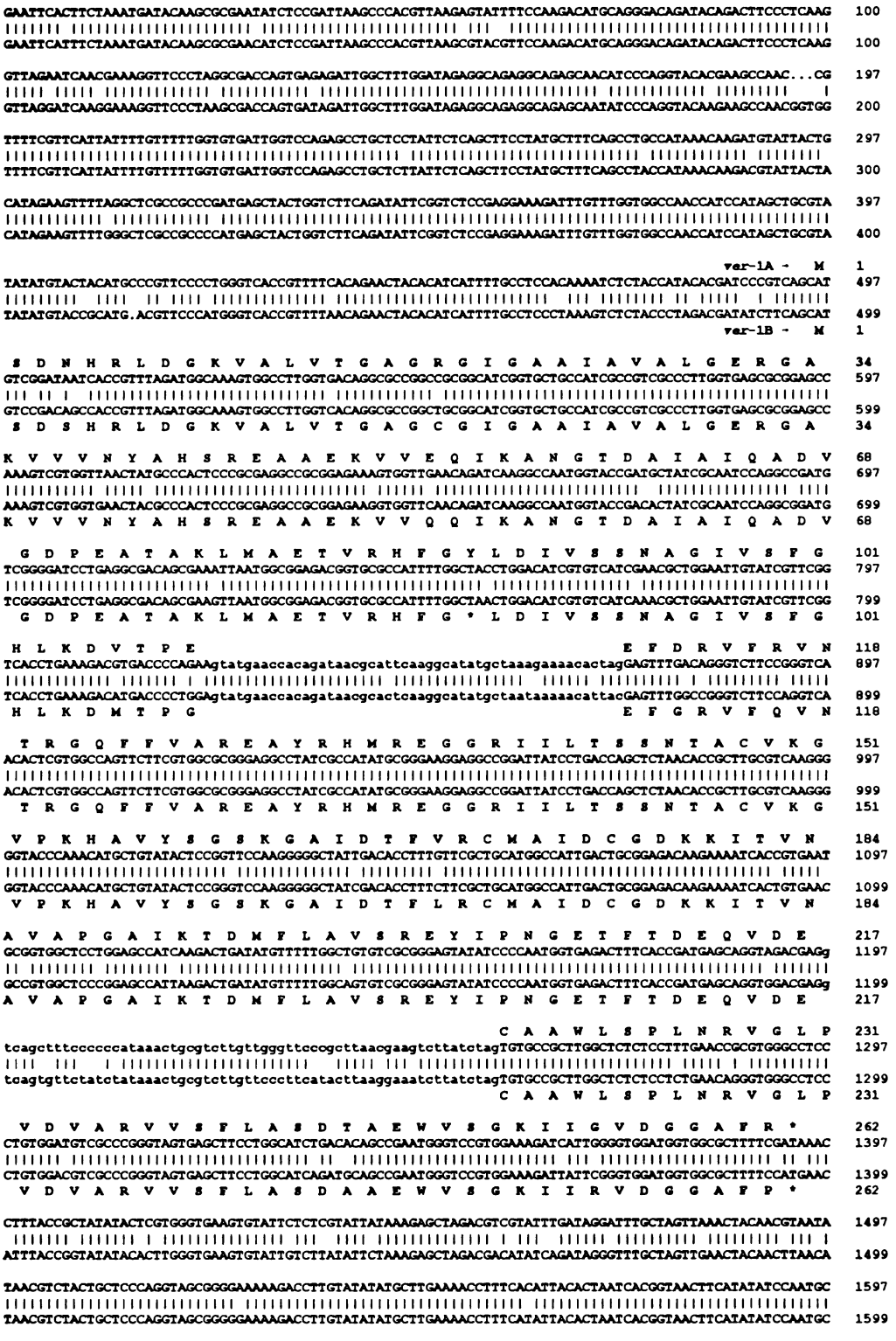
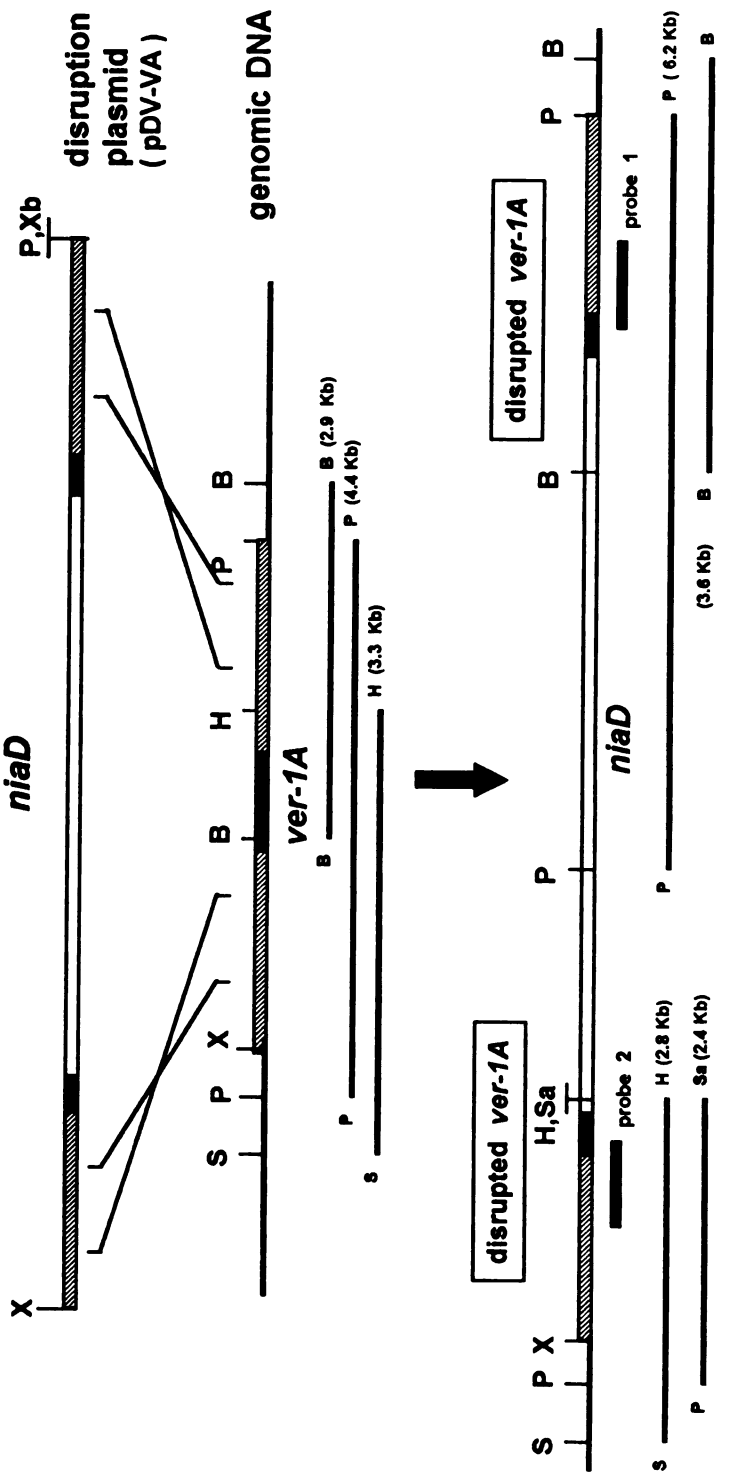


Figure 9. Schematic representation of the disruption of *ver-1A* by plasmid pDV-VA (gene replacement). The black boxes located below the restriction map of a putative recombinant clone are the predicted DNA fragments which would be observed using probe 1 and probe 2 in Southern hybridization analysis. B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; Sa, *Sac*I; S, *Sph*I; Xb, *Xba*I; X, *Xho*I.

Figure 9.


disruption plasmid (pDV-VA)
genomic DNA
P,Xb
niaD
ver-1A
X
S P X
B
H
P
B
B (2.9 Kb)
P (4.4 Kb)
H (3.3 Kb)
B
P
S
disrupted ver-1A
H,Sa
probe 2
probe 1
P (6.2 Kb)
(3.6 Kb)
H (2.8 Kb)
Sa (2.4 Kb)

total of 250 transformant colonies were generated. One clone, *A. parasiticus* VAD-102, was identified which lost the ability to produce aflatoxin but did accumulate a yellow pigment (presumptive VA) based on an initial screen on CAM. TLC analysis of cell extracts confirmed that VAD-102 did not produce AFB_1 or G_1 but instead accumulated VA (Figure 10).

In an attempt to identify the genetic recombination events which occurred in VAD-102, Southern blot analysis was conducted by cutting genomic DNA extracted from single spore isolates of VAD-102 with four different combinations of restriction enzymes : (1) *Bam*HI, (2) *Pst*I, (3) *Pst*I plus *Sac*I, or (4) *Hin*dIII plus *Sph*I. The Southern blots were probed separately with radiolabelled probes 1 or 2 shown in Figure 9. Based on the schematic (Figure 9) indicating the disruption of *ver-1A*, when genomic DNA was cut with *Bam*HI and probed with probe 1, the wild-type 2.9-kb *Bam*HI fragment would shift to a 3.6-kb *Bam*HI fragment. If *Pst*I was used to cut the genomic DNA and probed with probe 1, the wild-type 4.4-kb *Pst*I fragment would be replaced by a 6.2-kb *Pst*I fragment. In the same way, if the genomic DNA was digested with *Pst*I plus *Sac*I and probed with probe 2, the wild-type 4.4-kb *Pst*I fragment would be replaced by a 2.4-kb *Pst*I-*Sac*I fragment. The wild-type 3.3-kb *Sph*I-*Hin*dIII fragment would be replaced by a 2.8-kb fragment when genomic DNA was digested with *Sph*I and *Hin*dIII and probed with probe 2. The Southern blots demonstrated that the wild type *ver-1A* gene fragment was replaced by the predicted disrupted DNA fragment with all restriction enzymes used in the analysis (Figure 11). These data indicated that the *ver-1A* but not *ver-1B* gene was disrupted by gene replacement in VAD-102. However, additional DNA fragments not predicted for a simple disruption event also hybridized to the probes (Figure 11). The stronger hybridization signal (according to the

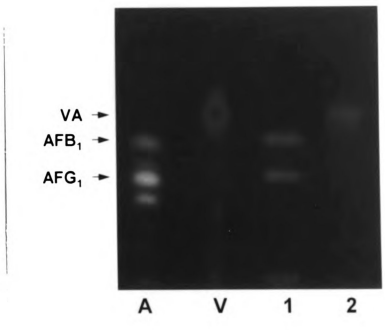


Figure 10. TLC analysis of the metabolites of *A. parasiticus* VAD-102. Aflatoxin B and G mixture (lane A) and VA purified from *A. parasiticus* ATCC 36537 (lane V) were used as standards. The chloroform-extract of mycelia and growth media from *A. parasiticus* VAD-102 (lane 2) was compared with that from the AFB_1 producing strain *A. parasiticus* NR-1(lane 1). Solvent system : chloroform-acetone (95:5).

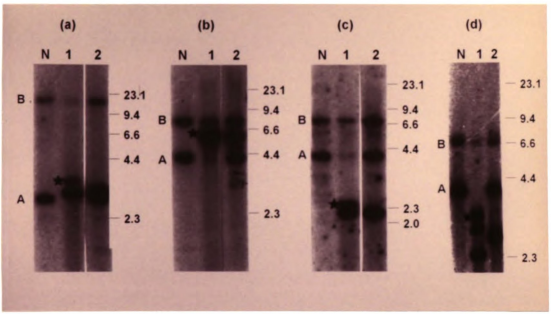


Figure 11. Southern hybridization analysis to confirm disruption of the *ver-1* gene. Genomic DNAs isolated from *A. parasiticus* NR-1 (lane N), *A. parasiticus* VAD-102 (lane 1), and a transformant which still produced aflatoxins (lane 2) were digested with *Bam*HI (blot a), *Pst*I (blot b), *Pst*I plus *Sac*I (blot c), or *Hin*dIII plus *Sph*I (blot d) and analyzed using standard procedures. Blot a and b were hybridized with probe 1 as indicated in Figure 9. Blot c and d were hybridized with probe 2. DNA fragments designated A, the DNA fragment containing *ver-1A*; B, the DNA fragment containing *ver-1B*. Stars indicate DNA fragments that are derived from gene replacement disruption of *ver-1A*. DNA size standards are *Hin*dIII-digested lambda DNA in kilobases.

intensity) of these additional fragments suggested that multiple integration of the disruption vector may have occurred in the genomic DNA. Southern hybridization analysis of these same DNAs using the *niaD* gene as a probe showed that the illegitimate recombination did not occur at the *niaD* gene locus (data not shown). Because multiple integrations of pDV-VA occurred, we could not rule out the possibility that loss of AFB_1 synthesis in VAD-102 was due to an event other than disruption of *ver-1A*. To solve this problem, an alternative hypothesis was proposed : if the accumulation of VA in strain VAD-102 is caused by disruption of the *ver-1A* gene, aflatoxin production will be restored after the functional *ver-1A* gene is put back into the genomic DNA.

In order to transform the *ver-1A* gene back into strain VAD-102, another selectable marker, *ben*^{r}, was used in fungal transformation to avoid generation of auxotrophic mutations in the VAD-102 strain. The *ben*^{r} gene is a mutated allele of the normal β-tubulin gene and confers resistance to the fungicide benomyl. The gene has been cloned in our lab from a benomyl resistant mutant of *A. parasiticus* (Wu *et al.*, 1996). Strain VAD-102 was transformed with pVer-Ben (Figure 5B) containing *ver-1A* and *ben*^{r}. Nine benomyl resistant transformants were obtained. TLC analysis of cell extracts of 5 transformants (*A. parasiticus* VAD-BV 1 to 5) demonstrated that they were able to produce aflatoxin although they still accumulated VA (Figure 12). Based on Southern hybridization analysis of their genomic DNAs, the five aflatoxin-producing transformants all contained at least one copy of the wild-type *ver-1A* gene (2.1-kb *Eco*RI fragment; see Figure 13). It was difficult to identify the site of integration of pVer-Ben in these transformants because the recipient strain already harbored multiple truncated *ver-1A* sequences. The different location and copy number of the wild-type *ver-1A* gene in the aflatoxin-producing transformants may explain different

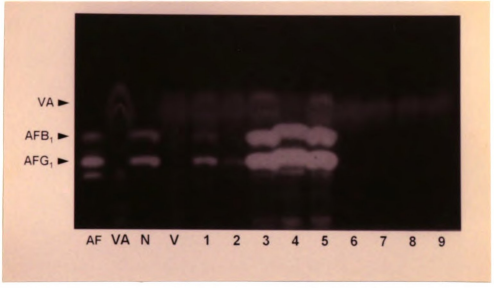


Figure 12. TLC analysis of the metabolites of transformants obtained by transformation of *A. parasiticus* VAD-102 with pVer-Ben. The chloroform-extract from *A. parasiticus* NR-1 (lane N) and *A. parasiticus* VAD-102 (lane V) were resolved on the same plate with those of the transformants *A. parasiticus* VAD-BV 1 to 9 (lanes 1 to 9). Aflatoxin B and G mixture (lane AF) and VA purified from *A. parasiticus* ATCC 36537 (lane VA) are used as standards. Solvent system : chloroform-acetone (95:5).

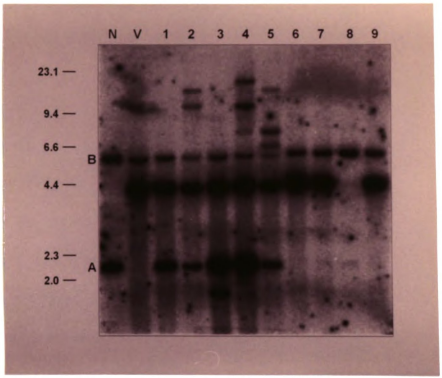


Figure 13. Southern hybridization analysis of *A. parasiticus* VAD-102 transformed with pVer-Ben. Genomic DNAs isolated from *A. parasiticus* NR-1 (lane N), *A. parasiticus* VAD-102 (lane V), and transformants (lanes 1 to 9) were digested with *Eco*RI and probed with a 0.8-kb *Sma*I/*Eco*RI DNA fragment containing *ver-1A* and analyzed by standard procedures. DNA fragments designated A, the DNA fragment containing *ver-1A*; B, the DNA fragment containing *ver-1B*. DNA size standards are *Hin*dIII-digested lambda DNA in kilobases.

levels of AFB_1 and VA observed. The remaining four benomyl resistant transformants (VAD-BV 6 to 9) did not produce detectable AFB_1 (Figure 12) nor did they contain wild type copies of *ver-1A* (Figure 13). These data suggested that disruption of *ver-1A* in VAD-102 accounted for the accumulation of the pathway intermediate VA and that a wild type *ver-1A* gene could complement this genetic block in the disruptant strain.

IV. DISCUSSION

The data confirm that *ver-1A* is directly involved in the conversion of VA to ST in AFB_1 biosynthesis in *A. parasiticus* SU-1. Of the two copies of *ver-1* present in this strain, only *ver-1A* is functional. A nonsense mutation occurred in the coding region of *ver-1B* which likely resulted in synthesis of a truncated polypeptide with no function. The direct involvement of a homolog of *ver-1A* in ST biosynthesis in *A. nidulans* was also confirmed by disruption of the *stcU* gene which was cloned based on sequence homology to *ver-1A* (Keller *et al.*, 1994). Disruption of *stcU* led to a block in ST production and accumulation of VA.

In this study, a linearized plasmid was used to disrupt *ver-1A* by gene replacement. A study performed by Tatebayashi *et al.* (1994), which analyzed the DNA fragments generated by illegitimate recombination in *Schizosaccharomyces pombe*, demonstrated that linearized DNA can recircularize and integrate into multiple sites in genomic DNA through homologous or nonhomologous recombination. These data may help explain the fact that not only was *ver-1A* disrupted by gene replacement but also the disruption plasmid recircularized and integrated (apparently multiple copies based on hybridization signal intensity) at one or several loci.

Recently, transcript mapping together with gene complementation and inactivation

experiments in *A. parasiticus* showed that the aflatoxin pathway genes *pksA*, *fas-1A*, *nor-1*, *aflR*, *norA*, *ver-1A*, and *omtA* are clustered in one linkage group (Trail *et al.*, 1995; Yu *et al.*, 1995). Based on the results obtained in this study, it appears that part of the gene cluster is duplicated in *A. parasiticus* SU-1. At least three previously identified genes, *ver-1A*, *norA*, and *aflR*, are located in this duplicated region. To date the presence of only one copy of any of these genes has been demonstrated in *A. flavus* (Skory, 1992). The duplication of a portion of the gene cluster in *A. parasiticus* (especially the duplication of one of the pathway regulators) but not in *A. flavus* may help explain the observation that nearly all *A. parasiticus* strains isolated produce high levels of aflatoxins, whereas many *A. flavus* isolates (up to 40% or more) produce no aflatoxins (Bennett and Papa, 1988; Cleveland and Bhatnagar, 1991).

The conversion of VA to ST in *Aspergillus* is a complex reaction which contains at least 5 enzymatic steps including deoxygenation, Baeyer-Villiger oxidation, lactone cleavage and rearrangement, oxidative decarboxylation, and methylation (Bhatnagar *et al.*, 1992; Dutton, 1988; see Figure 14). The enzymes that catalyze the conversion of VA to ST have not yet been identified. The lack of purified enzyme activities together with the absence of identified intermediates between VA and ST make it difficult to elucidate the exact function of the *ver-1* gene. Nucleotide sequence analysis of the *ver-1* gene suggested that it may encode a ketoreductase (Skory *et al.*, 1992). A comparison of the predicted amino acid sequence of *ver-1* with the published polypeptide sequence for the *Streptomyces coelicolor actIII* gene (Hallam *et al.*, 1988), which encodes a ketoreductase associated with biosynthesis of the polyketide actinorhodin, demonstrated a significant level of identity (~30%) between these proteins (Figure 15). Based on this result, the Ver-1 protein was proposed to be responsible for a deoxygenation reaction. Unfortunately, the timing of deoxygenation in

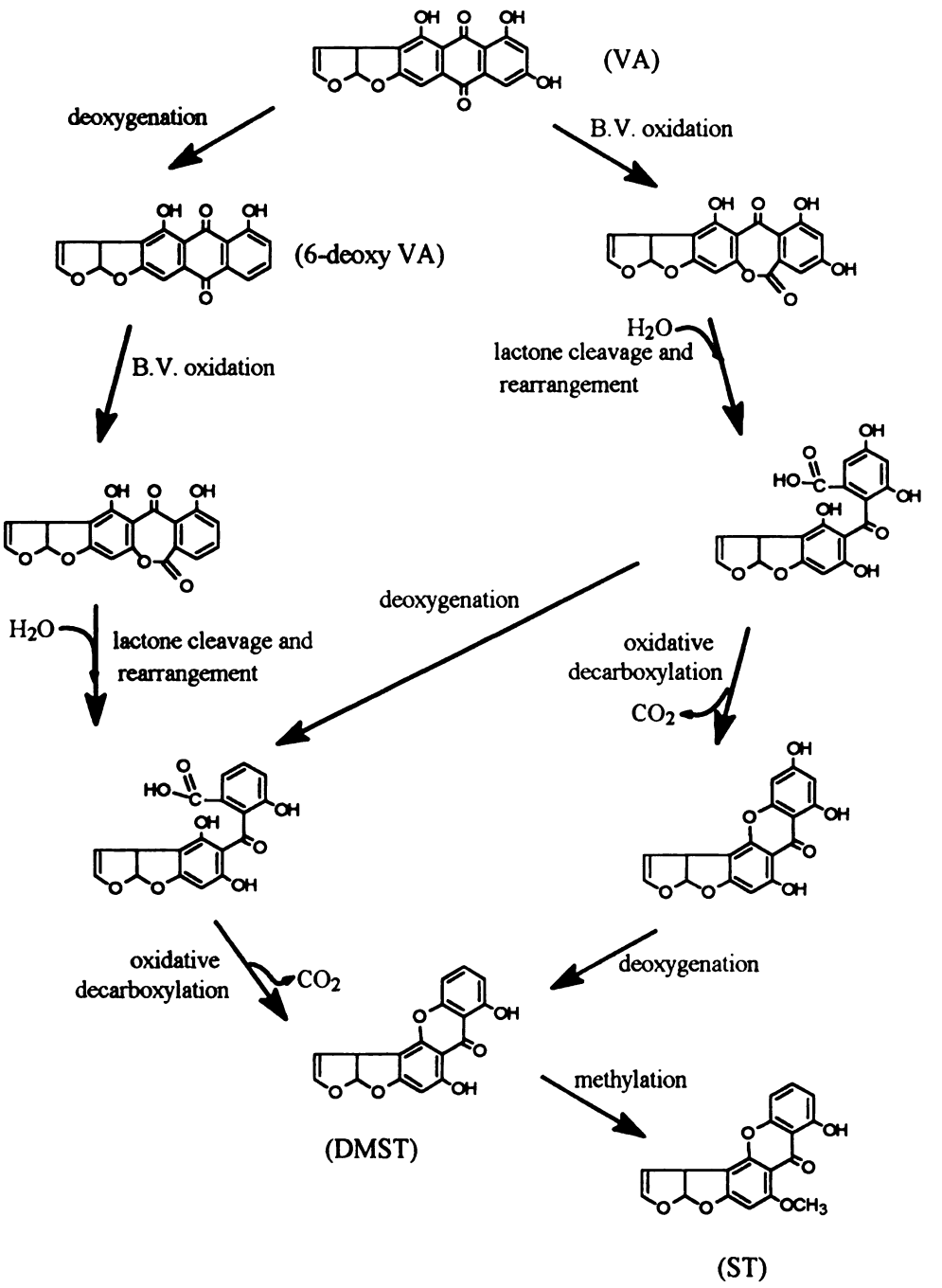
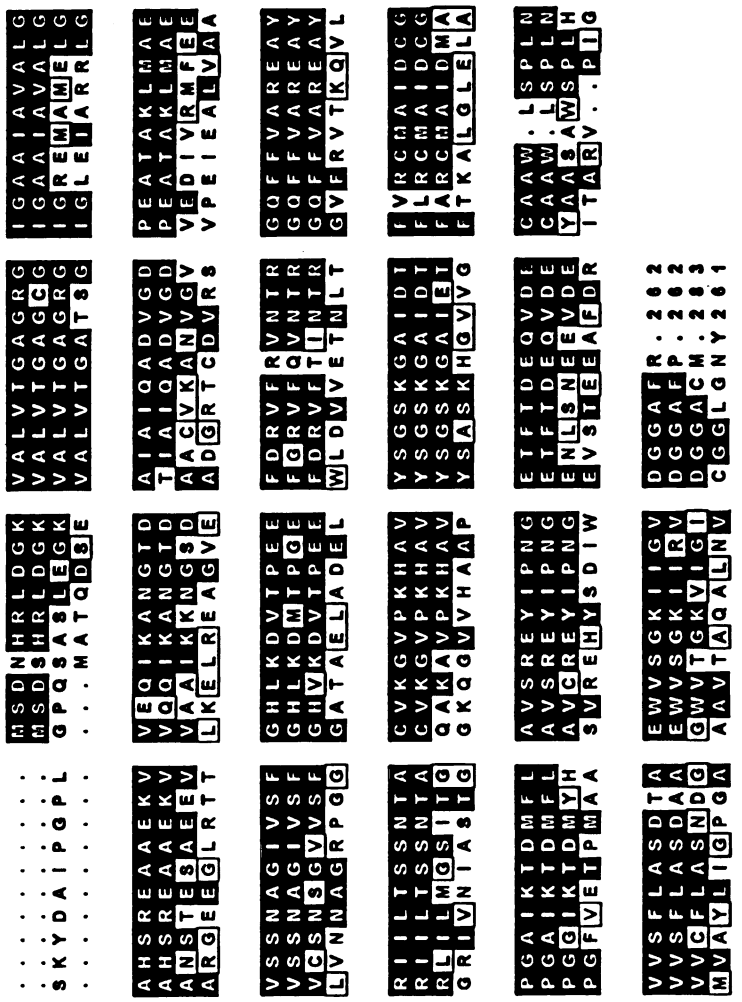


Figure 14. Proposed schemes for the enzymatic conversion of versicolorin A (VA) to sterigmatocystin (ST). 6-deoxy VA, 6-deoxyversicolorin A; DMST, demethyl-sterigmatocystin.

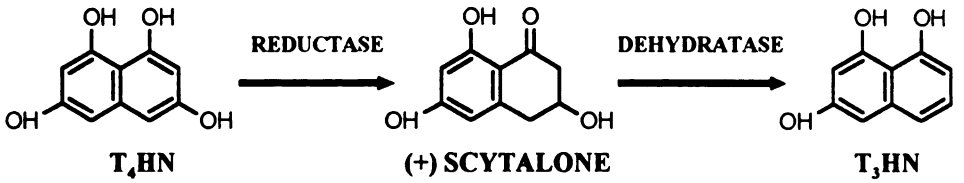
Figure 15. A comparison of the deduced amino acid sequences of *ver-1A, ver-1B*, *Streptomyces coelicolor actIII* (actIII; Hallam *et al.*, 1988), and *Magnaporthe grisea ThnR* (ThnR; Vidal-Cros *et al.*, 1994). The in-frame stop codon within the *ver-1B* polypeptide is indicated by an asterisk. Black squares represent amino acid identity. White squares represent amino acid similarity.

Figure 15.



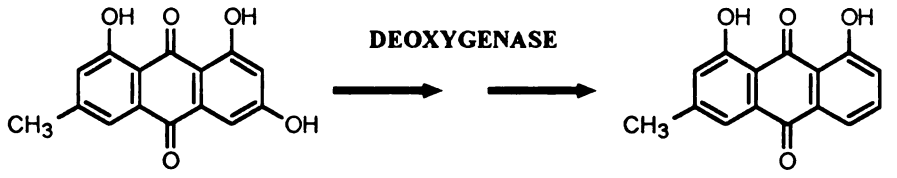
conversion of VA to ST is still ambiguous because no putative intermediates have been isolated from aflatoxin producing strains. 6-deoxyversicolorin A (6-deoxy VA), however, has been identified to be a metabolite of *Aspergillus versicolor* which produces ST (Elsworthy *et al.*, 1970). This prompted us to propose that the Ver-1 protein is involved in the deoxygenation of VA to form 6-deoxy VA. In support of this proposed scheme, a polyhydroxynaphthalene reductase (T_4HN reductase) involved in melanin biosynthesis in *Magnaporthe grisea* was recently purified to homogeneity (Vidal-Cros *et al.*, 1994). This reductase displays 66% identity and 82% similarity with the deduced amino acid sequence of the *ver-1A* gene product (Figure 15). The dehydroxylation reaction in part catalyzed by *M. grisea* T_4HN reductase is entirely analogous to the proposed deoxygenation of VA (Figure 16). The other analogous reaction is the reduction of emodin to chrysophanol at an early stage of the biosynthesis of ergochromes, fungal pigments produced by *Claviceps purpurea*. This conversion, mediated by NADPH, is believed to consist of two steps, reduction and dehydration (Ichinose *et al.*, 1993). Based on these observations, we hypothesize that VA is processed by two successively operating enzymes, the product of the *ver-1* gene and a dehydratase, to form 6-deoxy VA (Figure 16). Before completely understanding the conversion of VA to ST, however, it is necessary to clone several other genes involved in this complex reaction. Keller *et al.* (1995) have isolated a second gene, *stcS*, involved in the conversion of VA to ST in *A. nidulans*. *stcS* is located within 2-kb of *stcU* (*ver-1A* homolog) in the ST gene cluster. The close spatial relationship between these VA associated genes could lead to future studies focused on the isolation and characterization of other genes involved in the conversion of VA to ST in order to more clearly understand this process.

(1) MELANIN



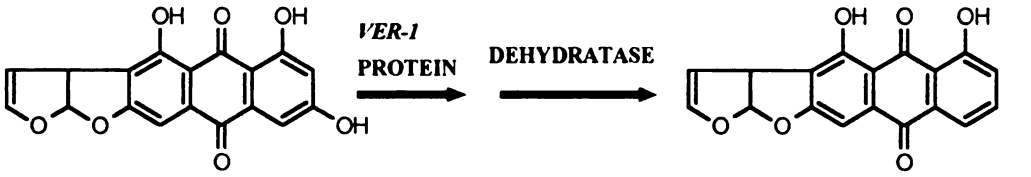
T_4HN (+) SCYTALONE T_3HN

(2) CYNODONTIN



EMODIN **CHRYSOPHANOL**

(3) AFLATOXIN



VERSICOLORIN A **6-DEOXYVERSICOLORIN A**

Figure 16. Proposed Ver-1 protein activity in a two-step dehydroxylation reaction (reaction 3) derived from analogous reactions in melanin (reaction 1; Vidal-Cros *et al.*, 1994) and cynodontin (reaction 2; Ichinose *et al.*, 1993) biosynthesis. T_4HN : 1,3,6,8-tetrahydroxynathalene; T_3HN :1,3,8-trihydroxynaphthalene.

CHAPTER 3

REGULATION OF *VER-1* GENE EXPRESSION IN FUNGAL CELLS

I. INTRODUCTION

Understanding the process of aflatoxin gene expression is one essential step toward elucidation of the molecular mechanisms which regulate aflatoxin biosynthesis. Two useful indicators for gene expression are the pattern of accumulation of the transcripts (mRNA) and proteins encoded by aflatoxin genes. Previous studies demonstrated that the appearance of several aflatoxin metabolic enzymes (Anderson and Green, 1994; Cleveland *et al.*, 1987; Lin and Anderson, 1992) and the accumulation of *nor-1*, *ver-1*, and *omtA* transcripts (Skory *et al.*, 1993; Yu *et al.*, 1993) coincide with the cessation of exponential growth of the fungus and the onset of aflatoxin production. In the current study, the expression of the *ver-1* gene was monitored by detection of the gene product, the Ver-1 protein. Since *ver-1* is directly involved in aflatoxin biosynthesis, it was hypothesized that the accumulation of the Ver-1 protein should parallel AFB_1 accumulation. Two complementary methods, batch fermentation analysis and nutritional shift assay, were performed to address this hypothesis. Because an enzyme activity assay of the Ver-1 protein was unavailable, a polyclonal antibody was generated for Western blot detection of the native Ver-1 protein in *A. parasiticus*. To generate the anti-Ver-1 antibody, a *ver-1A* cDNA was expressed in *Escherichia coli* using

the vector pMAL-c2. The maltose-binding protein/Ver-1 fusion protein produced by *E. coli* was used to generate polyclonal antibodies against the Ver-1 protein. After immunoaffinity purification, an anti-Ver-1 antibody was obtained to specifically recognize the Ver-1 protein (~28-kDa) in fungi by Western blot analysis.

The specific anti-Ver-1 antibody was also used to study the regulation of Ver-1 protein accumulation in fungal colonies grown on solid media. A temporal and spatial pattern of Ver-1 protein accumulation was observed using Western blot analysis of proteins extracted from different areas of the fungal colony. This observation was confirmed using the *ver-1*/GUS reporter strain (Wu, 1995) by monitoring GUS activity (β-glucuronidase). The *ver-1*/GUS reporter strains were also applied to study the expression of the *ver-1* gene in fungal mycelia using chromogenic and fluorescent substrates.

II. MATERIALS AND METHODS

A. Strains and plasmids

A. parasiticus NRRL 5862 (SU-1; ATCC 56775) was used as a wild-type aflatoxin-producing strain in the investigation of Ver-1 protein accumulation. *A. parasiticus* VAD-102, in which the *ver-1A* gene was disrupted, was used as a negative control for Ver-1 protein detection in Western blot analyses. *A. parasiticus* VAD-BV 1 to 5, five isolates of *A. parasiticus* VAD-102 which were transformed with a wild-type *ver-1A* gene, served as positive controls for Ver-1 protein detection. Several *ver-1*/GUS reporter strains were used in the analysis of Ver-1 protein accumulation in fungal colonies and mycelia. *A. parasiticus* pHD6-6 No. 1 and No. 4, in which the functional *ver-1* promoter/GUS fusion construct was integrated at the *ver-1* locus, were used as positive reporter strains. *A. parasiticus* pHD4-4

No. 3, in which a GUS construct without the *ver-1* promoter was integrated at the *niaD* gene locus, was used as a negative reporter strain (Wu, 1995). The pMAL-c2 vector (New England Biolabs) was used for expression of the maltose-binding protein (MBP)/Ver-1 fusion protein in *Escherichia coli* BL21 (Novagen Inc. Madison, Wisconsin).

B. Generation of polyclonal antibodies against the Ver-1 protein

The pMAL protein fusion and purification system (New England Biolabs) was used for Ver-1 protein production. The *Eco*RI/*Xho*I *ver-1A* cDNA fragment (kindly supplied by Dr. Jeff Cary, USDA-ARS, New Orleans, LA) was inserted between the *Eco*RI and *Sal*I sites in the polylinker of the pMAL-c2. This vector was then cut with *Eco*RI, treated with Klenow enzyme, and religated to generate the correct reading frame fused with *malE* which encodes the MBP. Expression of the MBP/Ver-1 fusion protein in *E. coli* was induced by adding IPTG (isopropyl β-D-thiogalactopyranoside) during active growth. The MBP/Ver-1 fusion protein was purified from the *E. coli* crude extracts using an amylose-resin column according to the manufacturer's instructions. The purified MBP/Ver-1 fusion protein [400 μg in 200 μl phosphate-buffered saline (PBS), pH 7.2] was mixed with 200 μl TiterMax adjuvant (Sigma) and used to immunize two rabbits to generate polyclonal antibodies. The rabbits were boosted with the same preparation of antigen at 28 days after the first injection. Sera were collected at 28, 58, and 65 days and the antibody titer was determined by ELISA using preimmune sera as controls. Microtiter wells were coated with MBP/Ver-1 proteins and then blocked with 3% BSA in PBS. After washing the plates with PBS containing 0.2% (v/v) tween 20 (PBS-Tween), the diluted serum was added and incubated for 60 min at 37°C. The plate was washed 6 times with PBS-Tween and the alkaline phosphatase conjugated goat anti-rabbit IgG was added to each well. After incubation for 60 min at 37°C, the plate was washed

6 times with PBS-Tween and the bound phosphatase activity was determined by adding 50 µl substrate solution consisting of 0.4 mM p-nitrophenyl phosphate, 10 mM diethanolamine, and 0.5 mM $MgCl_2$ in H_2O (pH 9.5). The reaction was stopped by adding 50 µl stopping solution containing 0.1 M EDTA in H_2O. Absorbance was read at 405 nm. Titers were defined as the highest dilution of serum resulting in absorbance greater than the control.

The IgG antibody fraction was purified by precipitation of the antiserum with 33% ammonium sulfate. After redissolving in PBS, the antibodies were passed through an affinity column in which the MBP and *E. coli* proteins were conjugated to a CNBr-activated Sepharose gel (Sigma). The antibodies which passed through the column were referred to as anti-Ver-1 antibodies.

C. Batch fermentation analysis

Conidia ($2x10^6$) of *A. parasiticus* SU-1 were inoculated in 250 ml Erlenmeyer flasks containing 100 ml YES broth (2% yeast extract, 6% sucrose; pH 5.8). The cultures were incubated at 30°C (in the dark) in an orbital shaker (150 rpm). Flasks were removed at appropriate time points after inoculation for the analysis of mycelial dry weight, aflatoxin concentration, and protein extraction.

D. Nutritional shift assay

The nutritional shift assay was performed as previously described (Skory *et al.*, 1993). Glucose-mineral salts medium (GMS), which is able to induce aflatoxin production, and peptone-mineral salts medium (PMS), a non-aflatoxin-supporting medium, were used to grow mycelia in this assay. Conidia ($2x10^7$) of *A. parasiticus* SU-1 were inoculated in 500 ml PMS and grown for 65 h at 30°C (in the dark) in an orbital shaker (150 rpm). The mycelia were harvested and equally distributed into fresh GMS or PMS media (30 ml for each sample).

The mycelia were collected at different time points (up to 48 h) after nutritional shift for protein extraction and the aflatoxin concentration in the growth media was measured.

E. Protein extraction and Western blot analysis

Mycelia for protein extraction were ground in liquid nitrogen using a mortar and pestle, suspended in TSA (2 mM Tris•Cl/40 mM NaCl/0.025% NaN_3; pH 8.0) buffer containing 1.0 mM phenylmethylsulfonyl fluoride (PMSF), and centrifuged at 20,000 x g for 15 min. The protein concentration in the supernatant was determined with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) based on the Bradford method (Bradford, 1976). 50-100 μg of protein from the supernatant were separated by electrophoresis (12 % SDS-PAGE), transferred to PVDF membrane, and probed with anti-Ver-1 antibodies. A Rad-Free chemiluminescent detection kit (Schleicher & Schuell, Keene, NH) was utilized to detect antibodies bound to proteins.

F. Analysis of mycelial dry weight and aflatoxin concentration

Fungal mycelia were harvested by filtration through Miracloth and the dry weight was measured after lyophilization or after complete drying at 70°C. The AFB_1 concentration in the filtrate was determined by an enzyme-linked immunosorbent assay (ELISA) described by Pestka (1988) with AFB_1 monoclonal antibodies (kindly provided by Dr. J. Pestka, Michigan State University).

G. Analysis of the accumulation of the Ver-1 protein in a fungal colony

Plate cultures were grown by inoculating conidiospores of *A. parasiticus* SU-1 onto the center of Petri dishes which contained YES. When the colonies had grown up to a point where well-defined sectors (different morphology in central, middle, and peripheral parts of the colony) could be observed (3 to 6 days of incubation), they were cut in concentric zones

with a sterile scalpel. Mycelia from each concentric zone were used for protein extraction and the same amount of protein was resolved by gel electrophoresis (12% SDS-PAGE), and the Ver-1 protein was analyzed by Western blot analysis.

In an alternative protocol, the *ver-1*/GUS reporter strains were grown the same way on solid YES medium. Instead of cutting out the mycelia, the colony was overlaid with a 1.5 % agar solution containing 0.5 mg/ml X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) and the plates were incubated at room temperature for 4 hr or until the blue color was well developed.

H. Analysis of the expression of the *ver-1* gene in mycelia

A modified method for fungal slide culture (Harris, 1986) was used in this experiment. The 1.5 % water agar was poured into Petri dishes and allowed to solidify. A 22-mm^2 cover glass was centered on the agar. One block of solid YES agar (5 to 8 mm^2) was placed on the center of the cover glass. The conidia of *ver-1*/GUS reporter strains were inoculated on each side of YES agar followed by placement of the second cover slip on top of the nutrient block. The slide cultures were incubated at 30°C from 24 to 65 hr (in the dark). After taking apart the assemblage, the cover glass with mycelia attached to it was immersed in liquid YES containing 0.5 mg/ml X-gluc and incubated for 4 hr. The preparation was then examined by a Nikon Labophot microscope for distribution of the blue color. A FITC-conjugated GUS substrate (C_{12}FDGlcU; Molecular Probes, Inc.) was also used to replace the X-gluc in this experiment, and the GUS activity was represented by the green fluorescence.

III. RESULTS

A. Specificity of anti-Ver-1 antibodies

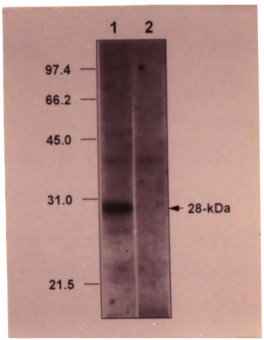
Previous data from nucleotide sequence analysis indicated that the *ver-1A* gene encodes a polypeptide composed of 262 amino acids (Skory *et al.*, 1992). The deduced molecular mass of the Ver-1 protein is 28-kDa. The specificity of the anti-Ver-1 antibodies raised against MBP/Ver-1 fusion protein was tested by Western blot analyses. The ability of this antibody preparation to detect the native Ver-1 protein in protein extracts of *A. parasiticus* NRRL 5862, a wild-type aflatoxin-producing strain, and *A. parasiticus* VAD-102, a non-aflatoxin-producing strain which accumulates versicolorin A (VA) was measured. Since the *ver-1A* gene was disrupted in strain VAD-102, it was predicted that the Ver-1 protein would not be present in the protein extract. A 28-kDa protein was detected in the protein extract of wild-type strain but not in that of strain VAD-102 (Figure 17A).

In the study described in Chapter 2, the *ver-1A* gene was transformed into strain VAD-102. Of the 9 transformants analyzed, 5 (transformants VAD-BV 1 to 5) restored *ver-1A* gene function and aflatoxin production. Protein extracts of these 9 transformants were employed in the Western blot analysis using anti-Ver-1 antibodies as the probe (Figure 17B). The data demonstrated that isolates which received the *ver-1A* gene and were restored in AFB_1 production (transformants VAD-BV 1 to 5), regained the production of the 28-kDa protein. These data confirmed that the anti-Ver-1 antibodies specifically recognize the native Ver-1 protein in *A. parasiticus*. Because of this specificity, the anti-Ver-1 antibodies raised against MBP/Ver-1 fusion protein were used to analyze Ver-1 protein accumulation.

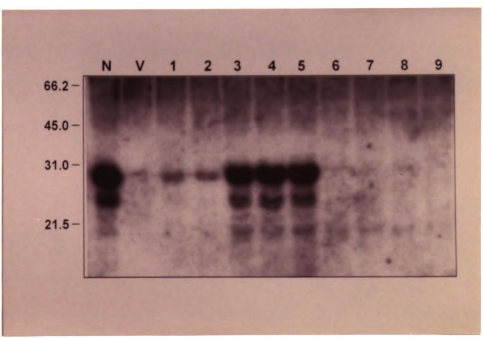
Figure 17. Western blot analysis to measure the specificity of anti-Ver-1 antibodies. Proteins were extracted from mycelia after 60 - 72 hr growth in YES media. 50 - 100 μg of protein were resolved by gel electrophoresis (12% SDS-PAGE), transferred to PVDF membrane, and probed with anti-Ver-1 antibodies raised against a MBP/Ver-1 fusion protein. (A) Western blot analysis of cell extracts from *A. parasiticus* NR1 (lane 1) and VAD-102 (lane 2). (B) Western blot analysis of cell extracts from *A. parasiticus* NR1 (lane N), *A. parasiticus* VAD-102 (lane V), and transformants (lane 1 to 9) obtained by transformation of VAD-102 with a plasmid containing the wild type *ver-1A* gene. Transformants 1 to 5 (lane 1 to 5) are aflatoxin producing strains. Transformants 6 to 9 (lane 6 to 9) are non-aflatoxin producing strains. The molecular mass of marker proteins is indicated on the left of the blots A and B.

Figure 17.

A.



B.



B. Accumulation of the Ver-1 protein in liquid cultures of *A. parasiticus*

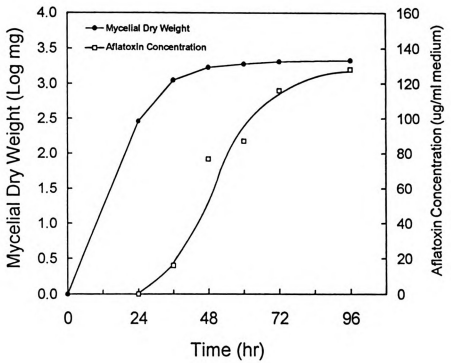
Two complementary experiments, batch fermentation analysis and nutritional shift assay, were performed to determine the relationship between Ver-1 protein accumulation and aflatoxin accumulation in *A. parasiticus*. In batch fermentation analysis, *A. parasiticus* NRRL 5862 was grown in YES medium for 5 days (conditions which induced aflatoxin production). The growth of the mycelia and aflatoxin production were measured at appropriate time intervals (Figure 18A). AFB_1 was first detected 36 hr after inoculation and accumulated rapidly between 36 hr and 60 hr. The rate of AFB_1 accumulation decreased after 60 hr. Western blot analysis (Figure 18B) revealed that the Ver-1 protein was not detected until 36 hr after inoculation, at approximately the same time as AFB_1 was detected. The quantity of the Ver-1 protein reached its highest level at 60 hr and decreased thereafter. These data indicated that the timing and rate of AFB_1 accumulation corresponded very well to the timing and rate of accumulation of the Ver-1 protein.

A nutritional shift assay was conducted to determine if the relationship between AFB_1 accumulation and Ver-1 protein accumulation was observed under a different set of conditions which induce AFB_1 synthesis. AFB_1 and the Ver-1 protein were not detected during initial growth of the wild-type strain in PMS medium (non-AFB1 inducing conditions) (Figure 19). After transferring the mycelia to fresh PMS and GMS media, AFB_1 was first detectable at 12 hr in GMS medium (AFB_1 inducing conditions) and increased up to 32 hr. On the contrary, no detectable AFB_1 accumulation occurred up to 48 hr after the shift to fresh PMS medium (Figure 19A). Western blot analysis (Figure 19B) showed that the Ver-1 protein could be clearly detected at 12 hr and the quantity peaked at approximately 36 hr after the shift to GMS. The Ver-1 protein was not detected in mycelia shifted to fresh PMS.

Figure 18. Batch fermentation analysis of Ver-1 protein accumulation in *A. parasiticus* SU-1. (A) Mycelial growth (dry weight) and aflatoxin production (measured by ELISA) after inoculation of conidiospores in YES media. (B) Western blot analysis of proteins extracted from mycelia from 24 to 120 h after inoculation in YES media using similar methods as in Figure 17. The molecular mass of marker proteins is indicated on the left of the blot.

Figure 18.

A.


Mycelial Dry Weight
Aflatoxin Concentration
Mycelial Dry Weight (Log mg)
Aflatoxin Concentration (ug/ml medium)
Time (hr)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
160
140
120
100
80
60
40
20
0
0
24
48
72
96

B.

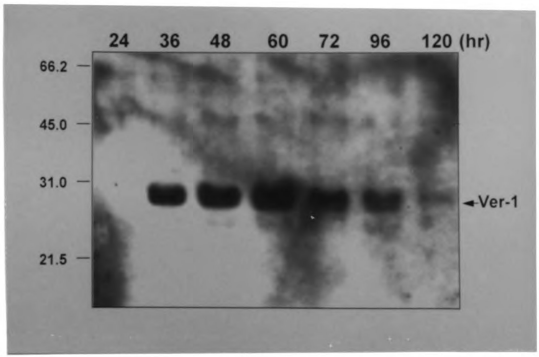
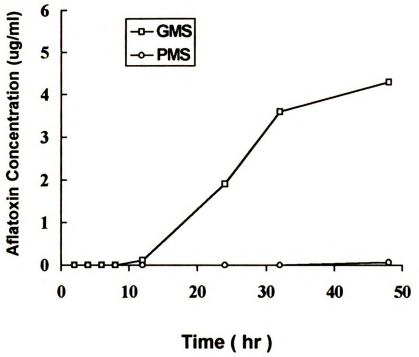

24
36
48
60
72
96
120 (hr)
66.2
45.0
31.0
21.5
Ver-1

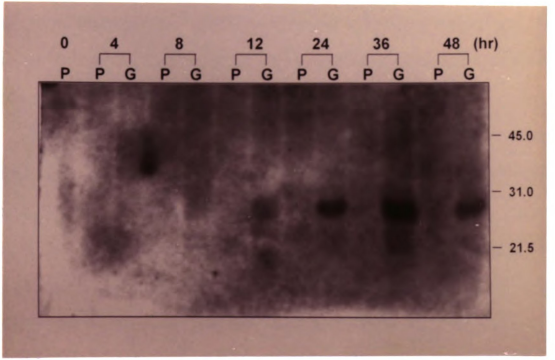
Figure 19. Nutritional shift assay for the identification of Ver-1 protein accumulation in *A. parasiticus* SU-1. (A) Aflatoxin accumulation in GMS and PMS media after nutritional shift (measured by ELISA). (B) Western blot analysis of proteins extracted from mycelia grown in GMS (G) and PMS (P) before (P at 0 h) and after nutritional shift. The molecular mass of marker proteins is indicated on the right of the blot.

Figure 19.

A.


GMS
PMS
Aflatoxin Concentration (ug/ml)
0
1
2
3
4
5
6
0
10
20
30
40
50
Time (hr)

B.


0
4
8
12
24
36
48
(hr)
P
P G
P G
P G
P G
P G
P G
45.0
31.0
21.5

These data confirm that Ver-1 protein accumulation correlates positively with AFB_1 accumulation in the wild-type aflatoxin-producing strain.

C. Accumulation of the Ver-1 protein in a fungal colony

Three concentric zones (center, middle, and periphery; see Figure 20), which differ in their morphology, could be clearly observed in colonies grown for 72 to 144 hr on solid YES medium. The central zone bears abundant pigmented conidia. The middle zone contains more aerial hyphae with scattered conidia. The peripheral zone may be divided into two parts, an outer, thin hyphal network border and a region where the hyphal network is piled up, with the formation of aerial hyphae. The width of these three zones increased constantly as growth proceeded. Colonies from 72 to 144 hr growth were cut in concentric zones and the Ver-1 protein was determined by Western blot analysis of proteins extracted from each zone (Figure 21). The data indicated that (1) the central part of the colony contains less Ver-1 protein than the middle and peripheral parts, and (2) the Ver-1 protein concentration in each concentric zone changed at different growth stages. In other words, there are spatial and temporal expression patterns of the *ver-1* gene in a fungal colony.

A *ver-1*/GUS reporter strain, pHD6-6 transformant 1, showed the same morphology as the wild-type aflatoxin-producing strain when grown on YES media. GUS expression in pHD6-6 transformant 1 had previously been shown to be under the control of the *ver-1* promoter (Wu, 1995) and thus could be used to monitor *ver-1* gene expression. By overlaying a colony of the pHD6-6 transformant 1 with agar containing X-gluc, it was observed that the blue staining was more intense in the peripheral part and the border between the peripheral and middle parts of the colony (Figure 22). These data confirmed the observation in Western blot analysis that the central part of the colony has a lower Ver-1

Figure 20. The morphology of a fungal colony grown on solid YES media. Conidiospores of *Aspergillus parasiticus* SU-1 were inoculated onto the center of the medium and grown for 120 hr at 30°C in the dark. Three concentric zones with different morphologies were designated C, central zone; M, middle zone; P, peripheral zone.

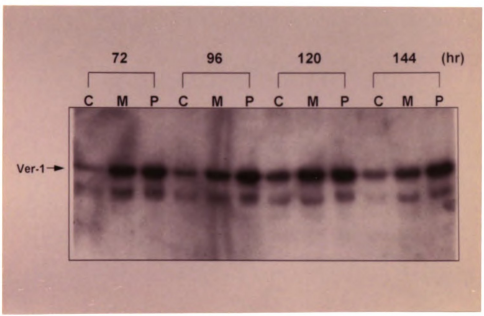


Figure 21. Western blot analysis of the proteins extracted from three concentric zones in fungal colonies. Plate cultures were grown by inoculating conidiospores of *A. parasiticus* SU-1 onto the center of Petri dishes (solid YES) and incubated for 72 to 144 hr. Central (C), middle (M), and peripheral (P) parts of the colonies were cut in concentric zones with a sterile scalpel and used for protein extraction. 50 μg of protein were resolved by gel electrophoresis (12% SDS-PAGE), transferred to PVDF membrane, and probed with anti-Ver1 antibodies.

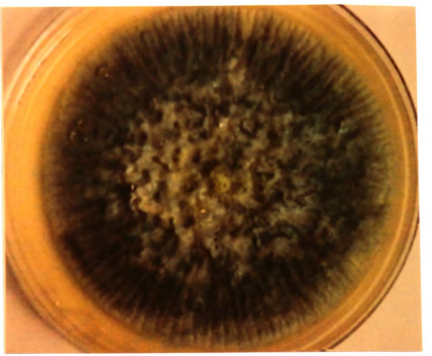


Figure 22. Analysis of *ver-1* promoter activity in a fungal colony using the *ver-1*/GUS reporter strain. Conidiospores of *Aspergillus parasiticus* pHD6-6 transformant 1 were inoculated onto the center of the YES medium and incubated for 120 hr at 30°C in the dark. The colony was overlaid with a 1.5 % agar solution containing 0.5 mg/ml X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) and the plates were incubated at room temperature for 4 hr. The blue staining indicates the location of GUS activity.

protein concentration.

D. Expression of the *ver-1* gene in mycelia

Using a slide culture method, the structure of fungi including vegetative mycelia, conidiophores, and conidiospores could be easily observed by bright field microscopy. For reference purposes, the structure of a typical conidiophore of *Aspergillus* is shown in Figure 23. Using pHD6-6 transformant 1 as the reporter strain, it was found that X-gluc staining was very weak in the 24 hr culture and relatively strong in the 48 and 64 hr cultures. The blue staining was well distributed in the vegetative mycelia (Figure 24A). In the conidiophore, the blue staining seemed to concentrate in the vesicle or the area near it (Figure 24D and E). It was also observed that the older hyphae had weaker blue staining than the younger hyphae. The same phenomenon was also observed when the fluorescent GUS substrate was used (Figure 24F and G).

IV. DISCUSSION

We have successfully generated polyclonal antibodies to detect the native Ver-1 protein in *A. parasiticus*. The results suggested that the antibodies generated against proteins expressed in *E. coli* possessed a high degree of specificity in detecting native Ver-1 proteins in *A. parasiticus*. The model we developed here for raising polyclonal antibodies to detect the aflatoxin proteins is very useful especially when the enzymatic activity of a protein involved in aflatoxin biosynthesis cannot be identified and hence cannot be purified. The protein encoded by the *ver-1* gene provides a good example. The enzymatic activity of the Ver-1 protein has not been identified although the *ver-1* gene has been confirmed to be directly involved in aflatoxin biosynthesis.

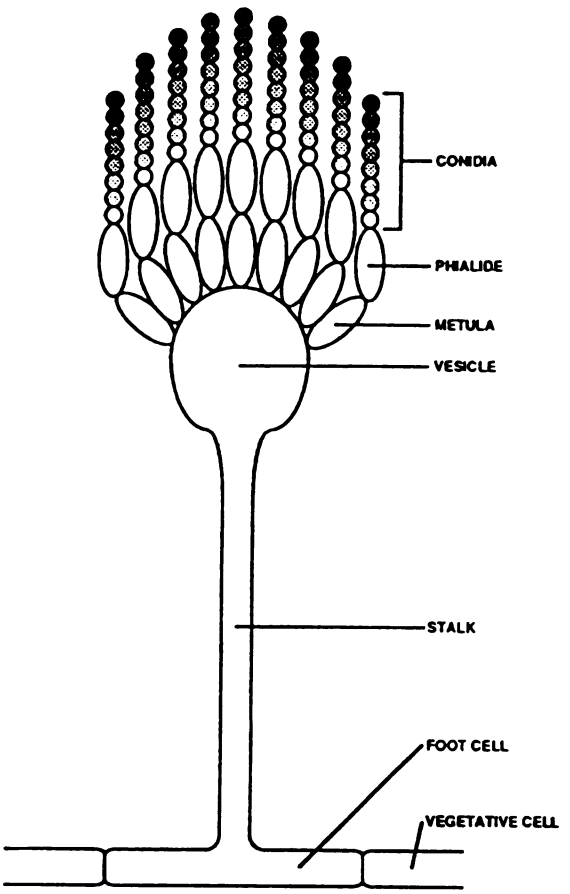
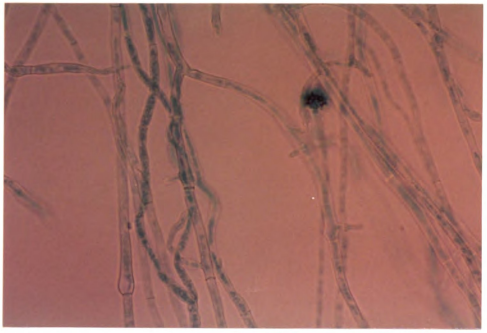


Figure 23. The structure of a typical conidiophore of *Aspergillus*. (Timberlake, 1993)

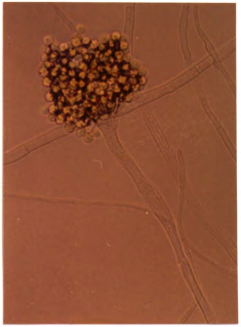
Figure 24. Microscopic images from the analysis of *ver-1* promoter activity in mycelia using the *ver-1*/GUS reporter strain. The fungi were grown on a cover glass for 48 or 65 hr using the slide culture method. The mycelia were stained by immersing the cover glass in liquid YES containing 0.5 mg/ml X-gluc (picture A - E) or C_{12}FDGlcU (picture F - H) and incubated for 4 hr. The preparation was then examined by a Nikon Labophot microscope for distribution of the blue color (bright field) or green fluorescence (UV). (A) *A. parasiticus* pHD6-6 transformant 1 incubated on YES media for 48hr. *x400*. (B) *A. parasiticus* SU-1 incubated on YES media for 48 hr (negative control). *x400*. (C) *A. parasiticus* pHD6-6 transformant 1 incubated on YES media for 48 hr (without X-gluc staining). *x400*. (D) and (F) A conidiophore (without conidial head) of *A. parasiticus* pHD6-6 transformant 1 incubated on YES media for 48 hr. *x400*. (E) and (G) A conidiophore with conidial head of *A. parasiticus* pHD6-6 transformant 1 incubated on YES media for 65 hr. *x400*. (H) *A. parasiticus* SU-1 incubated on YES media for 65 hr (negative control). *x100*.

Figure 24.

A.



B.



C.

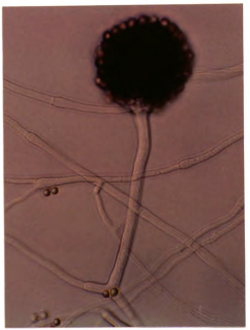
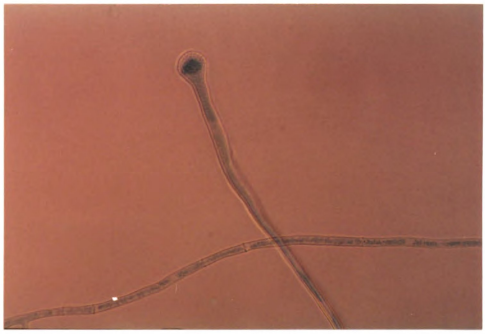


Figure 24. (cont'd).

D.



E.

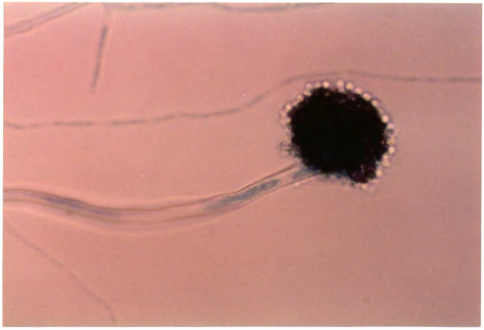
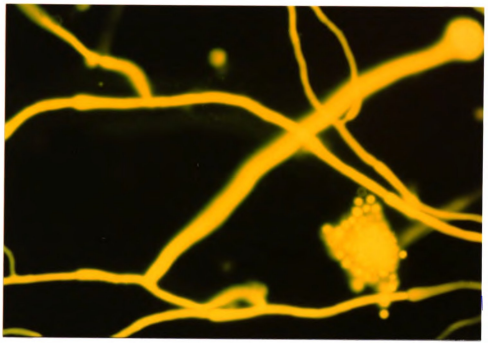
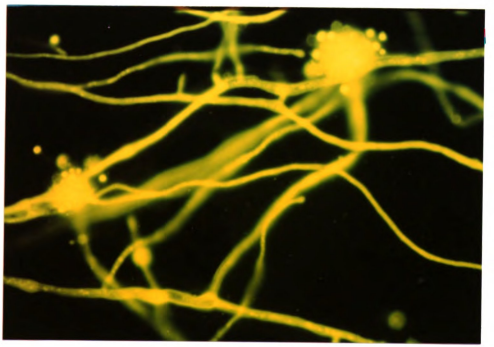


Figure 24. (cont'd).

F.



G.



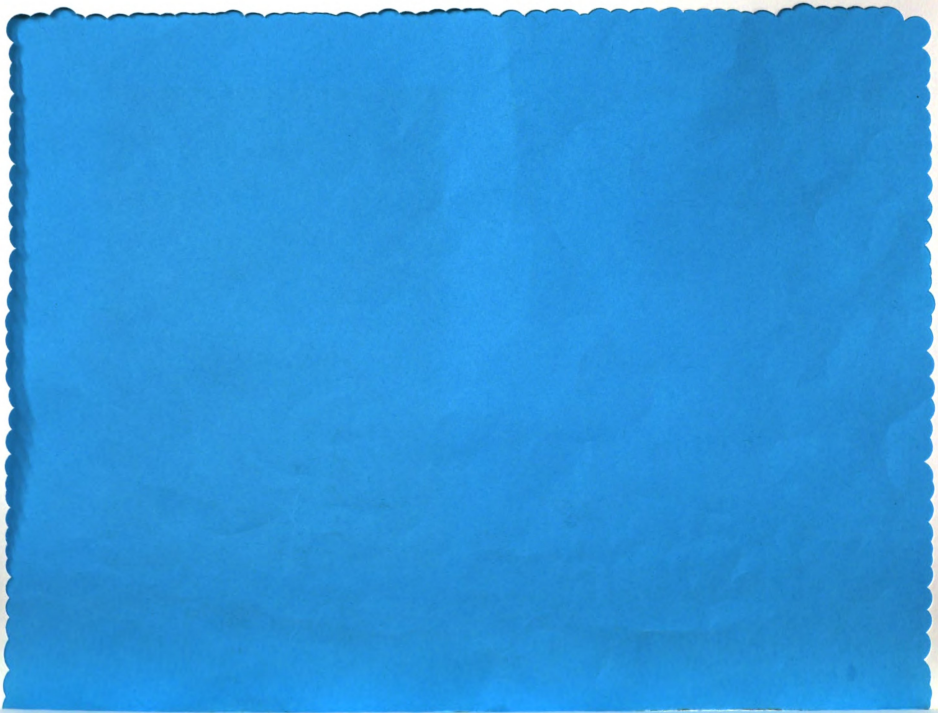
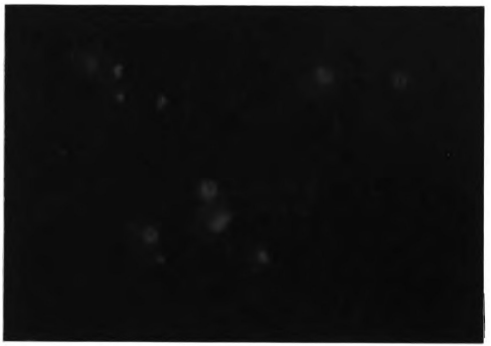


Figure 24. (cont'd)

H.



It was not possible to investigate Ver-1 protein accumulation in *A. parasiticus* until specific antibodies against the Ver-1 protein were generated in this study. The batch fermentation analysis demonstrated that the accumulation of the Ver-1 protein is consistent with a pattern predicted for a protein involved in secondary metabolism. These data indicated that the *ver-1* gene is expressed near the end of trophophase (approximately 36 hr after inoculation in YES medium) when aflatoxins begin to accumulate. The positive correlation between *ver-1* gene expression and aflatoxin accumulation was further confirmed in the nutritional shift assay in which Ver-1 protein accumulated to significant levels only in the medium (GMS) which supported aflatoxin biosynthesis. These data were consistent with our previous observation that the timing of *ver-1* RNA transcript accumulation corresponded to the timing of aflatoxin accumulation (Skory *et al.*, 1993).

To date, studies on the regulation of secondary metabolism are often based on submerged, agitated, and batch liquid cultures which clearly separate the trophophase and idiophase of fungal growth (Bu'Lock *et al.*, 1965). This model system, however, could potentially perturb the normal metabolic and physiological development of fungi and mask functionally significant information (Campbell *et al.*, 1981). In nature, the aspergilli grow on solid substrates and growth is far from homogeneous. In search of functional information on regulation of secondary metabolism, solid culture is a closer approximation to the natural growth conditions of fungi. In this study, Ver-1 protein accumulation was measured in a fungal colony grown on solid media. The morphology of the fungal colony we described here is very similar to that reported by Yanagita and Kogane (1962). In their study, the colonial structures of *Aspergillus niger* and *Penicillum urticae* were divided into four zones. These zones from outside to center are the extending zone, the productive zone, the fruiting zone,

and the aged zone. The extending zone is represented by the outermost periphery consisting of a thin hyphal network extending centrifugally. The productive zone is a piled-up hyphal mat with aerial hyphae. In the fruiting zone, fruiting bodies (conidiophore, conidia-bearing structure, and conidia) are being formed. The aged zone, the central part of the colony, consists of abundant conidia. Yanagita and Kogane also measured the basophilic substances such as RNA and DNA in each zone, and found that the productive zone contains more basophilic substances than any other part of the colonial tissue.

Using Western blot (protein accumulation) and GUS activity analysis (*ver-1* promoter function) in this study, it was determined that the quantity of Ver-1 protein and *ver-1* promoter activity are higher in the peripheral zone of the colony (Figure 21 and Figure 22). The morphology of the peripheral zone with the most intense blue staining (Figure 22) is identical to the productive zone described by Yanagita and Kogane. This means that the *ver-1* gene expression is more intense in the part of colony containing more basophilic substances. This spatial pattern of *ver-1* gene expression is likely based on the physiological differentiation in a mass of fungal cells developing on a solid medium.

Fungal cell differentiation can be observed by growing the fungi on solid substrates. When aspergilli grow on solid substrates, there are vegetative mycelia that grow in contact with the substrate. From vegetative mycelia, the aerial mycelia, a population of developing conidiophores, develop and eventually bear conidial heads (Bartman *et al.*, 1981). A slide culture method was applied in this study in an attempt to correlate aflatoxin biosynthesis and cell differentiation. Using the *ver-1*/GUS reporter strain, the activity of the *ver-1* promoter, which is directly involved in aflatoxin biosynthesis, was monitored indirectly by a GUS activity assay. The GUS activity (blue staining or green fluorescence) was distributed in both

vegetative and conidiophore structures. Since the blue stain has been reported to be insoluble and precipitates at the site of GUS activity (Jefferson, 1988), the distribution of GUS activity is not likely the result of migration of the blue dye between cells. These data suggested that aflatoxin gene expression and therefore aflatoxin biosynthesis can occur in both cell structures. However, it remains possible that the GUS protein could be transported from cell to cell.

To look more closely at this question, an alternative approach was utilized. The aerial mycelia were separated from vegetative mycelia by scraping the top layer of a fungal colony (pHD6-6 transformant 1) with a metal spatula after freezing the whole colony in liquid nitrogen. Microscopic examination of a scraped preparation indicated that the bulk of conidiophores and conidia had been removed. The remaining cell preparation (after scraping), however, still contained a significant number of conidiophores on them. Despite this lack of cleanliness, the scrape experiment is still capable of helping determine the distribution of aflatoxin gene expression. The proteins were extracted from scraped and residual cells and used for Western blot analysis for the Ver-1 protein and the GUS activity assay. The results indicated that accumulation of Ver-1 protein and *ver-1* promoter activity occurred at approximately equal levels in scraped and residual cells. These data confirm that the aflatoxin gene expression occurs throughout the fungal colony. The above data, however, still do not clearly establish the relationship between aflatoxin biosynthesis and cell differentiation because the experimental design does not allow us to record the timing of the onset of aflatoxin biosynthesis and cell development.

The role of secondary metabolism in the producing fungus remains unclear despite decades of study. One proposal is that secondary metabolism may be involved in cell

differentiation (Campbell, 1983). By experimental design, any attempt to relate cell differentiation to fungal secondary metabolism should show that the initiation of production of secondary metabolites is accompanied by the development of one or more specific fungal cell phenotypes. By detailed microscopic observation of cell growth and the measurement of the production of specific secondary metabolites, it has been reported that synthesis of some secondary metabolites is correlated to cell differentiation. Peace *et al.* (1981) have shown that **6-methylsalicylic acid** (6-MSA) is produced by *P. patulum* only after aerial mycelia have begun to form. It was not clear whether 6-MSA biosynthesis occurs in vegetative or aerial hyphae. Bartman *et al.* (1981) demonstrated that *P. brevicompactum* produces **mycophenolic acid** only after aerial mycelia have begun to form. The biosynthesis likely takes place in vegetative hyphae and the bulk of the metabolite is excreted into the growth medium. Bird *et al.* (1981) reported that **brevianamides** A and B are produced by *P. brevicompactum* after conidiation had begun. These metabolites are located in the upper part of conidiophores and conidial heads. An **ergosterol** formed by *P. brevicompactum* was also shown to be produced after aerial mycelia began to form and the metabolite was distributed in both vegetative and aerial hyphae (Bird and Campbell, 1982).

The involvement of aflatoxin biosynthesis in cell differentiation has been hypothesized based on the observation that aflatoxin biosynthesis is associated with the sclerotia formation (Trail *et al.*, 1995). To address this hypothesis, future studies will analyze aflatoxin gene expression together with the cell development during the growth of *A. parasiticus* on solid media.

CHAPTER 4

INTRACELLULAR LOCALIZATION OF THE VER-1 PROTEIN IN FUNGAL CELLS

I. INTRODUCTION

While a lot of effort has been focused on genetic studies, little information has been reported with regard to understanding AFB_1 biosynthesis at the cellular level. Specifically, understanding the process of localization of proteins involved in AFB_1 biosynthesis may generate sufficient information to allow the design of specific and efficient ways to block aflatoxin production in fungi. For those enzymes that have been identified to be involved in aflatoxin biosynthesis, two activities, averantin monooxygenase (AVN→5'-hydroxyaverantin; Yabe *et al.*, 1993) and *O*-methylsterigmatocystin oxidoreductase (OMST→AFB_1; Bhatnagar *et al.*, 1989; Yabe *et al.*, 1989), are localized to the microsomal fraction. Several other identified activities (Bhatnagar *et al.*, 1989; Matsushima *et al.*, 1994; Yabe and Hamaski, 1993), on the other hand, are found to be cytosolic or loosely bound to membranes. These data suggest that the biosynthesis of aflatoxins is carried out by a combination of membrane-associated and cytoplasmic enzyme activities. This could suggest that aflatoxins are synthesized at specific sites within the fungal cell because at least some of the enzymes may localize to closely related sites to allow aflatoxin synthesis to be an efficient process. To test this hypothesis, in this study we localized the Ver-1 protein by the protocols of subcellular

fractionation and immunolocalization.

The subcellular localization of the Ver-1 protein was determined by differential centrifugation of crude cell extracts. The proteins in each fraction were analyzed by Western blot analysis for the concentration of the Ver-1 protein. Analysis of marker enzymes for certain cell organelles was also employed to determine their distribution within the cell fractions.

Indirect immunofluorescence (IIF) microscopy was performed to localize the Ver-1 protein in hyphae and in individual cells of the fungus. A slide culture method was used to grow the fungi for IIF microscopy. The location of the Ver-1 protein was observed by the combination of anti-Ver1 antibodies and the goat-antirabbit polyclonal antibodies (secondary antibody) labeled with fluorescein isothiocyanate (FITC) using a microscope equipped with epifluorescent filters. Laser Scanning Confocal Microscopy (LSM) was also applied to increase resolution and create a three-dimensional image.

II. MATERIALS AND METHODS

A. Differential centrifugation of fungal cell extracts

The preparation of cell lysates for subcellular fractionation was performed by two different methods.

1. Grinding in liquid nitrogen

Conidia ($2x10^6$) of *A. parasiticus* SU-1 were inoculated into 100 ml of YES medium and grown in the dark for 60 hr at 30°C with shaking (150 rpm). The mycelia were filtered through Miracloth and frozen in liquid nitrogen. The frozen mycelia were ground using a mortar and pestle, suspended in TSA (2 mM Tris•Cl/40 mM NaCl/0.025% NaN_3; pH 8.0)

buffer containing the proteinase inhibitors phenylmethylsulfonyl fluoride (PMSF; 0.1 mM) and aprotinin (50 μg/ml). The cell debris was removed by three successive centrifugation steps at 1,000g for 10 min at 4°C. The resulting supernatant was fractionated into a pellet and a supernatant by centrifugation at 6,000g for 20 min. The same procedure was repeated by centrifugation of the subsequent supernatant at 20,000g for 30 min; 50,000g for 30 min; 100,000g for 60 min; and 140,000g for 60 min, all at 4°C. Each pellet was washed three times and resuspended in TSA buffer. The protein concentration of the cell fractions was determined with the Bio-Rad Protein Assay kit. 50-100 μg of protein from each pellet fraction and the final supernatant were separated by electrophoresis (12 % SDS-PAGE), transferred to PVDF membrane, and probed with anti-Ver1 antibodies.

2. Disruption of protoplasts by a homogenizer

The growth of *A. parasiticus* SU-1 and the harvest of the mycelia was performed as described above. The mycelia were digested with 1% Novozyme 234 in digestion buffer (0.6 M KCl, 0.1 M citric acid, pH 6.0) for 4 hr at 30°C. The protoplasts were collected by passage through a 29 μm nylon mesh, and washed three times in 0.1 M MES buffer [2(N-morpholino)ethane sulphonic acid, pH 7.5] containing 0.6 M KCl. The resulting protoplasts were resuspended in 0.1 M MES buffer containing 1.0 mM PMSF, 50 μg/ml aprotinin, 5 mM DTT, and 0.6 M KCl. The cells were disrupted by a Potter homogenizer (Auther H. Thomas Co.), and the resulting cell lysates were then treated as described above for differential centrifugation and Western blot analysis for the Ver-1 protein.

B. Calculation of the quantity of the Ver-1 protein in cell fractions

Western blot analysis of proteins of each cell fraction from differential centrifugation was used to determine the possible association of the Ver-1 protein with organelles or the

cytoplasm. The relative but not exact quantity of the Ver-1 protein could be determined by this method. Two sets (duplicate) of samples were loaded onto the same SDS-PAGE gel. After electrophoresis and transfer of the protein to PVDF membrane, one set of samples was stained with Coomassie blue to visualize the total protein. The other set of samples was used for Western blot analysis with anti-Ver1 antibodies. The Coomassie blue stained membrane and the Western blot were scanned to record the images using a scanner (Epson ES-1000C), and the intensity of the protein "bands" was analyzed by SigmaGel softwafe. The proportion of Ver-1 protein in each cell fraction was calculated by the following three steps.

(1) **Specific quantity (SQ)** of the Ver-1 protein (in each cell fraction)

= intensity of the Ver-1 protein (on Western blots) / intensity of the total protein loaded for analysis

(2) **Total quantity (TQ)** of the Ver-1 protein (in each cell fraction)

= (SQ) x total protein in each cell fraction

(3) **Proportion of the Ver-1 protein** in each cell fraction

= (TQ) / the sum of (TQ) of all cell fractions

C. Analysis of marker enzyme activities

Four marker enzymes were analyzed to determine the distribution of different organelles in cell fractions including succinate dehydrogenase, catalase, acid phosphatase, and glucose-6-phosphatase.

1. Succinate dehydrogenase (marker for mitochondria)

A spectrophotometric measurement based on reduction of ferricyanide was used according to Singer *et al.* (1965) with minor modifications. 0.6 ml Tris buffer (0.3 M, pH 7.6) was combined with 0.1 ml of 0.1 M potassium ferricyanide and 0.4 ml of 0.01 M

succinate. The final volume was adjusted to 3 ml by adding water. The optical density of this mixture was measured at 450 nm before adding the enzyme solution. 1~5 µl (20 to 30 µg protein) of cell fraction preparation was then added to the reaction solution. The reaction was incubated 30 to 60 min until the optical density no longer decreased and the final reading was recorded. A control reaction was prepared by including 0.05 ml of 1.0 M malonate to completely inhibit the succinate dehydrogenase. 2 µmoles of ferricyanide are reduced for each µmole of succinate oxidized. The decrease in succinate was calculated using 2.06 as the molar extinction coefficient of ferricyanide. 1 unit of succinate dehydrogenase was defined as the amount of enzyme causing a decrease of 1 µmole succinate in 1 min.

2. Catalase (marker for microbodies)

The catalase activity was determined as described by Cohen *et al.* (1970). The reaction mixture contained excess of H_2O_2 (1 ml of 2 % H_2O_2 solution) and 1~5 µl (20 to 30 µg protein) of cell fraction preparation. After incubation of the enzyme reaction for 10 to 30 min, the residual H_2O_2 was measured by reacting it with KMnQ and then measuring the residual $KMnO_4$ spectrophotometrically at 480 nm. One unit of catalase was defined as the amount of enzyme required to liberate half the peroxide oxygen from a hydrogen peroxide solution of any concentration in 100 sec (Lück, 1965).

3. Acid phosphatase (marker for lysosomes)

The activity of acid phosphatase was measured using an assay kit (Sigma) based on the method described by Andersch and Szczypinski (1947). The assay and determination of units of enzyme activity were performed according to manufacturer's instructions. The Sigma procedure depends on the hydrolysis of *p*-nitrophenyl phosphate by the acid phosphatase, yielding *p*-nitrophenol and inorganic phosphate. Under alkaline conditions, *p*-nitrophenol is

converted to a yellow complex readily measured at 400-420 nm.

4. Glucose-6-phosphatase (marker for endoplasmic reticulum)

Glucose-6-phosphatase activity was determined by measuring the quantity of inorganic phosphate formed after the reaction (Harper, 1965). The reaction mixture consisted of 1~5 µl (20 to 30 µg protein) of cell fraction preparation, 0.1 ml citrate buffer (0.1 M, pH 6.5), and 0.1 ml of 0.1M glucose-6-phosphate. A control was prepared by addition of the same amount of sample to 0.2 M citrate buffer without the substrate. The mixture was incubated at 37°C for 30 min, and 1 ml trichloroacetic acid (10 %) was added to stop the enzyme reaction. After centrifugation, 1 ml supernatant was mixed with 5 ml molybdate solution ($2x10^{-3}$ M) and 1 ml reducing agent ($4x10^{-2}$ M 1-amino-2-naphthol-4-sulphonic acid). A standard was prepared by replacing the sample supernatant with 1 ml of $5x10^{-4}$ M phosphate standard solution. The mixture was stored at room temperature for 15 to 30 min and the optical density was read at 660 nm. One unit of glucose-6-phosphatase was defined as the quantity of enzyme required to liberate 1 µmole phosphate in 1 min.

D. Indirect immunofluorescence microscopy

A. parasiticus SU-1 or VAD-102 were grown on cover slips as described in Chapter 3. The slide cultures were incubated in the dark at 30°C for 24 to 65 hr. The fungal cells were fixed for 4 hr at room temperature or 16 hr at 4°C in Histochoice Tissue Fixative (Ameresco; Solon, OH). For partial digestion of the cell wall, the fungal cells were subjected to a cell wall digestive enzyme solution containing 1 % Novozyme 234 in water for 1 hr at 30°C. The cell membrane of the hyphae was then permeabilized with 0.1% saponin in Tris-buffered saline (TBS, pH 7.3) for 30 min at room temperature. The hyphae were then exposed to anti-Ver-1 antibodies (10 µg/ml) in TBS containing 0.1 % saponin, and 1 %

bovine serum albumin (BSA) for 1 hr. This incubation was followed by thorough rinses with TBS (3 times, 10 min each). The slide preparation was then incubated with labeled secondary antibodies (100 fold dilution of FITC-conjugated anti-rabbit IgG; Sigma) for 1 hr. For negative control experiments, the hyphae were labeled only with the secondary antibodies. The hyphae were rinsed with TBS to remove the unbound secondary antibodies. After the final wash with water, the preparation was air dried, mounted on slides (using Sigma immunofluorescent mounting medium), and examined under a Nikon Labophot fluorescent microscope or a laser scanning confocal microscope (Zeiss 10) for FITC fluorescence.

III. RESULTS

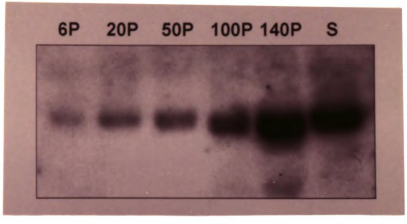
A. Subcellular localization of the Ver-1 protein in *A. parasiticus*

Western blot analysis (Figure 25) showed that the Ver-1 protein was detected in all pellets as well as supernatant fractions regardless of which method was used to prepare the cell lysates. However, the distribution of Ver-1 protein in cell fractions was different between these two methods (Table 1). When mycelia were ground in liquid nitrogen, almost all the Ver-1 protein (96.9 %) was in the final supernatant fraction. If the cell lysate was prepared by disruption of protoplasts, the proportion of Ver-1 protein in the supernatant fraction was decreased to 80.95 %, and a significant increase of Ver-1 protein was found in the 20,000g pellet (Table 1). The analysis of marker enzyme activity (Table 2) indicated that a very high percentage of organelle enzymes (e.g., 98 % of catalase activity and 98.24 % of acid phosphatase activity) were distributed to the final supernatant fraction when the cell lysate was prepared by grinding the mycelia in liquid nitrogen. The distribution of one marker enzyme (acid phosphate) in the final supernatant was decreased from 98.24 % to 69.78 %,

Figure 25. Western blot analysis of cell fractions from *A. parasiticus* SU-1. The cell lysates were prepared by grinding the mycelia in liquid nitrogen (panel A) or disruption of protoplasts by a homogenizer (panel B). Cell pellets (P) and the final supernatant fraction (S) were obtained by differential centrifugation of cell lysates at 6,000g (6P), 20,000g (20P), 50,000g (50P), 100,000g (100P), and 140,000g (140P). 50 μg of protein from each fraction were resolved by gel electrophoresis (12% SDS-PAGE), transferred to PVDF membrane, and probed with anti-Ver-1 antibodies.

Figure 25.

A.



B.

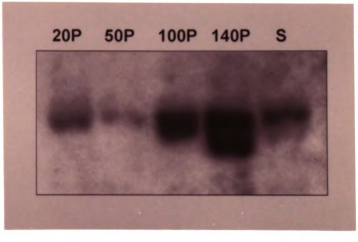


Table 1. Distribution of the Ver-1 protein in cell fractions obtained from *A. parasiticus* SU-1 by differential centrifugation

	6k x g pellet	20k x g pellet	50k x g pellet	100k x g pellet	140k x g pellet	140k x g supernatant
Mycelia ground in liquid nitrogen						
protein quantity in fractions (mg)	1.61	7.98	6.00	5.56	0.62	248.00
proportion of protein in fractions (%)	0.56	2.96	2.22	2.06	0.23	91.92
relative specific quantity of Ver-1 protein	36.15	20.96	25.42	61.68	99.77	100.00
proportion of Ver-1 protein in fractions (%)	0.23	0.65	0.59	1.34	0.24	96.90
Protoplasts disrupted by homogenizer						
protein quantity in fractions (ug)		2240	242	152	53	7500
proportion of protein in fractions (%)		22.00	2.38	1.49	0.52	73.62
relative specific quantity of Ver-1 protein		18.31	5.21	47.21	100	31.03
proportion of Ver-1 protein in fractions (%)		14.26	0.41	2.50	1.84	80.95

Table 2. Distribution of marker enzymes in cell fractions obtained from *A. parasiticus* SU-1 by differential centrifugation

Enzyme	Marker for		6k x g pellet	20k x g pellet	50k x g pellet	100k x g pellet	140k x g pellet	140k x g supernatant
Mycelia ground in liquid nitrogen								
Succinate dehydrogenase	mitochondria	activity (U)	0.25	0.56	0.16	0.07	n.d.	n.d.
		activity/total activity (%)	24.03	53.85	15.38	6.73	--	--
Catalase	microbodies	activity (U)	3.50	2.10	2.40	6.70	2.88	1116.00
		activity/total activity (%)	0.31	0.18	0.21	0.59	0.25	98.00
Acid phosphatase	lysosomes	activity (U)	217	390	818	604	204	124,930
		activity/total activity (%)	0.17	0.31	0.64	0.47	0.16	98.24
Glucose-6-phosphatase	endoplasmic reticulum	activity (U)	1.26	4.06	3.60	4.35	0.68	80.60
		activity/total activity (%)	1.33	4.29	3.81	4.60	1.72	85.00
Protoplasts disrupted by homogenizer								
Acid phosphatase	lysosomes	activity (U)	--	5.07	2.21	0.25	0.05	17.50
		activity/total activity (%)	--	20.21	8.81	1.00	0.20	69.78

n.d. : no detectable enzyme activity

-- : no data were analyzed

accompanied by a significant increase of enzyme distribution in the 20,000g and 50,000g pellets when disruption of protoplasts by a homogenizer was employed to prepare the cell lysate (Table 2). These data suggested that grinding the mycelia in liquid nitrogen significantly damaged cell organelles and resulted in a higher level of distribution of marker enzymes in the final supernatant fraction. Disruption of protoplasts using a homogenizer was a better way to preserve intact cell organelles. Using this method the distribution of marker enzymes and Ver-1 protein increased in cell pellets fractions indicating that the Ver-1 protein may locate in an "organelle-like" structure. This "structure" was distributed mainly in the 20,000g (or less then 20,000g) pellet fraction.

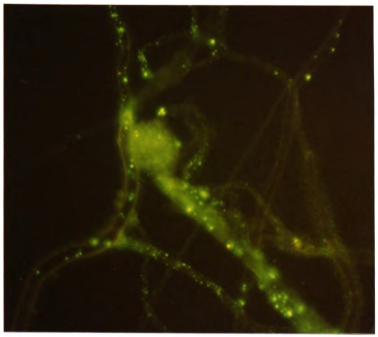
B. Intracellular localization of the Ver-1 protein in *A. parasiticus*

Immunofluorescence microscopy using the anti-Ver-1 antibodies demonstrated that the Ver-1 protein was tightly associated with particle-like fluorescent signals that were distributed to the hyphae and conidiophores of an aflatoxin-producing strain (Figure 26A and B). In contrast, a control strain, VAD-102 in which no Ver-1 protein was synthesized, showed no or very little fluorescent signal (Figure 26C). The negative control, when only secondary antibodies were utilized on aflatoxin-producing strain (SU-1), showed no fluorescent signal. Under low magnification (*x100*), it was observed that the overall fluorescent signal was stronger in conidiophores then in vegetative hyphae (Figure 26D and E). The fluorescent particles were then observed at higher magnification in 3 dimensions by confocal microscope (Figure 26F), and the particles were determined to be inside the cell wall (Figure 26G).

Figure 26. Localization of the Ver-1 protein in fungal cells by immunofluorescence microscopy and laser confocal microscopy. *A. parasiticus* SU-1 and VAD-102 were grown by the slide culture method for 65 hr on YES media. The cells were fixed, partially digested by Novozyme 234, and incubated with anti-Ver1 antibodies. The bound antibodies were then probed with the FITC-conjugated secondary antibodies. Preparations were examined in a Nikon Labophot microscope equipped with epifluorescence filters for FITC fluorescence (picture A ~ E) or a Zeiss 10 laser confocal microscope (picture F and G). The fluorescent particles were observed in the hyphae and conidiophores of *A. parasiticus* SU-1 (picture A, B; *x400*) but very little in that of VAD-102 (picture C; *x400*). A stronger fluorescent intensity was shown in conidiophores of *A. parasitius* SU-1 under lower magnification (picture D; *x100*). Picture E was a brightfield image of picture D. The fluorescent signals were also observed by the laser confocal microscope (picture F; *x4000*). A fluorescent particle was shown by confocal microscope to be inside the cell (picture G, top half).

Figure 26.

A.



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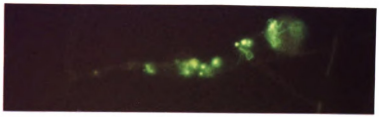


C.

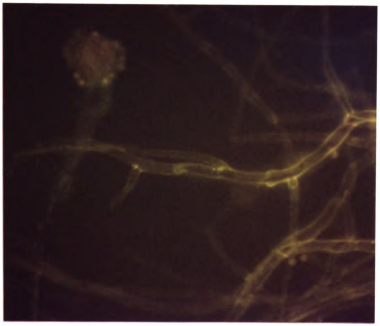
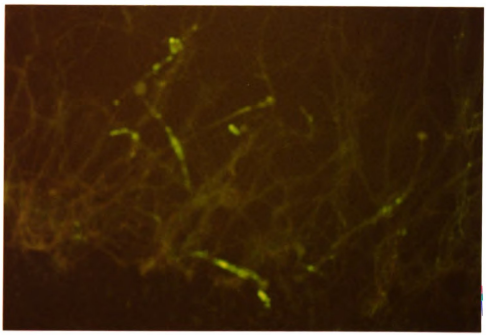


Figure 26. (cont'd)

D.



E.

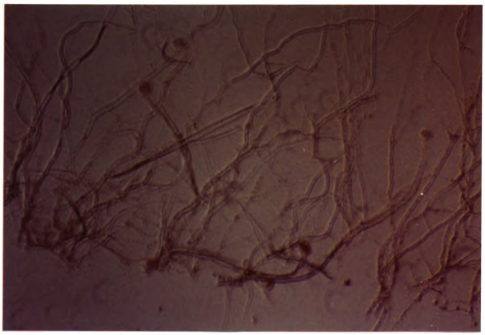
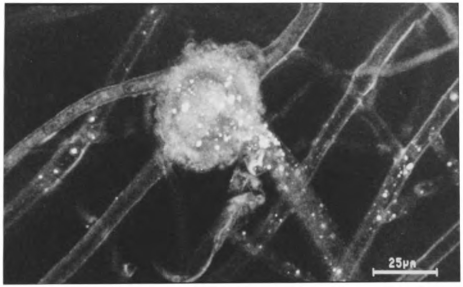
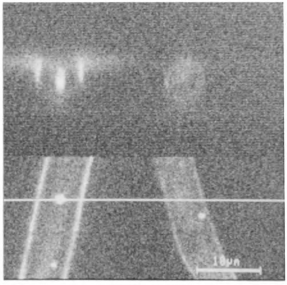


Figure 26. (cont'd)

F.



G.



IV. DISCUSSION

The location of one aflatoxin biosynthetic pathway protein, the Ver-1 protein, in the fungal cell was investigated in this study. Subcellular localization was initially conducted by differential centrifugation. Although Western blot analysis of cell fractions generated by grinding cells under liquid nitrogen showed that the Ver-1 protein was located in all pellet fractions, a high percentage of the Ver-1 protein was contained in the final supernatant fraction. It was thus not clear whether the Ver-1 protein is located in the pellets or if it is just carried-over contamination from the supernatant. The fact that a high percentage of marker enzyme activities were also distributed to the supernatant fraction indicated that cell organelles were damaged using the grinding procedure. Tolbert (1974) has reported that most organelles are damaged by the grinding procedure, which therefore must be as brief as possible. When the cell lysates were prepared by disruption of protoplasts by a homogenizer, it was found that the distribution of marker enzymes and the Ver-1 protein in the supernatant was significantly decreased suggesting that this procedure is superior to the grinding method for preparing samples for subcellular fractionation. The increase in the proportion of Ver-1 protein in pellet fractions, especially the 20,000g pellet, suggested that the Ver-1 protein was located in an organelle or membrane-bound structure and it was not due to contamination from the proteins in the supernatant. The detection of the particle structures in the fungal cell by immunofluorescent microscopy supported the observation resulting from the subcellular fractionation. These Ver-1 protein containing structures seemed to be more densely clustered in the conidiophore and conidial head indicating a potential association of aflatoxin biosynthesis with conidiophore development.

Differential centrifugation separates organelles on the basis of mass and shape. The

data here suggest that a relatively large organelle (in the pellet of 20,000g or less) may contain the Ver-1 protein inside, in the membrane, or tightly associated with the outside surface. In general, the distribution of organelles from low to high gravitational forces are nuclei (500 - 2,000g), mitochondria and microbodies (5,000 - 15,000g), lysosomes (20,000 - 50,000g), microsomes (90,000 - 120,000g), and ribosomes (140,000g) (Griffin, 1981). However, the shape of the organelles may vary from species to species (Markham, 1994). Even within a single species, the developmental stage and nature of the carbon and energy source can affect the number and the shape of organelles. Organelles such as microbodies and lysosomes bounded by a single bilayer membrane are more vulnerable to the breakage procedure of subcellular fractionation. The involvement of cell organelles in penicillin biosynthesis was analyzed (Müller *et al.*, 1991 and 1992) using immunological detection methods in combination with subcellular fractionation and electron microscopy. The results suggested that the three penicillin biosynthetic enzymes are located in different subcellular compartments. The first step enzyme, δ-(L-α-aminoadipyl)-cysteinyl-D-valine synthetase (ACVS) was found to be associated with membranes or small organelles. The next enzyme, isopenicillin *N*-synthetase (IPNS), appeared to be a cytosolic enzyme. The enzyme involved in the final step of penicillin biosynthesis, acyltransferase (AT), was located in microbodies. This model implicates several transport steps for β-lactam intermediates and products. However, it is still not clear why a specific enzymatic reaction is confined in an organelle. The speculation is that the organelle may provide a specialized environment for the reaction, for example, by providing a condition suitable for the existence of the substrate or the reaction product, by the absence of competing enzymes, or by providing a different pH. It is also possible that the organelle can protect cells from the toxicity of the products.

In eukaryotic cells, the sorting of proteins to their subcellular locations is often mediated through N-terminal signal sequences that lack conservation and are cleaved upon import (Blobel, 1980). This mode of protein segregation has been demonstrated for protein import into the nucleus (Kalderon *et al.*, 1984), mitochondria (Hase *et al.*, 1984), chloroplasts (Van den Broek *et al.*, 1985), lysosomes (Johnson *et al.*, 1987), and the endoplasmic reticulum (secretion as well as retention of proteins; Walter and Lingappa, 1986). In contrast, conserved tripeptide targeting signals (serine, alanine, or cysteine at the first position; lysine, histidine, or arginine at the second position; leucine at the third position) at the extreme C-terminus are necessary for directing proteins into microbodies (Gould *et al.*, 1989). The targeting signal, an alanine-arginine-leucine sequence, was found to be at the C-terminus of the acyltransferase involved in the penicillin biosynthesis confirming its location in microbodies (Müller *et al.*, 1992).

By analysis of the cellular distribution of enzymatic activity, it has been proposed that some enzymes involved in aflatoxin biosynthesis are membrane-bound while others appear to be cytosolic. In this study, we presented the first physical observation that an aflatoxin pathway enzyme, the Ver-1 protein, might be located in a cell organelle. The Ver-1 protein may not be located in microbodies because the protein lacks a consensus C-terminal signal sequence. However, there may be a unique system for import of proteins involved in aflatoxin biosynthesis. It is unclear if there is a N-terminal signal sequence in the Ver-1 protein. This question cannot be solved until N-terminal sequence analysis is performed on the Ver-1 protein purified from the fungus. To confirm and examine the possible structure that is associated with the Ver-1 protein, a future study using immuno-electron microscopy will be helpful. Also, using antibodies against other pathway enzymes for immuno-fluorescent

or immuno-electron microscopy will be useful in analyzing whether several membrane-associated enzymes are confined in the same organelle.

CONCLUSIONS

In this dissertation, we have accomplished the specific aims proposed. (1) We have established the structural and functional characteristics of two *ver-1* genes (*ver-1A* and *ver-1B*) involved in aflatoxin biosynthesis. It was determined that the previously cloned *ver-1* gene is *ver-1A* by restriction fragment length polymorphism (RFLP) analysis. A duplicated chromosomal region (approximately 12-kb) was identified upstream from *ver-1A* and *ver-1B* by Southern hybridization analysis. The nucleotide sequence of *ver-1B* was determined, and the predicted amino acid sequence of *ver-1B* shared 95% identity with *ver-1A*. A translational stop codon, found in the *ver-1B* gene coding region, indicated that it encodes a truncated polypeptide. To confirm the function of the *ver-1* genes in AFB_1 synthesis, a plasmid (pDV-VA) was designed to disrupt *ver-1A* and/or *ver-1B* by transformation of the AFB_1 producer *A. parasiticus* NR-1. One disruptant, VAD-102, was obtained which accumulated the pathway intermediate VA. Southern hybridization analysis of VAD-102 revealed that *ver-1A* but not *ver-1B* was disrupted. A functional *ver-1A* gene was transformed back into strain VAD-102. Transformants which received *ver-1A* produced AFB_1 confirming that *ver-1A* is the only functional *ver-1* gene. (2) An anti-Ver-1 antibody which specifically recognized the fungal Ver-1 protein was generated against the maltose-binding protein/Ver-1 fusion protein obtained by expression of the *malE/ver-1A* cDNA construct in *E. coli*. Batch fermentation and nutritional shift analyses indicated that the timing

of Ver-1 protein accumulation is positively correlated to aflatoxin accumulation. The expression of the *ver-1* gene in a fungal colony grown on solid media was shown to exhibit temporal and spatial patterns of regulation. Using the *ver-1*/GUS reporter strain, it was demonstrated that fungal vegetative hyphae, conidiophores, and conidial heads all actively transcribed the *ver-1* gene. (3) Localization of the Ver-1 protein using subcellular fractionation and immunofluorescence microscopy indicated that the Ver-1 protein may be located in a membrane-bound structure. Two lines of data suggest that AFB_1 biosynthesis may occur at higher levels during early stages of conidiophore development. First, the stronger or darker GUS staining (blue or fluorescent staining) was located to the younger conidiophore, especially in the vesicle structure, when *ver-1*/GUS reporter strains were used to examine *ver-1* promoter function. Second, a higher density of fluorescent signals in the conidiophore was observed when anti-Ver-1 antibodies were used for immuno-detection of the Ver-1 protein.

This research represents one important approach toward achieving the long term goal of identifying compounds which interfere with the expression of genes involved in AFB_1 biosynthesis. After *ver-1* gene function was confirmed to be directly involved in AFB_1 biosynthesis, the *ver-1*/GUS reporter strain can thus be utilized to monitor the effect of various natural or synthetic compounds on the expression of the *ver-1* gene both in the laboratory and the field. Using the *ver-1* gene and its product, the Ver-1 protein, as a model system, a preliminary understanding of aflatoxin biosynthesis at the cellular level has been achieved. Further exploration in this field may lead to an understanding of the possible function of aflatoxin biosynthesis in the fungus. The information presented here may eventually help in solving the worldwide aflatoxin problem.

APPENDIX

APPENDIX

PHYSICAL AND TRANSCRIPTIONAL MAP OF AN AFLATOXIN GENE CLUSTER IN *ASPERGILLUS PARASITICUS*

I. INTRODUCTION

A cooperative study in our lab was performed to analyze an aflatoxin gene cluster in *A. parasiticus* NRRL 5862. During the genetic characterization of *nor-1* and *ver-1* genes, the cosmid NorA was identified to contain both genes. This was the first finding that the aflatoxin genes may be physically linked together. A restriction enzyme map of the 35-kb genomic DNA fragment insert in cosmid NorA was generated. Based on these initial studies, a hypothesis was proposed : some other aflatoxin associated genes may also be located within this chromosomal region. To address this hypothesis, Northern hybridization analyses were performed on RNA isolated from a toxigenic strain of *A. parasiticus* using consecutive DNA fragments from the 35-kb genomic DNA fragment of cosmid NorA as the probes. The resulting transcriptional map of the genomic DNA insert in cosmid NorA revealed that 14 different RNA transcripts were localized to this region. Eight of these transcripts were proposed to be derived from aflatoxin genes because the timing of their expression (transcript accumulation) was similar to that of *nor-1* and *ver-1* genes. Two of these eight hypothesized aflatoxin genes, *pksA* (previously named gene-1; Trail *et al.*, 1995) and *fas-1A* (previously named *uvm8*; Mahanti *et al.*, 1996) have been recently shown to be directly involved in aflatoxin biosynthesis by gene disruption analysis.

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Physical and Transcriptional Map of an Aflatoxin Gene Cluster in *Aspergillus parasiticus* and Functional Disruption of a Gene Involved Early in the Aflatoxin Pathway

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Two genes involved in aflatoxin B_1 (AFB1) biosynthesis in *Aspergillus parasiticus*, *nor-1* and *ver-1*, were localized to a 35-kb region on one *A. parasiticus* chromosome and to the genomic DNA fragment carried on a single cosmid, NorA. A physical and transcriptional map of the 35-kb genomic DNA insert in cosmid NorA was prepared to help determine whether other genes located in the *nor-1–ver-1* region were involved in aflatoxin synthesis. Northern (RNA) analysis performed on RNA isolated from *A. parasiticus* SU1 grown in aflatoxin-inducing medium localized 14 RNA transcripts encoded by this region. Eight of these transcripts, previously unidentified, showed a pattern of accumulation similar to that of *nor-1* and *ver-1*, suggesting possible involvement in AFB1 synthesis. To directly test this hypothesis, gene-1, encoding one of the eight transcripts, was disrupted in *A. parasiticus* CS10, which accumulates the aflatoxin precursor versicolorin A, by insertion of plasmid pAPNVES4. Thin-layer chromatography revealed that gene-1 disruptant clones no longer accumulated versicolorin A. Southern hybridization analysis of these clones indicated that gene-1 had been disrupted by insertion of the disruption vector. These data confirmed that gene-1 is directly involved in AFB1 synthesis. The predicted amino acid sequence of two regions of gene-1 showed a high degree of identity and similarity with the β-ketoacyl-synthase and acyltransferase functional domains of polyketide synthases, consistent with a proposed role for gene-1 in polyketide backbone synthesis.

Aflatoxins are potent teratogenic, mutagenic, and carcinogenic secondary metabolites synthesized by certain strains of *Aspergillus parasiticus* and *A. flavus* (25). Under the proper environmental conditions, these ubiquitous fungi can produce aflatoxin upon infection of many agricultural crops, including peanuts, corn, cottonseed, and tree nuts (20). Because of the difficulty in effectively controlling aflatoxin contamination of food and feed by traditional agricultural practices, recent research efforts have focused on developing an understanding of the molecular biology of the aflatoxin biosynthetic pathway. This knowledge may lead to novel methods for control of this economically and agriculturally important problem.

Aflatoxins are polyketide-derived secondary metabolites. The carbon backbone of aflatoxin B_1 (AFB1) is synthesized from acetate and malonate in a process analogous to fatty acid synthesis (9, 24, 53). A generally accepted pathway for the synthesis of AFB1 has been proposed (reviewed in references 9 and 10). The first stable intermediate identified in the pathway is the decaketide norsolorinic acid (NA), an anthraquinone, which is converted to averufin (AVF) by a multistep series of reactions involving up to three alternative pathways (9, 56). AVF is then converted to versiconal hemiacetal acetate, versiconal, versicolorin B, versicolorin A (VA), demethylsterigmatocystin, sterigmatocystin (ST), *O*-methylsterigmatocystin, and finally, to AFB1. As many as 17 different enzyme activities are proposed to be involved in aflatoxin synthesis (9, 24). Several of these enzymes have been purified to homogeneity (1, 8, 11, 16, 28, 34, 40, 53).

Aflatoxin-blocked mutants (4, 32) and purified enzymes have been used to clone several genes involved in the aflatoxin biosynthetic pathway, including *nor-1* (15), encoding an activity which converts NA to averantin; *ver-1* (48), encoding an activity associated with the conversion of VA to ST; *uvm8* (36), encoding a putative fatty acid synthase involved in polyketide backbone synthesis; *omt-1*, encoding a methyltransferase which converts ST to *O*-methyl-ST (57); and *aflR* (13, 45), apparently involved in the regulation of pathway gene expression. The recombinational inactivation (gene disruption) of *nor-1* (54), *ver-1* (33), *uvm8* (36), and *pksA* (14) in *A. parasiticus* and *verA* (29) in *A. nidulans* (which synthesizes ST) firmly established the functional role of these genes in the AFB1 (or ST) biosynthetic pathway.

Parasexual analyses of eight aflatoxin-blocked mutants (including an NA-accumulating strain) in *A. flavus* suggested that all loci were genetically linked on linkage group VII (44). Attempts to demonstrate linkage of *nor-1* and *ver-1* genes in *A. parasiticus* by parasexual analyses, however, gave conflicting results (5, 12; reviewed in reference 7). The molecular genetic analysis presented in the current study clearly demonstrates the clustering (linkage) of *nor-1*, *uvm8*, and *ver-1* within a 35-kb region on one chromosome in *A. parasiticus* SU1. In addition, restriction endonuclease analysis and transcript mapping of this 35-kb region localized eight other transcripts that are expressed in a pattern similar to that of *nor-1*, *ver-1*, and *uvm8*, suggesting that the genes encoding them are also involved in aflatoxin production. To test this hypothesis, disruption of gene-1 (tentatively named because of its position at the far left end of the cluster) encoding a 7-kb transcript within the gene cluster (37) was accomplished in this study. Genetic and biochemical analyses of disruptant clones and nucleotide sequence analysis of extensive regions within gene-1 suggest that

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it encodes a polyketide synthase involved in AFB1 biosynthesis.

MATERIALS AND METHODS

Strains and culture conditions. *Escherichia coli* DH5α F' '[F'*endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyrA* (Nal') *relA1*Δ(*lacZYA-argF*)$_{U169}$ (*m80lacZ*Δ M15)] was used for propagating plasmid DNA. *A. parasiticus* NRRL 5862 (SU1), a wild-type aflatoxin-producing strain, was used for preparation of RNA for transcript mapping. *A. parasiticus* CS10 (*ver-1 wh-1 pyrG* [48]), derived from *A. parasiticus* ATCC 36537 (*ver-1 wh-1* [4]) was used as the host strain for the disruption of gene-1. Strains CS10 and ATCC 36537 are unable to convert VA to ST as a result of a *ver-1* mutation, and neither produces detectable levels of AFB1 in liquid or on solid growth media. The following strains of *A. parasiticus* were used to analyze sclerotium development: AFB1-producing strain SU1, AVF-accumulating strain ATCC 24551 (23), NA-accumulating strain ATCC 24690 (32), and VA-accumulating strain ATCC 36537.

Fungal strains were maintained as frozen spore stocks (approximately 10^8 spores per ml) in 20% glycerol at −80°C. Coconut agar medium (CAM [2]), an aflatoxin-inducing medium, was used for rapid screening of fungal strains for accumulation of AFB1 and VA by visualization of blue and yellow fluorescence, respectively, under long-wave UV light. YES broth (2% yeast extract, 20% sucrose; pH 5.5), a rich aflatoxin-inducing medium, was used to grow mycelia for DNA and RNA preparations and for thin-layer chromatography (TLC) assays. Reddy's medium, a chemically defined aflatoxin-inducing medium (47), also was used to grow mycelia for RNA preparations.

Isolation and analysis of RNA and DNA. Fungal cultures for DNA and RNA preparations were grown in YES (DNA and RNA) or Reddy's medium (RNA) at 29°C with shaking (175 rpm) and harvested at the times indicated in the figure legends (48 h for DNA). DNA was purified from *A. parasiticus* by a published modification (50) of a phenol-chloroform protocol developed for mammalian DNA (3). Total RNA was purified from aflatoxin-induced cultures of *A. parasiticus* SU1 (10^5 spores per ml), using a hot-phenol protocol previously described (39). Restriction endonucleases utilized in analysis of DNA were purchased from Boehringer Mannheim Biochemicals or New England BioLabs and were used according to the manufacturer's instructions. Northern (RNA) and Southern hybridization analyses were performed using published procedures (38), with a modified hybridization buffer and conditions recommended by Stratagene Cloning Systems, La Jolla, Calif. ^{32}P-radiolabelled DNA probes were prepared with a Random Primed DNA Labelling Kit from Boehringer Mannheim Biochemicals. After the final wash, nylon or nitrocellulose membranes were placed on X-ray film (Kodak-XAR5) at −81°C. DNA probes utilized in transcript mapping (Northern analyses) and Southern hybridization analyses were generated from DNA restriction fragments derived from cosmid NorA or cosmid subclones as shown in Fig. 1. The gene probes used as controls in transcript mapping consisted of a 4.3-kb *Eco*RI-*Sac*I DNA restriction fragment isolated from cosmid NorA containing the *pyrG* gene from *A. parasiticus* (48) and a 1.1-kb *Ace*I-*Sac*I fragment containing part of the coding region of the gene encoding β-tubulin in *A. parasiticus* (55a).

Plasmid and cosmid construction and purification. Cosmids NorA, NorB, Ver2, Ver3, and Ver4 were isolated by in situ colony hybridization (3) of a cosmid library containing *A. parasiticus* SU1 genomic DNA cloned into the cosmid vector pBZ5 (48). ^{32}P-labelled DNA restriction fragments containing the *nor-1* or *ver-1* genes (*nor-1*, 1.5-kb *Bgl*II-*Cla*I restriction fragment; *ver-1*, 0.6-kb *Avr*II-*Bam*HI restriction fragment [49]) were used as probes to screen the library.

Plasmid pAPNVES43 was constructed to disrupt gene-1 by single-crossover insertion into the homologous region of the chromosome (see Fig. 4). A 4.3-kb *Eco*RI-*Sac*I *A. parasiticus* genomic DNA restriction fragment subcloned from cosmid NorA, encompassing the entire *pyrG* coding region (part of the original pBZ5 vector) plus a 1.6-kb region within the coding region of gene-1, was subcloned into pUC19 cut with *Eco*RI and *Sac*I.

Plasmid minipreparations were performed by the boiling method (38); large-scale plasmid preparations were performed according to the alkaline lysis procedure of Maniatis et al. (38).

Restriction endonuclease analysis, transcript mapping, and physical linkage analysis of cosmid NorA. An *Eco*RI restriction endonuclease digest of cosmid NorA was prepared. The resulting fragments were subcloned into pUC19 or pBluescript SKII(−) except for a 10.6-kb *Eco*RI fragment containing the *nor-1* gene and a 4.6-kb *Eco*RI fragment immediately adjacent to the 10.6-kb fragment. The 10.6-kb *Eco*RI fragment was cut into two fragments with *Sac*I, and each resulting fragment (4.3 and 6.3 kb; clones 3 and 4, respectively) was subcloned into pBluescript SKII(−) cut with *Eco*RI and *Sac*I. The 4.6-kb *Eco*RI fragment was subcloned into pBluescriptSKII(−) as three fragments: two 1.8-kb *Eco*RI-*Hin*dIII fragments flanking one 0.6-kb *Hin*dIII fragment (see Fig. 2). From these subclones, a restriction map of the entire cosmid was prepared by mapping each subclone with *Kpn*I, *Apa*I, *Sma*I, *Sac*I, and *Xba*I (Fig. 1). The genes *nor-1*, *ver-1*, and *uvm8* were localized onto subclones by Southern hybridization analysis in conjunction with the restriction endonuclease analysis. The position of *aflR* was provided as part of a separate study (58).

Measurement of aflatoxin synthesis in *A. parasiticus* SU1. Measurements of mycelial dry weights in Reddy's and YES growth media were performed essentially as described previously (54). Direct competitive enzyme-linked immunosorbent assay analyses of AFB1 production were performed as described by Pestka (46) with AFB1 monoclonal antibodies and AFB1-horseradish peroxidase conjugate (both kindly provided by J. Pestka, Michigan State University).

Transformation of *A. parasiticus* and genetic disruption of gene-1. Transformation of protoplasts of *A. parasiticus* CS10 was conducted using minor modifications of the polyethylene glycol method (43), as previously described (50). *pyrG*$^+$ prototrophs were selected on Czapek Dox medium (Difco). pAPNVES43 (see Fig. 4) was used as the disruption vector. pPG3J, containing the *pyrG* gene only (50), served as a control plasmid to measure the rate of successful transformation. Transformant clones were transferred to CAM to screen for VA accumulation. Transformant clones were purified by single spore isolation three successive times.

Genetic and biochemical analysis of gene-1 disruptant clones. Erlenmeyer flasks (250 ml) containing 100 ml of YES broth were inoculated (10^6 spores) with gene-1 disruptant clones or ATTC 36537 (control; parental strain of transformation recipient strain CS10) and incubated without agitation at 30°C in the dark. After 72 h of growth, mycelial mats were removed and blotted dry. One-quarter (wet weight) of the mycelial mat was dried overnight at 60°C to determine dry weight. The remainder of each mycelial mat and the growth medium were extracted separately with acetone and then with chloroform as previously described (54). TLC analyses of the solvent extracts were performed on activated high-performance silica TLC plates (10 by 10 cm) in a chamber equilibrated with benzene-acetic acid (95:5). Purified VA (generously provided by Deepak Bhatnagar) was resolved on each plate as a standard. DNA was purified from mycelium grown separately in YES broth for 48 h as described above and was analyzed by Southern hybridization.

Sclerotium production. Gene-1 disruptant clones were tested for the ability to produce sclerotia. Aflatoxin-producing strain SU1, NA-accumulating strain ATCC 24690, VA-accumulating strain ATCC 36537, and AVF-accumulating strain ATCC 24551 were grown under identical conditions for comparison. Strains were center inoculated onto petri plates containing approximately 30 ml of CAM medium and incubated for 14 days in the dark at 30°C. Sclerotia were harvested and counted by a published modification (48) of a method previously described by Cotty (19).

Nucleotide sequence analysis. Nucleotide sequence analysis was conducted on cosmid NorA subclones (clones 3 and 4, two *Sac*I-*Eco*RI restriction fragments encoding a large portion of gene-1) at the Plant Research Laboratory at Michigan State University and by DNA Technologies Inc., Rockville, Md. Automated nucleotide sequencers (ABI robotic catalyst and 373A DNA sequencer) and fluorescent labelled T3 and T7 oligonucleotide primers were used to generate and analyze dideoxy sequence reactions. Nucleotide sequence data were analyzed with the Wisconsin Genetics Computer Group Package. The locations of introns and open reading frames were predicted with the software programs Frames, Testcode, and Codon Preference and the *A. nidulans* codon usage file described previously (35, 54). Comparisons of predicted amino acid sequences to EMBL and GenBank database libraries were conducted with TFastA and Gap and aligned with Pileup from the Wisconsin Genetics Computer Group Package.

RESULTS

Restriction endonuclease analysis of cosmid NorA and physical linkage of *nor-1* and *ver-1*. In screening an *A. parasiticus* SU1 genomic DNA cosmid library, four cosmid clones hybridized to the *ver-1* probe (NorA, Ver2, Ver3, and Ver4) and two clones hybridized to the *nor-1* probe (NorA and NorB). Cosmid NorA was of particular interest because it hybridized to both the *nor-1* and *ver-1* gene probes. A restriction endonuclease map of cosmid NorA was generated to allow localization of *nor-1*, *ver-1*, and *uvm8* genes (an *Eco*RI and *Xba*I restriction map is shown in Fig. 1). Since cosmid NorA hybridized to both *nor-1* and *ver-1*, it is suggested that either the two genes are physically linked in the genome of *A. parasiticus* or *nor-1* and *ver-1* were brought together on cosmid NorA due to recombination of normally unlinked chromosomal fragments. To distinguish between these possibilities, Southern hybridization analyses were performed on cosmid NorA and genomic DNA isolated directly from toxigenic *A. parasiticus* SU1 (Fig. 2A). The *nor-1* probe hybridized to identical 22-kb *Xba*I DNA restriction fragments in cosmid NorA and in genomic DNA. The *ver-1* probe hybridized to a 19-kb *Xba*I fragment in cosmid NorA and a 21-kb *Xba*I fragment in genomic DNA. A 3.2-kb *Sac*I-*Bam*HI subclone from cosmid NorA which spanned the junction between the two large *Xba*I fragments (22 and 19 kb) hybridized to the same 22- and 21-kb

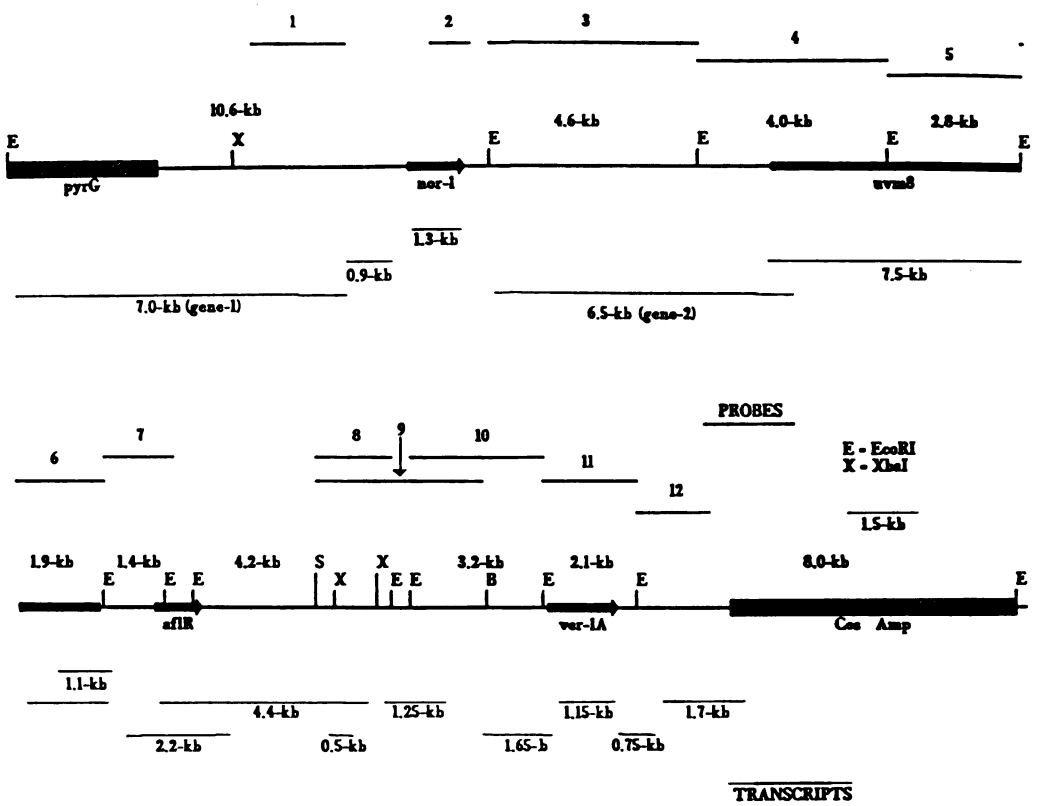

FIG. 1. Restriction endonuclease and transcript map of cosmid NorA. Sizes and locations of transcripts and *Eco*RI restriction fragments are shown. ^{32}P-labelled probes used to locate transcripts (see Fig. 3B) are numbered 1 through 12. Locations of genes and directions of transcription are indicated when information is available. Vector sequences are indicated by shaded blocks. The transcribed region of gene-1 continues beyond the end of cosmid NorA. All *Xba*I (X) and *Eco*RI (E) sites are shown. The locations of *Sac*I (S) and *Bam*HI (B) sites are only included to mark the probe used in linkage analysis; other sites are not included.

*Xba*I fragments in genomic DNA as the *nor-1* and *ver-1* gene probes (and to the 22- and 19-kb *Xba*I fragments in cosmid NorA). These results strongly suggest that the 22- and 21-kb DNA restriction fragments carrying *nor-1* and *ver-1*, respectively, are directly linked in the genome of *A. parasiticus* SU1. Since the *ver-1* and the 3.2-kb *Sac*I-*Bam*HI probes lie within a 12-kb duplication of the region containing *ver-1* and *aflR* in the genome of SU1 (33, 48), additional bands of the predicted size appeared in the genomic DNA analyzed with these probes (*ver-1* probe, 8.9-kb fragment; 3.2-kb *Sac*I-*Bam*HI probe, 8.9- and 6.5-kb fragments; see Fig. 2B for schematic).

Transcript map of cosmid NorA. The appearance of *nor-1* and *ver-1* transcripts occurs simultaneously in *A. parasiticus* SU1 under different growth conditions, suggesting that they are coordinately regulated in part at the transcriptional level (49). Since the two genes were found to be linked on the chromosome, a transcript mapping analysis of this 35-kb region was initiated to determine the size, location, and pattern of expression of other genes in the region. Genes with expression patterns similar to those of *nor-1* and *ver-1* would be studied further because of the potential for direct involvement in AFB1 synthesis. RNA was isolated at distinct time points from mycelia of aflatoxin-producing *A. parasiticus* SU1 grown in YES or Reddy's medium (which induces AFB1 synthesis). The time courses of aflatoxin production and accumulation of mycelial dry mass were qualitatively similar in the two media (Fig. 3C and D). The maximum rate of fungal growth occurred between 18 and 36 h after inoculation, whereas the maximum rate of aflatoxin synthesis occurred between 48 and 72 h, when growth had slowed considerably in a transition between active growth and stationary phase. Radiolabelled DNA probes (numbered 1 to 12 in Fig. 1) were used to analyze RNA isolated at various times during fungal growth in YES or Reddy's medium. Northern analysis identified the size, location, and pattern of accumulation of 14 transcripts in the region encompassed by cosmid NorA (Fig. 3B, Northern analyses; and Fig. 1, transcript map).

Transcript accumulation in Reddy's and YES media. The pattern of expression observed for genes known to be involved in AFB1 biosynthesis (*nor-1*, *ver-1*, and *uvm8*) in Reddy's medium (a chemically defined medium) showed very little transcript accumulation at the 18-h point and a high level of transcript accumulation between 36 and 84 h (Fig. 3A). Eight transcripts in the gene cluster (from left to right in Fig. 1:

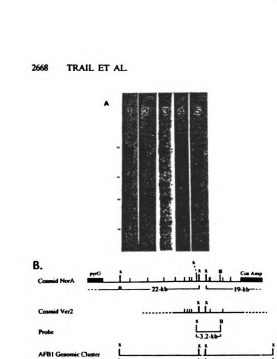

FIG. 2. Physical linkage between the *nor-1* and *ver-1* genes. (A) Southern hybridization analysis of *Xba*I-cut genomic DNA from *A. parasiticus* SU1 (lanes 2 to 4) or *Xba*I-cut cosmid NorA (lanes 1 and 5). The following radiolabelled DNA probes were used: *nor-1* (lanes 1 and 2), *ver-1* (lanes 4 and 5), and the 3.2-kb *Sac*I-*Bam*HI fragment which spans the adjoining *Xba*I fragments containing the *nor-1* and *ver-1* genes (lane 3; see panel B). The four dashes to the left of lane 1 represent (top to bottom) the positions of 23.1-, 9.4-, 6.6-, and 4.4-kb *Hin*dIII restriction fragments of lambda DNA used as a size standard. (B) *Xba*I restriction maps of cosmids NorA and Ver2, and the AFB1 genomic cluster showing the region of genomic DNA duplication. The location of the probe used in panel A is shown. The 19-kb *Xba*I restriction fragment of cosmid NorA is shown on both sides of the 22-kb fragment because of the circular nature of the cosmid. Dashed lines on cosmid Ver2 indicate unmapped regions. Abbreviations are given in the legend to Fig. 1. Unlabelled restriction sites are *Eco*RI sites.

gene-1, 0.9 kb; gene-2, 0.5, 1.25, 1.65, 0.75, and 1.7 kb) showed a pattern of accumulation similar to that of *nor-1*, *ver-1*, and *uvm8*. The data from *nor-1*, *ver-1*, *uvm8*, and gene-1 are shown in Fig. 3A to illustrate the transcript accumulation of these eight genes. In contrast, the accumulation of transcripts for *pyrG*, a gene associated with primary metabolism (UTP biosynthesis), was observed to be high at the 18-h time point. Transcripts of *benA* (encoding β-tubulin) showed nearly uniform accumulation at all time points, including 18 h, as would be expected of a housekeeping gene. The 1.1 and 2.2-kb transcripts (*aflR*) showed transcript accumulation patterns similar to that of *pyrG* (data not shown). It is not known whether the gene encoding the 1.1-kb transcript is involved in secondary metabolism; however, *aflR* has been reported to be a positive regulator of several genes in AFB1 synthesis (55), so the appearance of this transcript before those of *nor-1* and *ver-1* is not unexpected. The 4.4-kb transcript was present in very small quantities at all time points and encompassed the same region of DNA as *aflR*. The nature of this transcript is not clear.

To determine whether the type of growth medium influenced the relative expression of these two different groups of genes (i.e., AFB1-related genes versus primary metabolism or housekeeping genes), the time course of expression in YES, a "rich" AFB1-inducing medium, was also analyzed (Fig. 3B). The contrast between the pattern of expression of *nor-1*, *ver-1*, *uvm8*, and the eight AFB1-related genes and the expression of *pyrG* and *benA* is even more striking. Transcripts of the AFB1-related genes first appeared in the 40-h sample during a transition from active growth to "stationery phase" and decreased significantly by 72 h. In contrast, transcripts of *pyrG* and *benA* were expressed at the highest levels during active growth (first appearing in the 10-h sample) and did not decrease until 24 to 40 h after inoculation, as growth slowed. In both YES and Reddy's media, the appearance of transcripts of AFB1-related genes correlated well with the first appearance of AFB1 in the culture.

Recombinational inactivation of gene-1. The pattern of expression of eight transcripts in cosmid NorA (in addition to *nor-1*, *ver-1*, and *uvm8*) was observed to correlate well with AFB1 synthesis, suggesting that the genes encoding them are involved in AFB1 synthesis. To test this hypothesis, gene-1, encoding a 7-kb transcript (whose function was unknown), was disrupted by insertion of pAPNVES43 (schematic in Fig. 4), which contained an internal fragment of the transcribed region of gene-1. A single-crossover recombinational event between pAPNVES43 and the homologous region of gene-1 in the chromosome should result in insertion of the entire pAPNVES43 vector into gene-1, inactivating its function.

pAPNVES43 was used to transform *A. parasiticus* CS10, a VA-accumulating strain. In two separate experiments, 10% of the *pyrG*$^+$ colonies did not accumulate a yellow pigment (indicative of loss of VA production) on CAM. No transformants were obtained when DNA was not present in the transformation mixture, and no transformants lost their ability to produce the yellow pigment (VA) when pPG3J, carrying only the *pyrG* selectable marker, was used as a control plasmid.

TLC analysis of transformants. Three transformants that no longer appeared to accumulate VA on CAM and a known VA-accumulating strain, ATCC 36537, were grown in YES medium (aflatoxin inducing) for further analysis. TLC analyses of extracts of mycelial mats and the growth medium confirmed a loss of VA production in all three transformed strains (Fig. 5), whereas normal levels of VA were observed in the control strain ATCC 36537 grown under identical conditions. No aflatoxin production was noted in either the transformants or the control strain. No new pigments appeared to accumulate in the putative gene-1-disrupted transformants.

Genetic analysis of putative gene-1 disruptant clones. Southern hybridization analysis was performed on genomic DNA isolated from the parental strain, CS10, and five putative gene-1 disruptants (strains that no longer accumulated VA) (Fig. 6). A 10.2-kb *Eco*RI genomic DNA fragment hybridized to the pUC19 probe (0.8-kb *Sca*I-*Eco*RI), as expected, in four of five transformants (lanes 2, 4, 5, and 6) and to a 3-kb fragment in the fifth transformant (Fig. 6, lane 3; see also schematic in Fig. 4). The occurrence of the 3-kb DNA fragment is likely due to genetic rearrangement during or after integration of the disruption vector. The 10.2-kb fragment was absent in the parental strain (lane 1), as expected. An additional 8-kb fragment was present in two transformants (lanes 2 and 6), indicating that the disruption vector integrated at one other site. Identical DNA samples were also hybridized to a gene-1 probe (0.6-kb *Sma*I-*Sac*I fragment, shown in Fig. 4) located adjacent to the 1.6-kb gene-1 fragment carried on pAPNVES43. The expected 10-kb DNA fragment hybridized to this gene-1 probe in four of the transformed strains (Fig. 6, lanes 8, 10, 11, and 12), indicating insertion of pAPNVES43 by a single crossover at the homologous gene-1 locus on the

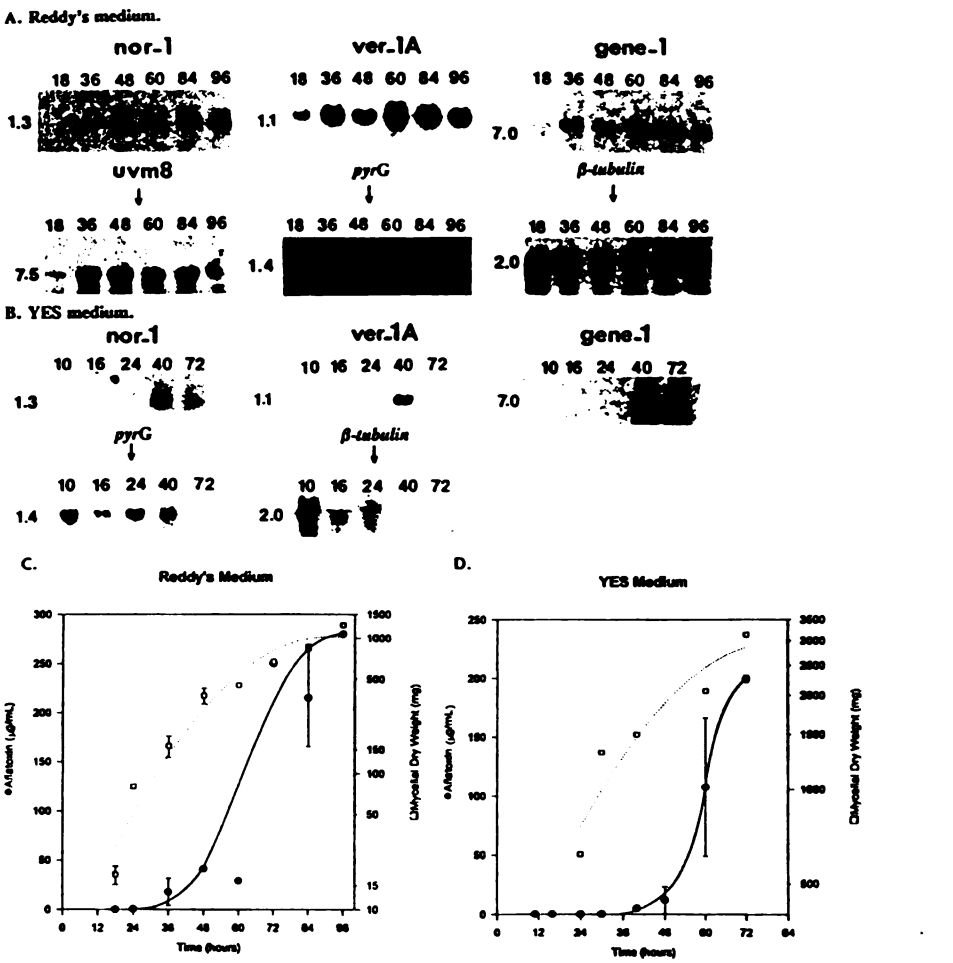

FIG. 3. Accumulation of transcripts of genes in the AFB1 cluster during batch fermentation. *A. parasiticus* NRRL 5862 was inoculated into Reddy's medium and YES medium (time zero) and grown with shaking at 29°C. Samples were removed at the indicated times for extraction of total RNA and analysis of mycelial dry weight and aflatoxin. Northern analyses of the RNA extracted from samples grown in Reddy's (A) and YES (B) media were done with the DNA probes for hybridization shown in Fig. 1 and described in Materials and Methods. Hybridization to *pyrG* and β-tubulin genes (controls) is also shown. Production of aflatoxin and mycelial dry weights are shown in panels C (Reddy's medium) and D (YES medium). Vertical bars indicate standard errors of the mean.

chromosome. The presence of a larger fragment in lane 9 and the absence of a 10-kb fragment support the genetic rearrangement argument proposed for the same DNA sample probed with pUC19 (lane 2). The probe hybridized to the expected 13.2-kb *Eco*RI genomic DNA fragment in CS10. DNA samples were also hybridized to a *pyrG* gene probe, which confirmed that the disruption vector was inserted by a single-crossover event into gene-1 (data not shown). The complete hybridization analysis was repeated with genomic DNA cut with *Eco*RV and *Sac*I (data not shown). These data confirmed the results observed for the *Eco*RI digests.

Sclerotium production. Two gene-1 disruptant clones (Tf1 and Tf2) as well as strains SU1 (aflatoxin accumulating), ATCC 24690 (NA accumulating; small quantities of AFB1 are also produced), ATCC 24551 (AVF accumulating), and ATCC 36537 (VA accumulating) were inoculated onto CAM and grown for 14 days at 30°C. These strains could be divided into three distinct groups on the basis of levels of sclerotia produced (Table 1). Gene-1 disruptants (which do not accumulate AFB1 or identifiable pathway intermediates) produced about three to six times the quantity of sclerotia produced by the wild type, SU1. The NA (an early pathway intermediate)-accumulating strain produced quantities of sclerotia similar to those produced by SU1, while the two strains that accumulated the pathway intermediates VA and AVF (intermediates near the middle of the pathway) and produced

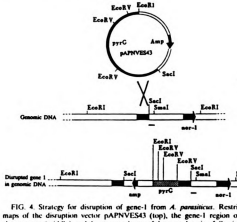

FIG. 4. Strategy for disruption of gene-1 from *A. parasiticus*. Restriction maps of the disruption vector pAPNVES43 (top), the gene-1 region of the chromosome (middle), and the proposed map of the gene-1 region following the disruption event (bottom) are shown. Also indicated are the pBluescriptSK(−) vector (with the *Amp*^{r} gene), the region of gene-1 included in the disruption vector (black bars), and flanking regions (open bars). The position of the *Sac*I-*Sma*I fragment used for Southern analysis is indicated by a solid line below the genomic DNA.

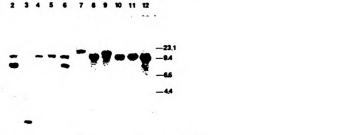

FIG. 6. Southern hybridization analysis of genomic DNA isolated from the disrupted transformants. DNA was cut with *Eco*RI and separated on a 0.8% agarose gel. Lanes 1 and 7, *A. parasiticus* 36537; lanes 2 to 6 and 8 to 12, transformants disrupted with pAPNVES43. A radiolabelled DNA fragment of pUC19 was used as a probe for lanes 1 to 6, and a *Sac*I-*Sma*I fragment (see Fig. 4) was used to probe an identical blot shown in lanes 7 to 12. The DNA size markers indicated on the right are from a *Hin*dIII digest of bacteriophage lambda. Film was exposed for 2 days at −81°C.

no detectable AFB1 failed to produce sclerotia or produced very few sclerotia.

DISCUSSION

Here, evidence is presented that several genes involved in AFB1 biosynthesis (*nor-1*, *ver-1*, *aflR*, *uvm8*, and gene-1) are physically linked on cosmid NorA and in the chromosome of *A. parasiticus* SU1. The *omt-1* gene has also been linked to this cluster of genes in *A. parasiticus* and *A. flavus* (57). Nucleotide sequence analysis of this entire gene cluster in *A. parasiticus* and a structurally similar (but not identical) gene cluster in *A. nidulans* is progressing. DNA sequence analysis of the entire region will allow identification of open reading frames, which may provide clues about the possible function of the seven other AFB1-related genes.

It is not surprising that the aflatoxin genes would be arranged in a cluster in the genome of *Aspergillus* organisms. Many genes involved in secondary metabolism in fungi have been found to be clustered. For example, genes involved in the biosynthesis of penicillin (21, 42), trichothecenes (26), and melanin (30) were all recently found to be clustered. What advantage gene clustering affords the producing organism is not clear, but one can imagine a selective advantage to having genes of like function clustered together on a chromosome if clustering is related to regulation of gene expression.

Recombinational inactivation of gene-1 provides the first indication that this gene is directly involved in aflatoxin biosynthesis and sets a precedent that other genes in the cluster, which are expressed in a pattern similar to those of *nor-1* and *ver-1* (AFB1-related genes), are also prime candidates to be involved in AFB1 synthesis. The activity of the product of gene-1 remains undescribed. However, data from two separate research approaches provide clues about its function. Nucleotide sequence comparisons between the proposed amino acid sequences in two distinct regions of gene-1 and proteins in the EMBL and GenBank database libraries were made, using computer-assisted analyses (Wisconsin Genetics Computer Group; TFastA and MOTIFS). High degrees of similarity (80%) and identity (64%) were observed between a 100-amino-acid domain in the gene-1 protein sequence and the β-ketoacyl-acyl carrier protein-synthase (Fig. 7A) functional domain of the *A. nidulans wA* gene (41), a polyketide synthase gene (PKS) involved in conidial pigment synthesis. The two other proteins that showed high identity in the same region were the *Streptomyces antibioticus* PKS (22) and the *Streptomyces erythrea* PKS (52), with identities of 29.0 and 25.0%, respectively (Fig. 7A). A significant level of identity (20 to 32%) was observed in the acyltransferase functional domains of the

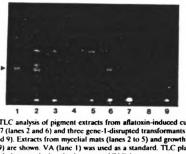

FIG. 5. TLC analysis of pigment extracts from aflatoxin-induced cultures of ATCC 36537 (lanes 2 and 6) and three gene-1-disrupted transformants (lanes 3, 4, 5, 7, 8, and 9). Extracts from mycelial mats (lanes 2 to 5) and growth medium (lanes 6 to 9) are shown. VA (lane 1) was used as a standard. TLC plates were observed and photographed under long-wave UV light.

TABLE 1. Sclerotium production in various strains of *A. parasiticus* grown on CAM for 14 days

Strain[a]	Intermediate accumulating	No. of sclerotia	
		Plate 1	Plate 2
TI1	ND[b]	5,020	3,104
TI2	ND	6,136	5,713
24690	NA	1,242	1,002
24551	AVF	0	0
36537	VA	36	5
SU1	AFB1	983	1,716

[a] Strain 24551 is derived from ATCC 15517 (24); all others are SU1 derivatives.

[b] ND, not detected.

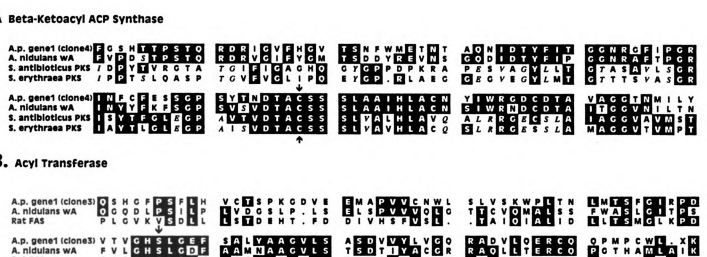

FIG. 7. Comparisons of peptide sequences in the (A) β-ketoacyl-acyl carrier protein (ACP)-synthase and (B) acyltransferase domains of gene-1 and polypeptides in the GenBank and EMBL databases. Amino acids identical in at least two of the presented sequences are shown as white letters on black backgrounds. When two different pairs of identical amino acids occur at the same residue, the pairs which do not match the *A. parasiticus* (A.p.) sequence are shown in italics. Arrows in panels A and B indicate active-site cysteines and serines, respectively. See text for analysis of comparisons.

wA PKS (32%) and rat fatty acid synthase (FAS) (20% [51]) enzymes and a distinct region in the gene-1 sequence (Fig. 7B). The identity of gene-1 in these two functional domains was higher with PKS than with FAS, suggesting that gene-1 encodes a PKS involved in AFB1 synthesis. However, these data do not rule out the possibility that gene-1 encodes an FAS.

Limited nucleotide sequence analysis of *uvm8* (37) identified a 180-amino-acid region with a high degree of sequence identity (48%) to a region of undefined function in FAS1 genes encoding the beta subunit of FAS in the yeasts *Saccharomyces cerevisiae* and *Yarowia lipolytica* (31). Townsend et al. (53) proposed that six-carbon hexanoate (two keto groups completely reduced to hydrocarbon) may serve as the starter unit for AFB1 synthesis and that hexanoate is extended by a PKS without further ketoreduction to form a decaketide, NA. This scheme would include at least one multifunctional enzyme, the FAS, with the necessary activities to reduce keto groups to hydrocarbons in order to synthesize the hexanoate starter. Another set of activities, the PKS, without keto reduction capability (27), would then extend hexanoate to generate the decaketide, NA. Our limited data are consistent with this scheme. *uvm8*, which has a high degree of identity to yeast FAS, could fill the hypothetical FAS role to produce hexanoate, which is extended by the product of gene-1 (*pksA*), a putative PKS. In support of this theory, Chang et al. (14) have independently disrupted and sequenced more extensive regions of gene-1, which they have called *pksA*. Several functional domains associated with polymerization of acetate (β-ketoacyl-synthase, acyltransferase, and acyl carrier protein) were identified, but the analysis strikingly failed to find evidence for a keto-reductase, dehydratase, or enoyl reductase involved in reduction of keto (carbonyl) groups to hydrocarbon.

A second approach, gene disruption, clearly demonstrated that gene-1 activity occurs prior to the *ver-1* gene in the pathway. Further evidence for gene-1 function is provided by the studies on sclerotium production in gene-1-disrupted transformants. The absence of production of sclerotia by strains that accumulate pathway intermediates between NA and VA suggests that gene-1 is involved at a step prior to *nor-1* activity. In a separate study it was shown that strains disrupted at *uvm8*, like strains disrupted in gene-1, produce sclerotia at levels higher than those produced by SU1 (36). Since it was demonstrated that *uvm8* activity occurs before *nor-1* in the pathway, this would argue that gene-1, like *uvm8*, is involved in some stage of polyketide backbone synthesis.

In previous research, an association between aflatoxin biosynthesis and sclerotium development has been observed (6, 18, 19). Using a molecular genetics approach, Skory et al. (48) observed that complementation of the *ver-1* mutation in strain *A. parasiticus* CS10, which accumulates VA and normally does not produce sclerotia on potato dextrose agar, restored wild-type levels of sclerotium production. The data presented here for the AVF- and VA-accumulating strains suggest that accumulation of pathway intermediates inhibits sclerotium development. Strains which accumulate early pathway intermediates (NA) or no pathway intermediates (gene-1 and *uvm8* disruptants) generate wild-type levels of sclerotia (or more), suggesting that elimination of accumulation of intermediates in the middle of the pathway allows sclerotial development to occur. When no intermediates accumulate, sclerotial development is apparently enhanced. Together, these observations support the hypothesis that the biosynthetic pathway for aflatoxin production strongly affects the development of sclerotia. Since secondary metabolism has long been considered a form of metabolic differentiation (7), it is not surprising that it may also be linked to morphological differentiation. Just how this link is structured remains to be elucidated.

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III. DISCUSSION

Gene clustering has been reported to be involved in numerous fungal secondary metabolic pathways and fungal development including the penicillin biosynthetic genes in *A. nidulans* and *P. chrysogenum* (Aharonowitz and Cohen, 1992; Montenegro *et al.*, 1992), the trichothecene biosynthetic genes in *Fusarium sporotrichioides* (Hohn *et al.*, 1993), and the spore-specific genes in *A. nidulans* (Orr and Timberlake, 1982). Besides fungi, clustering of genes involved in secondary metabolisms has been observed in several species of *Streptomyces* producing polyketide-derived antibiotics such as actinorhodin, erythromycin, tetracenomycin, streptomycin, griseusin, and granaticin (Ohnuki *et al.*, 1985; Martin and Liras, 1989).

The phenomenon of gene clustering was proposed to be a result of evolution (Aharonowitz and Cohen, 1992). This process may facilitate gene regulation by encompassing the positive or negative regulators within the gene cluster (Guilfoile and Hutchinson, 1992; Sophianopoulou *et al.*, 1993), or by providing a suitable chromatin structure for coordinate regulatory control (Cavalli and Thoma, 1993; Wolffe, 1994). The role of gene clustering in aflatoxin biosynthesis has yet to be elucidated. Our preliminary data (Wu, 1995) using the *ver-1*/GUS reporter strain demonstrated that an extremely high level of GUS expression resulted from the insertion of the *ver-1*/GUS construct at the *ver-1* locus. In contrast, a relatively low level of (500 to 1,000 fold less) GUS expression was obtained if the *ver-1*/GUS construct was inserted at another chromosomal locus, the *niaD* gene. This "local effect" may imply the importance of gene clustering in regulation of aflatoxin biosynthesis.

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