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Molecular analysis of HC-toxin biosynthesis
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MOLECULAR ANALYSIS OF HC-TOXIN BIOSYNTHESIS IN *COCHLIOBOLUS CARBONUM*

By

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ABSTRACT

MOLECULAR ANALYSIS OF HC-TOXIN BIOSYNTHESIS IN *COCHLIOBOLUS CARBONUM*

By

Yi-Qiang Cheng

HC-toxin is the molecular determinant in the interaction between the fungal pathogen *Cochliobolus carbonum* race 1 and its host plant, *Zea mays* L. (genotype *hm1/hm1*). HC-toxin exists as a small family of cyclic tetrapeptides. The major component of HC-toxin, HC-toxin I, has the structure cyclo(D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-9,10-epoxi-8-oxo-decanoic acid. The biosynthesis of HC-toxin is controlled by a genetic locus, *TOX2*, that is complex at the molecular level. Previous studies have identified four genes (*HTS1*, *TOXA*, *TOXC*, and *TOXE*; collectively called *TOX2*). These known *TOX2* genes each exist as two or three functional copies per genome and are present only in HC-toxin-producing isolates (Tox2$^+$) of *C. carbonum*. The *TOX2* genes are distributed over ~540 kb on a special chromosome in a standard lab strain SB111 (one copy of *TOXE* is located on a different chromosome). When all copies of any individual gene are mutated, the fungus loses the capability to produce HC-toxin and thus pathogenicity.

In this thesis research, two additional *TOX2* genes, *TOXF* and *TOXG,* have been cloned by using bacterial artificial chromosomes (BACs). *TOXF* and

TOXG are two tightly linked genes. Both have three copies in SB111, and two functional copies in isolate 164R10, both are exclusively present in $Tox2^+$ isolates of *C. carbonum*, both map to the *TOX2* locus, and both are regulated by *TOXE*. *TOXF* encodes a putative branched-chain amino acid aminotransferase that we hypothesize to aminate an α-keto acid in the pathway to make Aeo. A null mutant of *TOXF* failed to produce HC-toxin and lost the ability to causes severe leaf spot disease on maize. Therefore, *TOXF* can be regarded as a pathogenicity gene. *TOXG* encodes a novel alanine racemase that catalyzes the interconversion between L-alanine and D-alanine. D-alanine is a critical constituent in HC-toxin I, III and IV, but not HC-toxin II, which has glycine in place of D-alanine. A null mutant of *TOXG* failed to make HC-toxin I, III and IV, but still made HC-toxin II. Feeding the *TOXG* null mutant with D-alanine restored the normal HC-toxin production profile. Compared to wild type, the *TOXG* mutant caused a delayed disease phenotype that eventually resulted in full symptoms due to the presence of HC-toxin II. Therefore, *TOXG* can only be classified as a virulence factor. Genetic and biochemical experiments were successfully adopted to confirm that *TOXG* gene product functions as alanine racemase.

Another *TOX2* candidate gene, *TOXD*, was cloned based on its linkage to *TOXC*. *TOXD* shares similarities with other known *TOX2* genes, including physical linkage, copy number, gene distribution, and regulation by *TOXE*. However, HC-toxin production and fungal pathogenicity were unchanged in a *TOXD* null mutant. It appears that *TOXD* has no apparent role in HC-toxin production or fungal pathogenicity.

To my beloved parents.

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Aeo	2-amino-9,10-epoxi-8-oxo-decanoic acid
BAC	bacterial artificial chromosome
BCAT	branched-chain amino acid aminotransferase
bp	base pair
CHEF	contour-clamped homogenous electric field
FAS	fatty acid synthase
GSP	gene-specific primer
HST	host-specific toxin
HTS	HC-toxin synthetase
kb	kilobase
kDa	kilodalton
Mb	megabase
NRPS	non-ribosomal peptide synthetase
ORF	open reading frame
PCR	polymorase chain reaction
PLP	pyridoxal-5'-phosphate
RFLP	restriction fragment length polymorphism
TLC	thin layer chromatography

CHAPTER 1

SECONDARY METABOLISM AND FUNGAL TOXINS

Introduction

It has been estimated that the Mycota, the Fifth Kingdom, has about one and a half million species existing in almost every ecological niche on earth; nearly 70,000 of them have been described (Hawksworth, 1991; Kendrick, 1992). This vast pool of biological and genetic resources represents a considerable portion of all living things on the planet and has a profound effect on human life in many different ways. Since ancient times, human beings have been eating mushrooms and consuming yeast-fermented food and drinks. Ergotism, resulted from the consumption of grain contaminated with ergot alkaloids of *Claviceps purpurea*, has been responsible for numerous epidemics throughout human history (Beardle and Miller, 1994). In 1970, a widespread plant disease called Southern corn leaf blight, caused by *Cochliobolus heterostrophus* race T, which produces a highly specific phytotoxin known as T-toxin, resulted in substantial crop losses in the U.S. (Ullstrup, 1970). The most noteworthy event in contemporary fungal research has been the discovery of pharmaceutically active compounds. Nobel laureate Alexander Fleming observed that *Penicillium notatum* can inhibit the growth of the human pathogenic bacterium *Staphylococcus aureus* and subsequently discovered the first and still very important antibiotic, penicillin (Fleming, 1929; 1946). Later research showed that penicillins are also produced by *P. chrysogenum* and a variety of other organisms (Lechevalier, 1975). The immunosuppressant drug cyclosporin was first discovered as an antifungal agent produced by *Tolypocladium inflatums* and *Cylindrocarpon lucidum* (Dreyfus et al., 1976). It was later found to have excellent immunosuppressive activity and has been widely used during organ transplantation to suppress immune rejection (Goodman Gilman et al., 1985). Since its initial discovery, cyclosporin has been reported from many strains of *Tolypocladium inflatum*, *T. geodes*, and *T. niveum*, as well as from species of *Acremonium*, *Beauvaria*, *Fusarium*, *Paecillomyces*, and *Verticillium* (Sanglier et al., 1990). Among the most successful drugs derived from fungal sources are the cholesterol biosynthesis inhibitors related to lovastatin, which were initially reported by researchers at Merck & Co. from *Aspergillus terreus* (Vagelos, 1991). Related compounds were also reported from *Penicillium brevicompactum* and *P. citrinum* (Brown et al., 1976; Endo et al., 1976).

Concepts of *secondary metabolism* and *secondary metabolite*

Collectively, the above examples of fungal products with beneficial or hazardous biological activities are called *secondary metabolites*, which are generally unique to the producing strains but have no confirmed roles in growth and development. *Secondary metabolite* was first termed by plant natural product chemists in 1891 to describe the materials that were relatively unimportant in the overall physiology of plants (Czapek, 1921). It was not until 1961 that *secondary metabolite* was applied to microbial products (Bu'Lock, 1961). Many scientists had defined *secondary metabolite* and *secondary metabolism* in subtly different ways. Martin and Demain (1978) stated that "Secondary metabolites are those metabolites which are often produced in a phase subsequent to growth, have no function in growth (although they may have a survival function), are produced by certain restricted taxonomic groups of microorganisms, have unusual chemical structures, and are often formed as mixtures of closely related members of a chemical family". Campbell (1983) paraphrased that "materials that occur uniquely in a single strain or species, that are found in two or more closely related members of a single genus, or that are found sporadically in a limited number of evolutionarily unrelated species in different genera, families, orders, classes, phyla or kingdoms" should be called secondary metabolites (other terms are *shunt metabolite*, "*special*" metabolite, or *idiolyte*). Bennett and Bentley (1989) proposed a definition for secondary metabolite: "A metabolic intermediate or product, found as a differentiation product in restricted taxonomic groups, not essential to growth and life of the producing organism, and biosynthesized from one or more general metabolites by a wider variety of pathways than is available in general metabolism". Lately, Vining (1992) summarized three criteria of secondary metabolites: "1. Secondary metabolites are not essential for growth and tend to be strain specific; 2. They have a wide range of chemical structures and biological activities; 3. They are derived by unique biosynthetic pathways from primary intermediates and metabolites".

Characteristics of fungal secondary metabolites and their biological activities

With few exceptions, secondary metabolites are small molecules with diverse structures. They can be as simple as aliphatic acids (e.g., itaconic acid, $C_5H_6O_4$), or as complex as alkaloid toxins (e.g., palytoxin, $C_{129}H_{223}N_3O_{54}$)

(Bentley, 1999). Because secondary metabolites are deeply implicated in human life as antibacterial, antifungal, and antitumor compounds, antihelmintics, immunosuppressants, cholesterol-lowering agents, carcinogenic and tumorgenic chemicals, food contaminants, etc. (Table 1), they have a major impact on health, nutrition and economics.

Table 1. Biological activities of selected fungal secondary metabolites. Data adapted from Monagnan and Tkacz (1990), Vining (1990), Walton (1996), and Pearce (1997).

Name of metabolite	Biological activity	Producing genus
Aflatoxin	Carcinogen	*Aspergillus*
Apicidin	Antiprotozoal	*Fusarium*
Auvericin	Insecticidal	*Beauveria*
Calphostins	Antineoplastic	*Cladosporium*
Cephalosporin	Antibacterial	*Cephalosporium*
Cornexistin	Herbicidal	*Paecilomyces*
Cyclosporin	Immunosuppressant	*Tolypocladium*
Ergotrate	Vasosuppressant	*Claviceps*
Fusarielin	Antimitotic	*Fusarium*
Gibberellic acid	Plant growth stimulating	*Gibberella*
HC-toxin	Phytotoxic	*Cochliobolus*
Ibotenic acid	Narcotizing	*Amanita*
Lachnumon	Antinematode	*Lachnum*
Lovastatin	Antihypercholesterol	*Aspergillus*
Paraherquamide	Antihelmintic	*Penicillium*
Penicillin	Antibacterial	*Penicillium*
Viridiofungin	Antitumor	*Trichoderma*
Zaragozic acid	Antifungal	*Leptodontidium*
Zearalenone	Estrogenic	*Fusarium*

Biosynthesis of fungal secondary metabolites

Fungi are prolific producers of secondary metabolites. Early compendia recorded a total of about 5000 fungal metabolites (Shibata et al., 1964; Turner 1971; Turner and Aldridge, 1983). The accelerating pace of new discoveries nowadays has simply made such encyclopedia works practically impossible.

Secondary metabolism utilizes a limited number of metabolites from primary metabolism in novel ways. The structural diversity of fungal secondary metabolites reflects the complexity of their biosynthetic routes. In summary, there are four major pathways in secondary metabolite biosynthesis (Figure 1-1) (Adapted from Bentley, 1999).

The "isoprene pathway" starts with mevalonic acid derived from acetyl-CoA. The subsequent steps occur by head-to-tail condensation of the "isoprene" unit -C-C(C)-C-C-, often with further biochemical modifications. Trichothecenes (discussed later in this Chapter), which share a trichodiene core made of three isoprene units (called sesquiterpene), are examples of fungal secondary metabolites derived from this pathway.

The "polyketide pathway" typically uses acetyl-CoA as starter unit, and the Co-A forms of lower fatty acids and their carboxylated derivatives, such as malonyl-CoA, propionyl-CoA, methylmalonyl-CoA, butyryl-CoA, and ethylmalonyl-CoA, as extender units. Polyketides vary from each other in the length of backbone, degree of reduction, and numbers of modifications. The polyketide family is the largest known group of secondary metabolic compounds

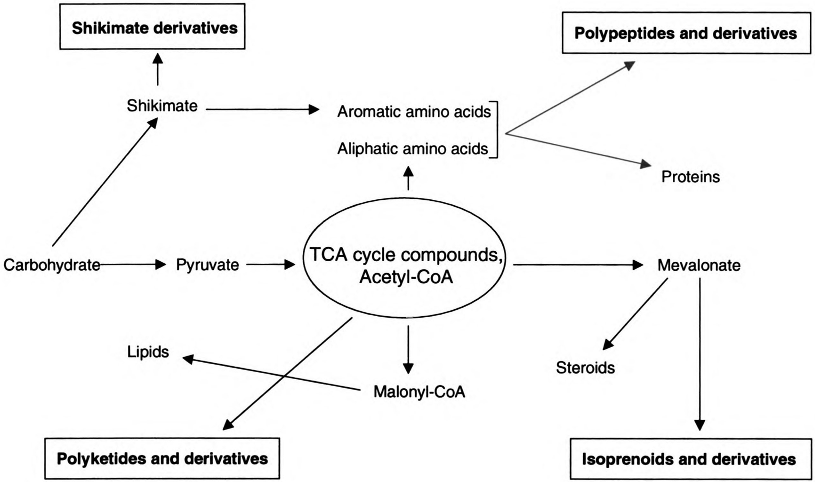


Figure 1-1. The four major pathways for secondary metabolite biosynthesis.

in fungi. Aflatoxins/sterigmatocystins, fumonisin and T-toxin (discussed in the later part of this Chapter) are polyketides produced by fungi.

The "shikimate pathway" uses shikimic acid itself as the starter material, or an intermediate involved in shikimate formation, or a primary metabolite derived from shikimate (e.g., isochorismate). Many plant aromatic products originate in this way, as well as some nonaromatic, cyclohexane structures. Fungal toxins derived from this pathway, however, are not common.

The fourth "polypeptide pathway" includes secondary metabolites synthesized from amino acids and amino acid derivatives in a non-ribosomal fashion. This class of compounds is synthesized by multifunctional (cyclic)-peptide synthetases. HC-toxin and victorin (discussed later in this Chapter) are examples of this pathway.

While these four pathways are the major biosynthetic routes to secondary metabolites, there are still other possibilities, such as compounds derived directly from carbohydrates without cleavage of the carbon chain and those derived from intermediates of the tricarboxylic acid cycle. Moreover, many secondary metabolites are biosynthesized by a mixed process involving a combination of two or more of these major pathways. A classic example is ergot alkaloids, which consist of both amino acid units and isoprene units (Bentley, 1999).

Functions of fungal secondary metabolites

Why do fungi produce secondary metabolites? Alternatively, what are the biological functions of secondary metabolites to the producing fungi? There is yet no definitive answer to this question. On the one hand, as defined, metabolites produced through secondary metabolic pathways are not essential for the growth and development of the producing organisms. Early hypotheses favored the conclusion that the secondary metabolism was merely an overflow of energy and intermediates (Bu'Lock, 1961; Woodruff, 1966). On the other hand, from the Darwinian point of view, the presence in an organism of the genetic information and biosynthetic machinery to carry out a complex synthesis implies positive selection. Not only does the process require energy and therefore introduce a growth disadvantage, but also the occurrence of deleterious mutations in a multi-step pathway would be expected to eventually terminate the normal functions, unless the metabolic processes or its products were somehow beneficial to the organism (Vining, 1990). Many putative functions for secondary metabolites have been proposed. The simplest idea is that they dispose of metabolic waste to ease the toxic effects of shunt primary metabolites that are formed in abnormal amounts under nutritional stress (Dhar and Khan, 1971). Use of the biochemical machinery of secondary metabolism was postulated to keep the primary pathways, which supply energy and precursors, functional during times when they would otherwise be brought to a standstill and deteriorate. By maintaining its system in an idling mode, the organism would be in readiness to resume growth when the opportunity arose (Woodruff, 1980). Secondary metabolites functioning as chemical signals or sex hormones are generally thought to play roles in modulating cellular differentiation such as mating, sporulation, and dormant spore formation (Demain, 1984; Beppu, 1992). As to those that produce antibiotics, the secretion of such compounds may promote their own survival by inhibiting other antagonistic organisms in a competing environment (Vining, 1990). Secondary metabolites owe their antibiotic activities to their ability to inhibit essential primary metabolic processes. As a classical example, penicillin inhibits the synthesis of bacterial cell walls (Monaghan and Tkacz, 1990). Zaragozic acid is a strong inhibitor of squalene synthase of all yeasts and fungi, while the producer *Leptodontidium elatius* itself possesses a self-resistance mechanism (Pearce, 1997). Some symbiotic fungi associated with grasses produce neurotoxic compounds which serve to deter consumption by worms, birds, or animals (Clay and Cheplick, 1989; Rowan, 1993). Phytotoxins, especially host-selective toxins, produced by plant fungal pathogens, allow the colonization of healthy plant tissue and therefore confer an ecological advantage to the producers (Walton, 1996; Desjardins and Hohn, 1997).

Origin/evolution of secondary metabolism

It is argued that microorganisms have evolved the ability to produce secondary metabolites because of the selective advantages thereby acquired (Vining, 1992). In general, only those organisms that lack an immune system are prolific producers of secondary metabolites, which are proposed to act as an alternative defense mechanism (Stone and Williams, 1992). Genetically, it is a widely accepted hypothesis that genes involved in secondary metabolism evolved from those of primary metabolism though gene-duplication, random mutation, and horizontal-gene-transfer. Zahner et al. (1983) described the biochemical arena in which secondary metabolic pathways arise as a "trial and error" mode in which a reasonable level of low-cost inventive evolution is tolerated. As useful products appear, the genes for their synthesis are adopted into the genotype and the process is re-examined by selection. Pathways that prove to have no value are eventually discarded. Genes for the pathways that prove advantageous may be further transferred horizontally and thereby placed in a different, less confining regulatory setting (Hutter, 1986). Molecular genetic data obtained though gene sequence analysis supports the concept that secondary metabolism has arisen by modification of existing primary metabolic reactions. Although amino acid sequence identity deduced from nucleotide sequences of genes from related primary and secondary metabolic pathways is often sufficient to indicate a common ancestry, the match is often better when genes in different rather than in the same species are compared. The information so far available suggests that gene transfer between organisms has been an important factor in the evolution of secondary metabolism. This notion has been exemplified by the discovery of a striking similarity in gene organization, protein structure, and enzymatic functions between polyketide synthases of the secondary metabolic pathways and fatty acid synthases of the primary metabolic pathways (Hopwood and Sherman, 1990; Vining, 1992).

The existence of gene clusters for fungal secondary metabolite biosynthesis

Gene clustering can be broadly defined as the close linkage of two or more genes that participate in a common metabolic pathway (Keller and Hohn, 1997). Studies have shown that genes involved in fungal secondary metabolic pathways are, in most cases, clustered. The discovery of clustered genes for a secondary metabolic pathway in fungi was first demonstrated in the penicillin biosynthesis pathway (Diez et al., 1990; MacCabe et al., 1990). It was the clustering of pathway genes, coupled with the high level of sequence similarity between unrelated organisms, that facilitated the rapid isolation and cloning of genes in the cephalosporin pathway (Skatrud, 1991; Turner, 1992). The most striking examples of clustered genes for fungal secondary metabolism are those of aflatoxins/sterigmatocystins. Brown et al. (1996) identified 25 transcripts within a 60-kb region of the sterigmatocystin gene cluster in *Aspergillus nidulans*. The majority of genes could be assigned to the biochemically-defined metabolic pathway. Comparative analysis of the aflatoxin gene cluster in *A. parasiticus* and *A. flavus* has revealed many genes that are functionally and structurally similar to homologs in *A. nidulans* (Trail et al., 1995; Yu et al., 1995). Gene clusters for fungal secondary metabolism have also been identified in *Alternaria alternata* for the biosynthesis of melanin (Kimura and Tsuge, 1993), in *Fusarium sporotrichioides* and *Myrothecium roridum* for trichothecenes (Hohn et al., 1995; Trapp et al., 1998), in *Gibberella fujikuroi* for gibberellin (Tudzynski and Hölter, 1998), and in *Aspergillus terreus* for lovastatin (Kennedy et al., 1999). Slightly distant from this "doctrine" is that, in *Cochliobulus carbonum,* genes involved in HC-toxin biosynthesis, export, and regulation are not tightly clustered; instead, they are scattered over a region of at least 540 kb on a single chromosome (Ahn and Walton, 1996, 1998).

Three functional categories of genes are commonly found in secondary metabolic pathway gene clusters. These are genes encoding enzymes and transporters, regulatory genes, and genes conferring self-resistance. The clustering of functionally related genes on a chromosome implies that at least part of their evolution has occurred as a unit. Stone and Williams (1992) rationalized that, "if the genes had occurred initially at distant positions on the chromosome, then the production of a secondary metabolite would be favored if the genes were moved closer together, since this would increase the probability for them being passed on as a unit to subsequent generations, or to other species via horizontal gene transfer, and would facilitate mechanisms for their simultaneous or perhaps coordinated expression. Such a clustering of genes would only be selected for if the product conferred a selective advantage to the organism". Therefore the occurrence of gene clusters is evidence that the products have been advantageous to the producers.

Selected fungal toxins: Molecular biosynthesis and roles in pathogenesis

Among the secondary metabolites produced by fungi, there is a class of compounds that are notoriously toxic to animals (mycotoxins) or/and to plants (phytotoxins). Since the discovery of aflatoxins and their causal role in Turkey X disease in 1960 in England, over 100 toxigenic fungi and more than 300 mycotoxins have been identified from various sources (Wang and Groopman, 1999). These toxins are mainly produced by five genera of fungi: *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, and *Claviceps*. The major mycotoxins are: aflatoxins, sterigmatocystins, ochratoxin, and cyclopiazonic acid made by *Aspergillus*; patulin, ochratoxin, citrinin, penitrem, and cyclopiazonic acid made by *Penicillium*; deoxynivalenol, nivalenol, zearalenone, T-2 toxin, diacetoxyscirpenol, fumonisins, moniliformin, and trichothecenes by *Fusarium*; tenuazonic acid, alternariol, and alternariol methyl ether by *Alternaria*; and ergot alkaloids by *Clavieps* (Steyn, 1995). Dozens of phytotoxins including nearly 20 host-specific toxins (HSTs) have also been purified from fungal cultures (reviewed by Scheffer and Livingston, 1984; Kohmoto and Otani, 1991; Walton, 1996). HSTs are produced mainly by two genera of fungi, *Cochliobolus* (*Helminthosporium*) and *Alternaria*. The best known HSTs are: HC-toxin from *C. carbonum*, T-toxin from *C. heterostrophus*, victorin from *C. victoriae*, AM-toxin from *A. alternata* Apple pathotype, AAL-toxin from *A. alternata* Tomato pathotype, AF-toxin from *A. alternata* Strawberry pathotype, AK-toxin from *A. alternata* Japanese pear pathotype, ACR-toxin from Rough lemon pathotype, ACT-toxin from *A. alternata* Tangerine pathotype, and AT-toxin from *A. alternata* Tobacco pathotype.

The studies of fungal toxin toxicity, biogenesis, and mode of action not only have significance for crop protection, post-harvest storage, and food-process decontamination, but also have implications in carcinogenic and medicinal biochemistry research. The remaining part of this Chapter will be devoted to several of the most important fungal toxins, with an emphasis on the molecular genetics of their biosynthesis, mechanisms of toxicity, and roles in phytopathogenesis.

1. Aflatoxins/sterigmatocystins

Aflatoxins and sterigmatocystins are a group of polyketide mycotoxins produced by several genera of fungi, mainly the genus *Aspergillus*. They are derived from highly homologous metabolic pathways (Keller and Hohn, 1997). Aflatoxins are the end products in *A. flavus* and *A. parasiticus*; whereas sterigmatocystins are the end products in *A. nidulans*. The four naturally occurring aflatoxins are B_1, B_2, G_1 and G_2, with B_1 (Figure 1-2) usually being found at the highest concentration in contaminated food and feed (Sweeney and Dobson, 1998). Aflatoxins are potent teratogenic, mutagenic, and carcinogenic agents. Aflatoxin B_1 is by far the most toxic form of the family and the most studied in mammalian toxicity. Upon entering the mammalian liver, the unsaturated terminal furan ring of aflatoxins is converted into an epoxide group by a cytochrome P450. Covalent binding of this epoxide to DNA bases to form a drug-DNA adduct is the nature of aflatoxin tocixity (Wang and Groopman, 1999).

Aflatoxins/sterigmatocystins are synthesized by condensation of acetate units, a typical polyketide-forming process. It is estimated that at least 19 gene products are required for aflatoxin biosynthesis, and at least 17 for sterigmatocystins (Brown et al., 1999). The sterigmatocystin biosynthetic pathway in *A. nidulans* is one of the best-studied pathways of fungal secondary metabolism. A sequencing and transcript mapping study of the 60-kb region of the gene cluster revealed 25 coordinately regulated genes (Brown et al., 1996). Most of those genes are predicted to encode enzymes with roles in sterigmatocystin biosynthesis. Among them, one gene encodes a polyketide synthase, two encode a pair of fatty acid synthase α and β subunits, five encode P450 monooxygenases, several encode oxido-reductases and dehydrogenases, one encodes a methyltransferase and another an esterase. Specially, one gene (*aflR*) encodes a transcription factor that regulates the expression of the most, if not all, of the genes in the cluster. There are still a number of genes having no significant homology to sequences in the database. Comprehensive reviews of the biochemistry, genetics, molecular biology and physiology of aflatoxin/sterigmatocystin biosynthesis are available (Minto and Townsend, 1997; Payne and Brown, 1998; Woloshuk and Prieto, 1998; Brown et al., 1999).

Aflatoxin production occurs on maize kernels, nuts, and cotton bolls when infected with *Aspergillus*, mainly *A. flavus*. However, aflatoxins do not increase fungal virulence to crops because both toxigenic and atoxigenic strains of the fungus colonize plant tissues equally well (Cotty et al., 1994). Therefore, aflatoxins seem to have no role in pathogenesis.

2. Fumonisins

Fumonisins are amino polyalcohol mycotoxins produced by several species of the genus *Fusarium*. Among the most common and highest producing

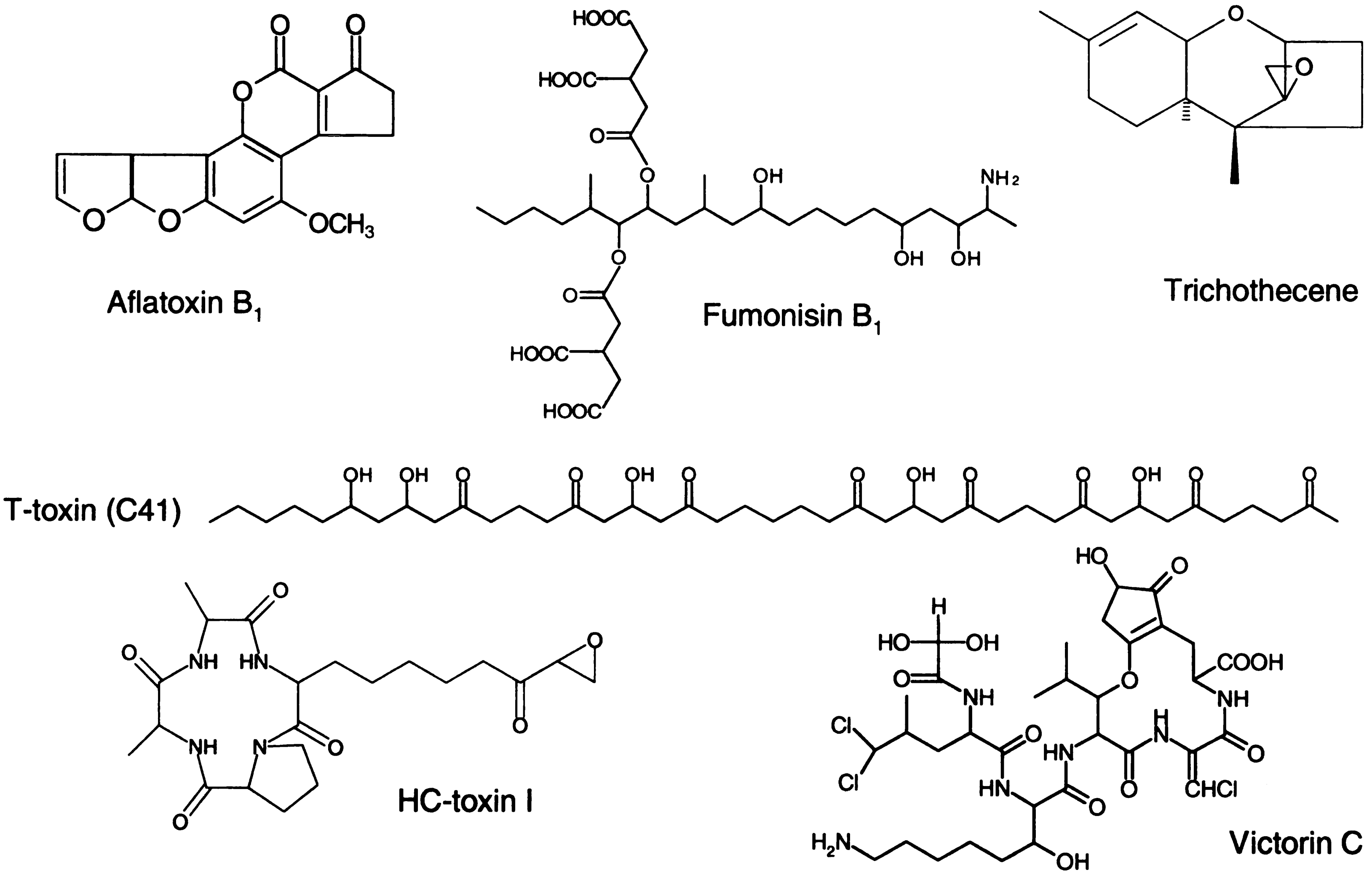


Figure 1-2. Structures of selected fungal toxins.

species are *F. moniliforme* (teleomorph *Gibberella fujikuroi* mating population A) and *F. proliferatum*, both of which are frequently found on maize (Ross et al., 1990). Fumonisin B_1 (Figure 1-2) was first isolated from cultures of *F. moniliforme* by Gelderblom et al. (1988) and structurally characterized by Benzuidenhout et al. (1988). Fumonisin B_1 is the most prevalent component of fumonisins in naturally contaminated maize. Fumonisins B_2, B_3, and B_4 were later identified as minor forms (Plattner et al., 1992; Marasas, 1996). Fumonisins induce a wide range of adverse effects in experimental animals. Among the observed toxicological symptoms are equine leucoencephalomalacia (Kellerman et al., 1990), porcine pulmonary edema (Colvin and Harrison, 1992), and hepatic cancer in rats (Gelderblom et al., 1993). Epidemiological study has suggested a linkage between high rate of esophageal cancer in humans and high levels of consumption of fumonisins in maize products (Marasas, 1995). The mechanism of action of fumonisins appears to involve binding to proteins, probably thereby causing the conversion of an amino acid side chain to the anhydride followed by covalent coupling to other proteins (Shier et al., 1997).

Fumonisins are structurally similar to the long-chain backbones of sphingolipids. Studies have found that fumonisins inhibit the activity of sphingosine N-acetyltransferase, which results in the accumulation of toxic sphingoid bases. Sphingolipids are essential components of biological membranes. Sphingosine, the backbone of sphingolipids, acts as an intracellular regulator and second messenger mediating normal cellular development (Shiel et al., 1997). Therefore, the correlation between fumonisin toxicity and tumorogenesis is rationalized, but more study is needed to verify the current working model.

The biosynthesis of fumonisins is poorly understood. The structural similarity between fumonisins and sphingosine prompted the idea that they may be biosynthetically related (Plattner and Shackelford, 1992). Sphingosine biosynthesis begins with the condensation of palmitoyl-CoA with serine. Fumonisin B_1 has an alanine instead of serine, and an 18-carbon fatty acid instead of 16-carbon fatty acid. Isotope-feeding studies demonstrated that alanine is indeed a biosynthetic precursor of fumonisin B_1, and that the polyalcohol moiety is derived from acetate (Blackwell et al., 1996). Genetic studies indicated that at least some of the genes involved in fumonisin production are closely linked to form a putative gene cluster on chromosome 1 of *G. fujikuroi* (Desjardins et al., 1996; Xu and Leslie, 1996). Very recently, Proctor et al. (1999) cloned a polyketide synthase gene (*FUM5*) by degenerate PCR approach, and showed that the new gene is involved in fumonisin biosynthesis in *Gibberella fujikuroi* mating population A. *FUM5* has an ORF of 7.8 kb. The predicted gene product is highly similar to bacterial and fungal Type I polyketide synthases. Transformation of a cosmid clone carrying *FUM5* into *G. fujikuroi* enhanced production in three strains and restored wild-type production in a fumonisin non-producing mutant. Disruption of *FUM5* reduced fumonisin production by over 99%. *FUM5* is the first fumonisin biosynthetic gene obtained.

Fumonisin-producing *Fusarium* species are routinely found in maize tissues, such as root, stalks, kernels, and seeds. Species of *Fusarium* have also been found in sorghum, millet, rice, wheat, cotton, etc. The high frequency of fumonisin contamination in maize suggests the possibility that fumonisins play a role in pathogenesis (Desjardins and Hohn, 1997). Low concentrations (1-100 μM) of pure fumonisins have been shown to cause necrosis and other disease symptoms in maize and other plant seedlings (Lamprecht et al., 1994). Genetic analysis has also shown an association between production of fumonisin and high levels of virulence on maize seedlings (Desjardins et al., 1995). Additionally, the structural similarity of fumonisins to AAL-toxin, the host-specific toxin of *Alternaria alternata* Tomato pathotype, suggests a role for fumonisins in pathogenesis (Winter et al., 1994). The future cloning of the fumonisin biosynthesis genes and creation of null mutants will unambiguously define the role of fumonisins in this regard.

3. Trichothecenes

Trichothecenes are a large family of sesquiterpenoids produced by several genera of fungi, mainly *Fusarium* and *Myrothecium*, and by at least two species of the plant genus *Baccharis* (Jarvis, 1991; Desjardins et al., 1993). All trichothecenes contain a tricyclic nucleus named trichothecene (Figure 1-2) and usually have an epoxide at C-12 and C-13 that is essential for toxicity. The total number of naturally occurring trichothecenes known today exceeds 60 (Desjardins et al., 1993). Structurally, trichothecenes can be classified into two distinct categories. Linear trichothecenes, such as T-2 toxins, diacetoxyscirpenol and deoxynivalenol, are produced by *Fusarium* species and are the major source of trichothecene contamination in agricultural products. Macrocyclic trichothecenes, such as verrucarin A and baccharinoid B7, are produced by *Myrothecium* species and the plant species *Baccharis* (Trapp et al., 1998). The discovery that some higher plant species make highly similar trichothecenes prompted the hypothesis that plants originally obtained the genes for trichothecene biosynthesis though horizontal-gene-transfer from a trichothecene-producing fungus (Jarvis, 1991).

Trichothecenes are potent inhibitors of protein synthesis in eukaryotic cells (McLaughlin et al., 1977). They bind to the peptidyl transferase active site within the 60S subunit of the ribosome, thereby inhibiting the initiation, elongation and termination reactions (Feinberg and McLaughlin, 1989). Macrocyclic trichothecenes are about 10-fold more toxic than the *Fusarium* trichothecenes. In general, trichothecenes induce toxic effects in animals, such as vomiting, oral lesions, dermatitis, and hemorrhaging, and they have been implicated in mycotoxicosis in both humans and animals (Sharma and Kim, 1991).

Trichothecene biosynthesis starts with the cyclization of the isoprenoid biosynthetic intermediate farnesyl pyrophosphate to trichodiene, catalyzed by the central enzyme trichodiene synthase. Subsequent steps involve a variety of oxygenations, isomerizations, cyclizations, and esterifications (Desjardins et al., 1993). Genes for trichothecene biosynthesis in *F. sporotrichioides* and *M. roridum* are clustered. A total of 10 clustered trichothecene biosynthesis genes have been identified within a 25-kb region in *F. sporotrichioides*. Among them, eight are predicted to encode biosynthetic enzymes (but only three have been biochemically studied), one gene encodes a pathway transcription factor, and another encodes a transport protein. Apparent homologs of several *Fusarium* pathway genes have been found within a 40-kb region in *M. roridum*. However, the distances between genes in *M. roridum* are greater, compared to *F. sporotrichioides*. The gene order in *M. roridum* is also different from that in *F. sporotrichioides* (Hohn et al., 1993; Keller and Hohn, 1997; Trapp et al., 1998).

Trichothecenes appear to contribute to the virulence of certain phytopathogenic *Fusarium* species on some host plants (Desjardins et al., 1989, 1992). At very low concentrations (1 μM to 1 mM), trichothecenes cause wilting, chlorosis, necrosis, and other disease symptoms in a wide variety of plants (Cutler, 1988). For example, simple trichothecenes such as T-2 toxin and deoxynivalenol inhibit protein synthesis in maize leaf disks and kernel sections and the growth of wheat coleoptiles (Casale and Hart, 1988; Wang and Miller, 1988). Complex macrocyclic trichothecenes of *M. roridum* induce chlorotic and necrotic lesions on muskmelon leaves (Kuti et al., 1992). UV-induced mutants of *F. sporotrichioides* that lost trichothecene production were much less virulent than the wild type strain on parsnip root (Desjardins et al., 1989). However, molecular genetic studies generated unexpected results. When a critical gene (*Tox5*) encoding trichodiene synthase was disrupted in *F. sporotrichioides*, the virulence of toxin-nonproducing mutants on parsnip roots was significantly reduced when compared with the virulence of the parental strain; on potato tubers, in contrast, the virulence of mutants was unchanged (Desjardins et al., 1992). A plausible explanation for these results is that protein synthesis or some other trichothecene target sites in parsnip and potato cells have different degree of sensitivity to trichothecenes.

4. T-toxin

T-toxin is a group of linear long-chain (C_{35} to C_{41}) (Figure 1-2) polyketide molecules produced exclusively by *Cochliobolus heterostrophus* race T. Race O of *C. heterostrophus* does not produce T-toxin. Along the methylene backbone in T-toxin, there are repeated β-oxydioxo groups that are regularly spaced (Kono and Daly, 1979; Kono et al., 1981).

Cochliobolus heterostrophus was first described in 1925 as a weak fungal pathogen of maize (Drechsler, 1925). An extremely virulent race (named race T, in contrast to the original race O) first appeared in the USA in 1969 and subsequently caused a severe epidemic on Texas male sterile (T) cytoplasm maize that resulted in a 15% crop loss in 1970 (Ullstrup, 1970). T-toxin is the disease determinant of race T and is regarded as a host-specific toxin (HST) due to its specific toxicity toward T-cytoplasm maize, not to other cytoplasmic types of maize.

Extensive studies have demonstrated that T-toxin binds to a protein (URF-13) unique to the inner mitochondrial membrane of T-cytoplasm maize (Dewey, et al., 1987, 1988). Expression of URF-13 in *Escherichia coli*, yeast, tobacco, or insect cells causes the cells to become sensitive to T-toxin, providing definitive evidence that URF-13 is responsible for T-toxin sensitivity (Dewey et al., 1988; Glab et al., 1990; von Allmen et al., 1991; Korth and Levings, 1993). Physical biochemical studies and modeling indicate that URF-13 forms oligomeric pores in mitochondrial membranes in the presence of T-toxin (Korth et al., 1991; Levings et al., 1995). After 1971, T-cytoplasm maize was replaced by normal (N) cytoplasm maize, and *C. heterostrophus* race T virtually disappeared as a severe plant pathogen in the field.

Classical genetic analysis showed that progeny from a cross between race T and race O of *C. heterostrophus* segregate in a 1:1 ratio with regard to T-toxin production and hence high virulence toward T-cytoplasm maize (Yoder and Gracen, 1975). The conclusion was then drawn that a single genetic locus (named *TOX1*) controls the production of T-toxin (Leach et al., 1982; Bronson, 1992). However, recent studies revealed that T-toxin production is actually governed by two loci (named *TOX1A* and *TOX1B*) that reside on two different chromosomes (Turgeon et al., 1995; Kodama et al., 1999). Additional genetic mapping indicated that a total of ~1.2 Mb of extra DNA is present in race T but not in race O. This stretch of DNA is likely present at both the *TOX1A* and *TOX1B* loci (Rose, 1996). To clone the T-toxin biosynthesis genes, restriction enzyme mediated integration (REMI) was used to randomly mutagenize *C. heterostrophus.* Among approximately 1300 transformants recovered from the procedure, two no longer produced T-toxin (Lu et al., 1994). Subsequently, plasmid rescue was used to clone two genes that were mutated by insertions and were shown to be involved in T-toxin biosynthesis. The gene *PKS1* (*TOX1A*) encodes a polyketide synthase and is thought to synthesize the backbone of T-toxin (Yang et al., 1996) and a second gene *DEC1* (*TOX1B*) encodes a putative decarboxylase that probably decarboxylates the product of the polyketide synthase (Rose et al., 1996). Targeted inactivation of either gene resulted in loss of T-toxin production and reduced virulence on T-cytoplasm maize. *PKS1* and *DEC1*, and a putative reductase-encoding gene (*RED1*) that is linked to *DEC1* but has no apparent role in T-toxin biosynthesis, appear to be single-copy genes and are all found only in race T, but not in race O, of *C. heterostrophus*. It is likely that these genes locate within the ~1.2 Mb of extra DNA (Kodama et al., 1999).

5. Victorin

Victorin is a collection of closely related cyclic pentapeptides produced by *Cochliobolus victoriae*, which causes Victoria blight of oats (Meehan and Murphy, 1946, 1947). The amino acids in victorin are extensively modified. The most abundant form of victorin is victorin C (Figure 1-2), which consists of 5,5-dichloroleucine, 3-hydroxylysine, chloroacrylic acid, and a cyclic alpha-amino acid derivative called victalanine. Additional minor forms of victorin have been identified and named as victorin B, D, E, and victoricine; they differ from victorin C in the degree of chlorination and hydroxylation of various side chains (Macko et al., 1985; Wolpert et al., 1985, 1986).

The toxicity of victorin is host-specific. Only oat lines containing Victoria-type resistance to crown rust (caused by *Puccinia coronata*) are susceptible to *C. victoriae* and sensitive to the toxin. Sensitivity to victorin and susceptibility to *C. victoriae* cosegregate and are controlled by a single dominant nuclear gene (*Vb*) (Litzenberger, 1949). *Vb* has been postulated to encode a receptor for victorin

(Scheffer and Livingston, 1984), but definitive proof of this hypothesis is still lacking. Great progress in elucidation of the mode of action of victorin has been achieved during the past decade. An *in vivo* assay identified a 100-kD protein that binds to ^{125}I-labeled victorin C in a ligand-specific manner, and only protein extracts from oats carrying the *Vb* gene bind (Wolpert and Macko, 1989). This *in vivo* binding is competitive, covalent, and appears to be correlated with the biological activity of victorin. The gene encoding this 100-kD protein was cloned and the deduced protein sequence indicated that it encodes the P-protein component of the glycine decarboxylase complex (GDC), which is located in the mitochondrial matrix (Wolpert et al., 1994). Victorin also binds to the 15-kD H-protein component of GDC in both susceptible and resistant oat protein extracts. The binding of victorin to two components of GDC inhibits the normal function of GDC during the photorespiratory cycle in susceptible oat tissues, which results in a series of senescence-like responses, such as the site-specific proteolytic cleavage of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), loss of chlorophyll, and DNA laddering (Navarre and Wolpert, 1995, 1999). These results suggest that GDC is the target of victorin toxicity, but whether P- or H-protein or neither component of GDC is the product of the *Vb* gene still remains unclear.

Genetic analysis indicated that victorin production in *C. victoriae* is controlled by a single genetic locus, *TOX3* (Scheffer et al., 1967; Bronson, 1992). Considering that victorin is a cyclic pentapeptide, it is likely to be synthesized by a multifunctional cyclic peptide synthetase and many other enzymes responsible for the chemical modifications. However, no protein has been purified or gene cloned for the biosynthesis of victorin.

6. HC-toxin

HC-toxin is a small family of cyclic tetrapeptides produced by *Cochliobilus carbonum* race 1, the causal pathogen of Northern corn leaf spot. The major and most toxic component of HC-toxin, HC-toxin I (Figure 1-2), has the structure cyclo(D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-9,10-epoxi-8-oxo-decanoic acid (Pringle and Scheffer, 1967, 1971; Gross et al., 1982; Walton et al., 1982; Pope et al., 1983; Kawai et al., 1983). The biogenesis of the Aeo backbone as a fatty acid has been proved by ^{13}C-acetate labeling and NMR analysis (Cheng et al., 1999; See Appendix). Three minor components (HC-toxin II, III, and IV) differ slightly in amino acid composition and have significant differences in toxicity (Rasmussen, 1987; Rasmussen and Scheffer, 1988). HC-toxin II is about 50% as toxic as HC-toxin I and has glycine in place of D-alanine; HC-toxin III has about 10% relative activity and contains *trans*-3-hydroxyproline instead of proline; HC-toxin IV has only 1% relative activity and has a hydroxyl group instead of a carbonyl group at position C8 in Aeo. Those HC-toxin components can be separated by TLC or reverse phase HPLC due to their differences in polarity.

Northern corn leaf spot disease was first reported in 1938 on an inbred maize line, Pr, in Indiana. Disease symptoms include grayish tan necrotic spots with concentric rings on the leaves and ears. Longer development often results in complete desiccation of leaves and plant death (Ullstrup 1941; Multani et al., 1998). Among four described races of *C. carbonum* that can be distinguished on the basis of their host range and lesion morphology, only race 1 produces HC-toxin and only race 1 is exceptionally virulent on maize lines that are homozygous recessive at the nuclear *Hm1* locus (*hm1/hm1*). HC-toxin appears to be the sole determinant of fungal pathogenicity, although the various cell-wall-degrading enzymes secreted by the fungus might play a complementary role in fungal virulence. Genetic variants that do not produce HC-toxin are unable to colonize much beyond the site of initial penetration and, therefore, cause only small necrotic flecks on leaves (Comstock and Scheffer, 1973). The correlation between HC-toxin production and high virulence has been proved by gene-knockout experiments (Ahn and Walton, 1997, 1998; Panaccione et al., 1992; Chapters 3 and 4 in this dissertation).

Physiologically, HC-toxin inhibits maize root growth and chlorophyll biosynthesis (Rasmussen and Scheffer, 1988), but does not inhibit protein, RNA, or DNA synthesis (Ciuffetti et al., 1995). Biochemically, HC-toxin inhibits histone deacetylase, perhaps thereby suppressing host defense responses. Acetylated isoforms of histone H4 and H3 (but not H2A and H2B) accumulate in HC-toxin-treated maize tissues in a host-specific manner (Brosch et al., 1995; Ransom and Walton, 1997). Both the 8-carbonyl and the 9,10-epoxide groups are necessary for toxicity (Walton and Earle, 1983; Kim et al., 1987). HC-toxin IV with the carbonyl group hydrolyzed has only 1% toxicity compared with HC-toxin I (Rasmussen, 1987).

Although race 1 can be extremely virulent, it is normally not a serious economic problem because modern maize breeding programs have nearly eliminated the recessive allele *hm1*. *Hm1* encodes HC-toxin reductase (HCTR), which reduces the 8-carbonyl group of Aeo to the alcohol (i.e., 8-hydroxyl as in HC-toxin IV) thereby detoxifies HC-toxin (Meeley and Walton, 1991; Meeley et al., 1992; Johal and Briggs, 1992). Homologs of *Hm1* are present in many other plant species, such as wheat, barley, rice, sorghum, and oats, and all monocots tested except the susceptible maize inbreds possess HCTR activity. It has been postulated that monocots might share a common ancestor *Hm1* gene for protection against fungal pathogens that used HC-toxin or related cyclic tetrapeptides as pathogenicity factors. However, it is unknown how dicots defend themselves against HC-toxin since no HCTR activity has been detected in them so far (Multani et al., 1998; Meeley et al., 1992).

Pathogenicity of *C. carbonum* race 1 is determined by a single Mendelian locus, *TOX2*, which also confers the ability to produce HC-toxin (Nelson and Ullstrup, 1961; Scheffer et al., 1967; Ahn and Walton, 1996). A list of biochemical activities required for HC-toxin production has been proposed (Table 2). Prior to the work presented in this dissertation, there were four genes (collectively called *TOX2*) that had been cloned and demonstrated to be involved in HC-toxin biosynthesis, export, or regulation. The first *TOX2* gene, cloned by a reverse genetic approach, was *HTS1*, which encodes a 570-kDa tetrapartite non-ribosomal peptide synthetase (NRPS) called HC-toxin synthetase (HTS) (Panaccione et al., 1992; Scott-Craig et al., 1992). HTS is the central enzyme in

Table 2. Biochemical Activities Required for HC-toxin Production.

Activity	Gene	Reference
Cyclic peptide synthetase (CPS)	*HTS1*	Scott-Craig et al., 1992; and
Proline racemase	*HTS1*	Panaccione et al., 1992
Alanine racemase	*TOXG**	Cheng and Walton, submitted
Aeo synthesis		
- FAS** α subunit	?	
- FAS** β subunit	*TOXC*	Ahn and Walton, 1996
- Amino group	*TOXF**	Cheng et al., submitted
- Epoxide group	?	
- Carbonyl group	?	
Efflux pump	*TOXA*	Pitkin et al., 1996
Regulatory protein 1	*TOXE*	Ahn and Walton, 1998
Other regulatory proteins	?	

* Research presented in this thesis. See Chapters 3 and 4.
** FAS, fatty acid synthase.

HC-toxin biosynthesis. It activates and cyclizes the four amino acid constituents of HC-toxin, and epimerizes L-proline to D-proline; *TOXA*, which is clustered with *HTS1* but transcribed divergently, encodes a putative HC-toxin transporter of the major facilitator superfamily (Pitkin et al., 1996). The product of *TOXC* is predicted to be a fatty acid synthase β subunit that works together with an unknown α subunit to synthesize the decanoic acid backbone of Aeo (Ahn and Walton, 1997). *TOXE* encodes a regulatory protein required for the expression of *TOXA* and *TOXC* but not *HTS1* (Ahn and Walton, 1998). All of the known *TOX2* genes are present only in HC-toxin-producing (Tox2$^+$) isolates of *C. carbonum* in multiple functional copies, are dedicated to HC-toxin production, and, with one exception, are linked over a ~540 kb region on one chromosome (Ahn and Walton, 1996, 1998). No homologous sequences to those genes have been detected in HC-toxin-non-producing (Tox2$^-$) strains of the fungus. When all copies of an individual gene are mutated (except *TOXA*, which would be lethal), the mutants grow and develop normally but lose the ability to produce HC-toxin and no longer cause disease on susceptible maize.

The study of HC-toxin also has medical implications. There are five classes of HC-toxin analogs: chlamydocin, Cyl-2, WF-3161, trapoxin and apicidin, all of which are produced by fungi and share striking similarity in structure (Walton, 1990; Itazaki et al., 1990; Darkin-Rattray et al., 1996). Both chlamydocin and HC-toxin have demonstrated cytostatic and antimitotic activities against transformed mammalian cells (Walton et al., 1985; Shute et al., 1987). Cyl-2 and WF-3161 have some degree of antitumor activity (Hirota et al., 1973; Umehara et al., 1983). Apicidin exibits potent antiprotozoal activity against *Apicomplexan* parasites (Darkin-Rattray et al., 1996). Specially, trapoxin induces morphological reversion of v-*sis* oncogene-transformed NIH3T3 fibroblast cells, as well as human tumor cells such as HeLa and T24 cells (Itazaki et al., 1990; Yoshida et al., 1992; Kijima et al., 1993). Mammalian cell lines treated with trapoxin accumulate highly acetylated core histones (H3 and H4). Nanomolar concentrations of trapoxin irreversibly inhibit deacetylation of acetylated histones. Later, Taunton et al. (1996) successfully used trapoxin as an affinity ligand to purify human histone deacetylase and subsequently cloned the gene. Since then, studies of histone deacetylase and acetylase in mediating chromatin structure and gene expression have surged (Davie, 1996; Pazin and Kadonaga, 1997; Tsukiyama and Wu, 1997; Kouzarides, 1999).

The genetically development of novel non-ribosomal peptide drugs for cancer chemotherapy is in the spotlight. As NRPSs are organized into distinctive functional domains, rational design of hybrid genes by the exchange of domain-coding regions among NRPSs leads to novel enzymes and the production of novel products (Stachelhaus et al., 1995; Jung et al., 1997; Cane et al., 1998). However, there is still much to learn about NRPSs.

Objectives of this dissertation

This century has witnessed the emergence and disappearance of three severe plant diseases all caused by *Cochliobolus* species (Scheffer and Livingston, 1984; Walton, 1996). The constant and frequent evolution of fungal genomes within and among species deserves close attention from fungal geneticists and plant pathologists, as well as from crop breeders (Dobinson and Hamer, 1993; Walton and Panaccione, 1993). The leading study of the interaction between *C. carbonum* and maize, in regard to aspects of host-specific toxin biosynthesis, molecular basis of toxicity and host resistance, has set a model for studying other plant fungal pathogens (Walton and Panaccione, 1993; Walton, 1996). However, there are still many unknown details with regard to the HC-toxin biosynthesis pathway. Based on the accumulated knowledge prior to the studies covered in this dissertation, we did not know how D-alanine was synthesized, what route led to Aeo biogenesis, or how many extra genes are needed for HC-toxin production and hence pathogenicity. The objectives of this study were to contribute my effort to bring us one step closer to completely elucidating the biochemical and molecular nature of HC-toxin biosynthesis.

CHAPTER 2

CLONING AND ANALYSIS OF *TOXD*, A GENE THAT BELONGS TO THE *TOX2* LOCUS BUT HAS NO APPARENT ROLE IN HC-TOXIN PRODUCTION

The gene sequence reported in this Chapter has been deposited in GenBank with accession number X92391.

ABSTRACT

Due to the production of a host-specific toxin, HC-toxin, race 1 isolates of the phytopathogenic fungus *Cochliobolus carbonum* are exceptionally virulent on maize lines that are recessive at the *Hm1* locus (genotype *hm1/hm1*). Genetic analysis showed that a single Mendelian locus, *TOX2*, controls HC-toxin production. The *TOX2* locus is complex in that it spans more than 540 kb on a single chromosome, and contains multiple genes, each with multiple copies. Previous studies identified four genes (*HTS1*, *TOXA*, *TOXC*, and *TOXE*) that are dedicated to HC-toxin production. All of them are present only in HC-toxin-producing (Tox2$^+$) isolates of *C. carbonum*. When all copies of any individual gene were mutated by targeted-gene disruption or replacement, the mutants lost HC-toxin production and no longer caused disease on susceptible maize. A new gene, named *TOXD*, was cloned based on its unique presence in Tox2$^+$ isolates of *C. carbonum*. *TOXD* is present in three copies in the standard lab strain SB111, also but only one copy in strain 164R10. *TOXD* is regulated by *TOXE*, a regulatory gene that also controls *TOXA* and *TOXC* expression. *TOXD* is predicted to encode a 297-amino acid-protein that has sequence homology to a protein involved in lovastatin biosynthesis in *Aspergillus terreus*. *TOXD* was disrupted in strain 164R10 to create a null mutant. The null mutant had normal phenotypes in growth and development. Disruption of *TOXD* had no apparent effect on HC-toxin

production or fungal pathogenicity. The biochemical function of *TOXD* is unknown.

INTRODUCTION

Interactions between plants and their fungal pathogens are complicated by the fact that pathogens possess multiple ways to infect and colonize their host plants, and because plants can mount multifaceted defense strategies against pathogens. As a result, the occurrences of plant diseases in nature are relatively low, compared to the frequent encounters between plants and potential pathogens such as viruses, bacteria, fungi, and nematodes (Heath, 1991). However, when a severe plant disease does occur, the consequences can be serious. In human history, the outburst of late blight of potato in the 1840s in Northern Europe caused the death of hundreds of thousands of human lives and forced millions to desert their homes. In 1970, the sudden appearance of Southern corn leaf blight caused the loss of 15% of that year's maize production in the United States (Agrios, 1988).

In general, fungal pathogens may use one or more of the following strategies for pathogenesis: mechanical penetration of plant cell epidermis with special infection structures such as appressoria and penetration pegs, secretion of cell wall-degrading enzymes to chemically dissolve plant cell walls, production of toxic chemicals to condition plant tissues for colonization, and detoxification of plant defensive compounds (Yoder, 1980). If a single strategy employed by a pathogen is necessary and sufficient to cause disease on a certain plant host, that strategy can be called a "pathogenicity factor"; otherwise, collectively such factors are called "virulence factors" because their actions are important but not decisive (Yoder, 1980). Cell wall-degrading enzymes are typically virulence factors, whereas host-specific toxins (HSTs) are generally regarded as pathogenicity factors.

HSTs are low molecular weight compounds produced exclusively by fungi, mainly the two genera *Cochliobolus* and *Alternaria* (Walton and Panaccione, 1993). They are the determinants of host range and disease specificity. A fungus that produces a HST causes more disease on its host plant than one that is otherwise identical but does not produce the HST. A good example is victorin, the most phytotoxic and most selective HST known, which is produced by *C. victoriae*. Victorin inhibits the growth of sensitive oats at picomolar concentrations, but does not affect resistant oats or any other plants even at a one million-fold higher concentration (Walton and Earle, 1984). Well-studied HSTs also includes T-toxin and HC-toxin, produced by *C. heterostrophus* and *C. carbonum*, respectively (Walton, 1996). Classical genetic analysis indicated that the production of HSTs by species of *Cochliobolus* is controlled by single Mendelian loci, called *TOX1* for T-toxin in *C. heterostrophus*, *TOX2* for HC-toxin in *C. carbonum*, and *TOX3* for victorin in *C. victoriae* (Scheffer et al., 1967; Bronson, 1992). More recent studies revealed that T-toxin production is actually governed by two loci (*TOX1A* and *TOX1B*) that reside on two different chromosomes (Turgeon et al., 1995; Kodama et al., 1999), and *TOX2* is a complex locus containing duplication of multiple genes dedicated to HC-toxin production (Ahn and Walton, 1996, 1998). At the molecular level, two genes (*PKS1*, *DEC1*) have been identified and proven to participate in T-toxin biosynthesis (Rose et al., 1996; Yang et al., 1996), and four genes (*HTS1*, *TOXA*, *TOXC*, *TOXE*) for HC-toxin biosynthesis, export and regulation (Panaccione et al., 1992; Scott-Craig et al., 1992; Pitkin et al., 1996; Ahn and Walton, 1997, 1998). All of these genes are dedicated to toxin production and are present only in toxin-producing (Tox$^+$) strains of the fungal species. Toxin-non-producing (Tox$^-$) strains do not possess these genes or homologous DNA.

A total of ~1.2 Mb DNA is present only in race T (T-toxin-producing) but not in race O (T-toxin-non-producing) isolates of *C. heterostrophus* (Rose, 1996), and the *TOX2* locus of *C. carbonum* encompasses at least ~540 kb on a single chromosome (Ahn and Walton, 1996). These facts lead to some interesting questions. On the one hand, are genes devoted to toxin production all unique to toxin-producing strains? On the other hand, does every gene unique to toxin-producing strains have a role in toxin production? In this Chapter, I describe the cloning and characterization of a gene, named *TOXD*, which belongs to the *TOX2* locus and is unique to HC-toxin-producing strains of *C. carbonum*, yet has no apparent role in HC-toxin production or fungal pathogenicity.

MATERIALS AND METHODS

Fungal Strains. Strains of *C. carbonum* used in this study were maintained as glycerol stocks at –80 °C. Growth conditions for fungal cultures on solid V8-plates or in modified Fries' liquid medium were previously described (Van Hoof et al., 1991). SB111 (ATCC 90305) and SB114 are standard Tox2$^+$ (HC-toxin-producing) and Tox2$^-$ (HC-toxin-non-producing) laboratory strains of *C. carbonum*, respectively. 164R5 though 164R18 are random progeny from a cross between SB111 and SB114 (Ahn and Walton, 1997). Field isolates 81-64 (race 1), 1368 (race 2) and 1309 (race 3) were originally obtained from K. Leonard (University of Minnesota, St. Paul, MN). Field isolates 141R, 151, 161, 171, 181, 1101 and 1111 were generous gifts from L. D. Dunkle (Purdue University, West Lafayette, IN; Jones and Dunkle, 1993).

Nucleic Acid Manipulations and Sequence Analysis. *C. carbonum* DNA and RNA isolation was previously described (Pitkin et al., 1996). DNA and RNA blotting, probe labeling, hybridization, cDNA and genomic library screening, and DNA subcloning were done following standard procedures (Sambrook et al., 1989). Oligonucleotides were synthesized at the Michigan State University (MSU) Macromolecular Structure Facility. Automated DNA sequencing was done at the MSU DNA Sequencing Facility. Sequences were assembled and analyzed with the *DNAStar* Software package (DNAStar Inc., Madison, WI). Protein sequence alignments were generated with the *ClustalW* Program (Thompson et al., 1994). The transcriptional start site of *TOXD* was determined by 5' RACE (Rapid Amplification of cDNA Ends) using a kit from Gibco-BRL (Frohman et al., 1988). The primer for reverse transcription (GSP1) was 5'-GTCGTCGGGTATGGCTGG-3' and the nested primer for PCR amplification (GSP2) was 5'-GAATATCACCCTTGACGG-3'.

Pulsed-field Gel Electrophoresis. Agarose gel-embedded intact chromosomal DNA was prepared as described (Ahn, 1996). Contour-clamped homogenous electric field (CHEF) electrophoresis was performed in a CHEF-DR II apparatus (Bio-Rad, Richmond, CA). Running conditions were: 0.8% chromosomal-grade agarose gel (Bio-Rad), 50 V with a 15 to 30 min switching interval for 72 hr, and a 10 to 20 min switching interval for 72 hr, in 0.5 x TBE buffer (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM EDTA, pH 7.6) at 14 °C. The gel was stained with ethidium bromide for 30 min and destained in distilled water for 30 min before being photographed under UV light and blotted to a nylon membrane.

Disruption of *TOXD* in Strain 164R10. Fungal protoplast preparation, transformation, selection, and single-spore isolation of transformants were done as described (Panaccione et al., 1992; Pitkin et al., 1996). To make the gene disruption vector pTOXDD4, a 611-bp internal fragment (from 540 bp to 1151 bp in the ORF) was amplified by PCR, filled-in, and digested with *Sac* I to generate a 320-bp fragment (from 540-bp to 860-bp in the ORF) that contains a unique *Msc* I site in the middle. This 320-bp DNA was subcloned into the *Sac I/Sma* I (filled-in) sites of pGEM-72 (Promega, Madison, WI) to make an intermediate construct pGEMTOXD. A 350-bp *Xba I/Sac* I fragment was released from pGEMTOXD and subcloned into the *Xba I/Sac* I sites of pHYG1 (Sposato et al., 1995) to make the final disruption vector pTOXDD4 (5.8 kb). pTOXDD4 was linearized with *Msc* I before transformation. Strain 164R10 was transformed with linearized pTOXDD4. Tramsformants (named T492) were selected from medium containing 120 μg/ml hygromycin.

Analysis of HC-toxin and Pathogenicity Test. HC-toxin was extracted with chloroform from 28 d-old fungal culture filtrates as described (Walton et al., 1982). Extracts were separated on thin layer chromatography (TLC) plates (Si250-PA, J. T. Baker, NJ) and detected using an epoxide-specific spray as described (Meeley and Walton, 1991). Pathogenicity was assayed by spraying 2-week-old maize (*Zea mays* L.) inbred Pr (*hm1/hm1*) with a spore suspension (1 x 10^4/ml in 0.1% Tween-20). Plants were observed daily for a week and representive leaves were photographed at 4-d post-inoculation.

RESULTS

Discovery of *TOXD*. A study of restriction fragment length polymorphisms (RFLP) of *TOXC* revealed that when two subclones of *TOXC* (pA4-1 containing the 5'-region of *TOXC* and pA6-4 containing the 3'-region of *TOXC*) were used as probes against genomic blots of SB111 DNA digested with *Eco*RV, *Sal* I, *Xba*

I, or *Xho* I, an extra band appeared to the hybridization with pA4-1 (Figure 2-1; Ahn and Walton, 1997). The 6.5-kb band from the *Eco*RV digestion was subsequently subcloned and used as a probe to obtain a 1.1-kb cDNA clone (pTOXDC4). The corresponding gene was later named as *TOXD.* A genomic clone, pTOXDG5, containing a 3.1-kb *Xho* I insert (Figure 2-6D, lane 1), was also obtained.

Characteristics of *TOXD*. A survey by Southern hybridizations showed that *TOXD* was present only in Tox2$^+$ (race 1) isolates of *C. carbonum* and not in any tested Tox2$^-$ (race 2 or 3) strains (Figure 2-2). *TOXD* cosegregated with HC-toxin production. *TOXD* has three copies in most strains surveyed, but only one copy in 164R10 (Figure 2-2A, lane 8). A 1.1-kb message was detected by Northern hybridization (data not shown). Chromosomal mapping determined that the three copies of *TOXD* are physically tightly linked to the known *TOX2* genes in strain SB111 within *TOX2* locus on a 3.5-Mb chromosome (Figure 2-3; Ahn and Walton, 1996). Like *TOXA* and *TOXC*, the expression of *TOXD* is regulated by *TOXE* (Ahn and Walton, 1998). Near-full-length cDNA (pTOXDC4) and genomic (pTOXDG5) clones were sequenced on both strands. The 5'-end of the transcript was amplified by RACE and also sequenced. Sequences were assembled using the *DNAStar* program (Figure 2-4) and deposited in GenBank with accession number X92391. There are no introns in *TOXD,* and the experimentally determined transcriptional start site is only 16 bp upstream of the translational start site. The *TOXD* open reading frame encodes a predicted 32.5-kDa protein

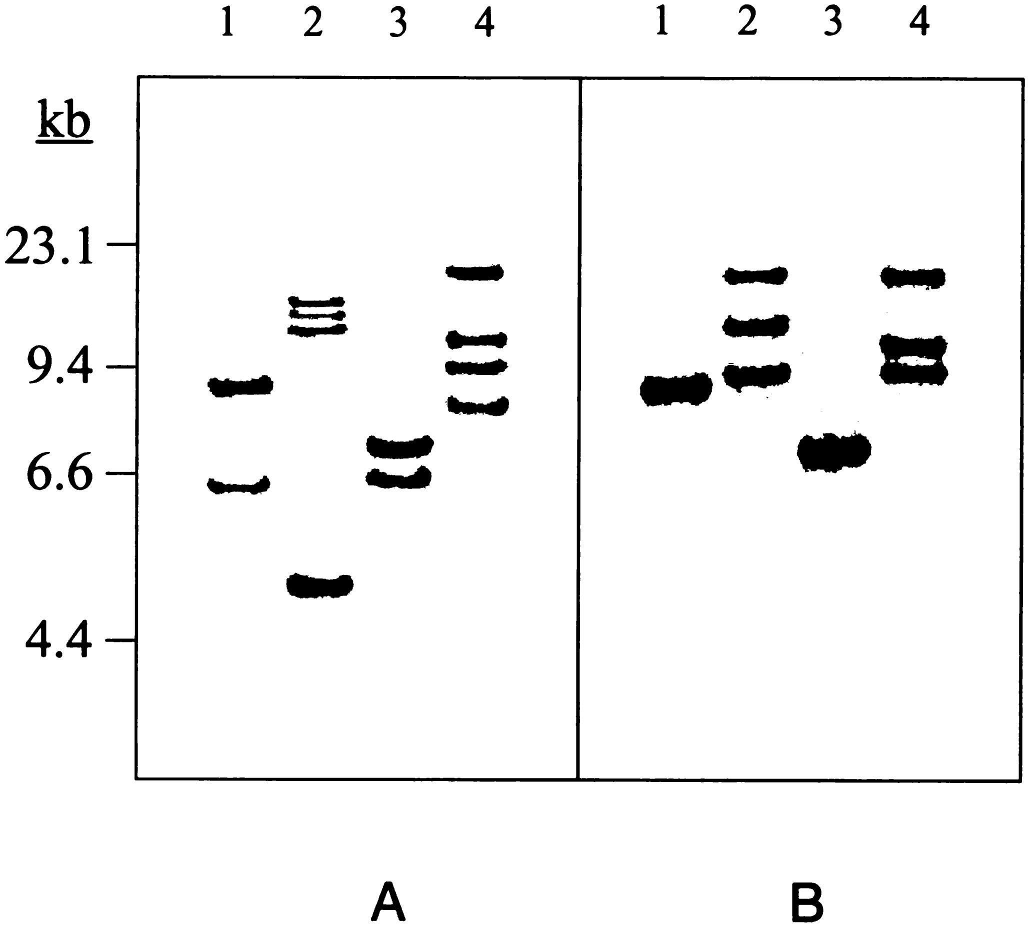


Figure 2-1. Restriction fragment length polymorphism (RFLP) of *TOXC*. Genomic DNA of SB111 was digested with (1) *Eco*RV, (2) *Sal* I, (3) *Xba* I, or (4) *Xho* I. Two identical blots were hybridized with probes of (A) pA4-1 containing the 5'-region of *TOXC* and (B) pA6-4 containing the 3'-region of *TOXC*. *Eco*RV and *Xba* I digestions could not distinguish between the three copies of *TOXC* in SB111.

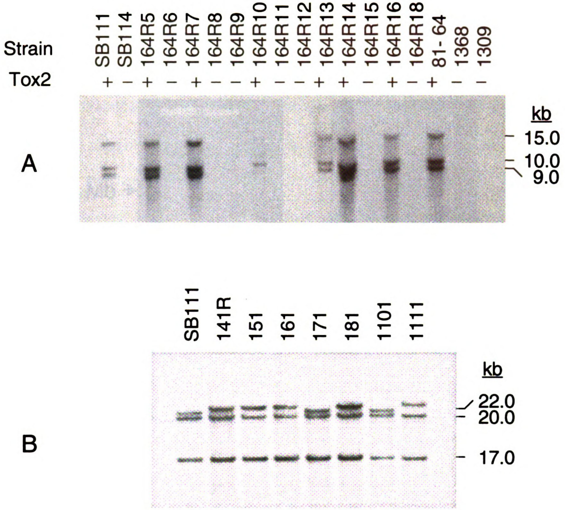


Figure 2-2. Presence of *TOXD* only in HC-toxin-producing (Tox2⁺) isolates of *C. carbonum*. Genomic DNA was digested with (A) *Bam*HI, or (B) *Eco*RI and blots were probed with *TOXD* cDNA. Strain 164R5 though 164R18 are random progeny from a cross between SB111 ($Tox2^+$) and SB114 ($Tox2^-$). Strain 81-64, 1368 and 1309 are independent race 1 ($Tox2^+$), race 2 ($Tox2^-$) and race 3 ($Tox2^-$) isolates, respectively. Strains used in blot (B) are all independent isolates of race 1 ($Tox2^+$) of *C. carbonum*.

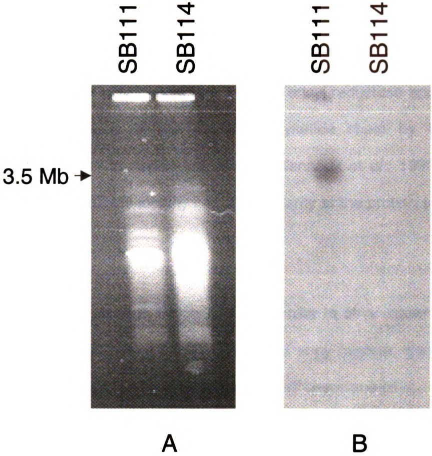


Figure 2-3. Chromosomal location of *TOXD*. Agarose gel-embedded chromosomes from SB111 (Tox2$^+$) and SB114 (Tox2$^-$) were separated in a CHEF gel, stained with ethidium bromide (A) or blotted to nylon membrane and hybridized with a *TOXD* cDNA probe (B).

with a pI of 7.72. The deduced *TOXD* protein sequence had no homology to any known protein in the public database for many years. The closest match was to three yeast hypothetical proteins (GenBank P53912, P25608, and P54009). Recently, a related gene called *lovC* (GenBank AF141925) of filamentous fungal origin was described. *lovC* encodes a putative enoyl reductase and is necessary for correct processing of the growing polyketide chain by the lovastatin nonaketide synthase in *Aspergillus terreus* (Kennedy et al., 1999). *TOXD* and *lovC* share overall 37.4% identity / 47.8% similarity at the protein sequence level (Figure 2-5).

Creation of *TOXD* Mutant: Since *TOXD* is similar to other known *TOX2* genes (*HTS1*, *TOXA*, *TOXC*, and *TOXE*) in terms of copy number, gene distribution, chromosomal location, and expression pattern (Panaccione et al., 1992; Pitkin et al., 1995; Ahn and Walton, 1996, 1998), we investigated its potential role in HC-toxin production by creating a loss-of-function mutant. Strain 164R10 has only one copy of *TOXD* and was therefore used as the recipient for targeted-gene disruption (Figure 2-6). Disruption vector pTOXDD4 (Figure 2-6A), linearized with *Msc* I, was introduced into fungal protoplasts by polyethyleneglycol (PEG)-mediated transformation. A transformant (designated as T492) was selected with hygromycin. Homologous integration of pTOXDD4 resulted in the disappearance of the 10.5-kb band of *TOXD* and appearance of two new band of 8.75 kb and 5.05 kb (Figure 2-6B, C and D). The disruption of *TOXD* was verified by RNA hybridization (Figure 2-6E). Morphologically, T492 grew and developed normally

```
   1 CACGTCAATACCGCGGTCATCCAAGAGTGCTTTGACTTTATATCCTTATATAGTTATGGT
  61 TCCTTAGTGTTTTCGATCTCTAGGCTATATTTATCTAAGACGGTGAAACATTATTGCATA
 121 AACGTTGGCTATTCTCAGATATTTGTCAGGCTCGTATTAGGTGATGCTTTCTGCTTATGC
 181 AACATGTTGTCAGTCCTCACGTCAGACAACGATATACTCATATTCACTATTTATTCTTGG
                                    |→ Transcriptional start site
 241 TCTATCATCAACCTTAGATAGGTTTAGAATCATACACATTGAACTAAGATGACCTTTCAA
   1                                                  M  T  F  Q
 301 AAGGCTATCGTCACTGAAGCCCCGCACCGTGCAAGACTCGTATCGGATCGACTGATTCCC
   5  K  A  I  V  T  E  A  P  H  R  A  R  L  V  S  D  R  L  I  P
 361 AAGTTGAGAGACGATTACATACTCGTCAGGACGGTAAGTGTTGCTCTTAACCCCACTGAT
  25  K  L  R  D  D  Y  I  L  V  R  T  V  S  V  A  L  N  P  T  D
 421 TGGAAGCATATTTTGCGTCTCTCACCCCCAGGCTGTCTGGTTGGCTGCGACTACGCTGGC
  45  W  K  H  I  L  R  L  S  P  P  G  C  L  V  G  C  D  Y  A  G
 481 ATCGTTGAGGAAGTAGGCAGGAGTGTTAAAAAACCGTTCAAAAAAGGTGATCGAGTATGC
  65  I  V  E  E  V  G  R  S  V  K  K  P  F  K  K  G  D  R  V  C
 541 GGCTTTGCTCACGGGGGTAATGCAGTTTTTTCAGATGATGGAACATTTGCCGAAGTAATT
  85  G  F  A  H  G  G  N  A  V  F  S  D  D  G  T  F  A  E  V  I
 601 ACCGTCAAGGGTGATATTCAAGCATGGATACCAGAGAACTTGAGTTTTCAGGAAGCAGCG
 105  T  V  K  G  D  I  Q  A  W  I  P  E  N  L  S  F  Q  E  A  A
 661 ACTCTCGGGGTGGGCATTAAAACCGTGGGGCAGGGGCTCTACCAAAGCCTAAAACTCTCT
 125  T  L  G  V  G  I  K  T  V  G  Q  G  L  Y  Q  S  L  K  L  S
 721 TGGCCAACGACTCCCATCGAGCACGCCGTACCTATCCTCATTTATGGTGGTTCCACAGCC
 145  W  P  T  T  P  I  E  H  A  V  P  I  L  I  Y  G  G  S  T  A
 781 ACAGGAACACTCGCAATACAGCTGGCGAAGCTATCTGGGTATCGCGTCATTACAACGTGC
 165  T  G  T  L  A  I  Q  L  A  K  L  S  G  Y  R  V  I  T  T  C
 841 TCTCCCCACCACTTTGAGCTCATGAAGTCGCTAGGAGCGGATTTGGTGTTCGATTATCAC
 185  S  P  H  H  F  E  L  M  K  S  L  G  A  D  L  V  F  D  Y  H
 901 GAAATAACATCAGCCGACCATATTCGACGGTGCACGCAAAACAAACTCAAGCTGGTTTTC
 205  E  I  T  S  A  D  H  I  R  R  C  T  Q  N  K  L  K  L  V  F
 961 GATACGATTTCGATTGACGTTAGTGCCCGGTTCTGTGACAGGGCTATGTCGACAGAAGGC
 225  D  T  I  S  I  D  V  S  A  R  F  C  D  R  A  M  S  T  E  G
1021 GGGGAATACAGCGCCTTACTCGATGTATCTATTGCGCGCACCAATATATCGAGCCGATGG
 245  G  E  Y  S  A  L  L  D  V  S  I  A  R  T  N  I  S  S  R  W
1081 ACGTTAGCCTACACCGTCCTTGGAGAGGGTTTTACTTCGGAACAGATCGTTTTCCAGCCA
 265  T  L  A  Y  T  V  L  G  E  G  F  T  S  E  Q  I  V  F  Q  P
1141 TACCCGACGACTATGAGTTCGGGAAGAAATTTTGGATGTAGCTATGGAGTTTTGTGGCCG
 285  Y  P  T  T  M  S  S  G  R  N  F  G  M  *
1201 ATGGTAGTGTCAAAGTGCATCCCCCTAGAGTTATGTCTGGTGGTTTGGCAGGGGTTTTGG
1261 ACGGGTTGGAGTTGTTACAAAAGGGGCTTGTTAGTGGGGAGAAACTTGTTTATAATGTCT
1321 GAGCCTCAATAGACCAAAGATAAATTAGTAGTCTAGTTACCAATTTCGTCGCTTCTAAGA
1381 AATTGTTTATACAGCCGGTTGATAACTATATATTATTGCTAGTATTAATGCAGCAGAACA
      †
1441 GGTAGTTGAATATAAATAATAGATAGCTAGTTAAGAACTTCATTAGCATGCAAAATGTGT
1501 TACGGG
```

Figure 2-4. Sequence of *TOXD*. The *TOXD* ORF encodes a 297-amino acid protein. The experimentally determined transcriptional start site is 16-bp upstream of the predicted translational start site. Three in-frame stop codons upstream of the translational start codon are underlined. The polyadenylation site is indicated with "†" below the appropriate nucleotide. A unique *Msc* I site in the middle of the ORF is underlined.

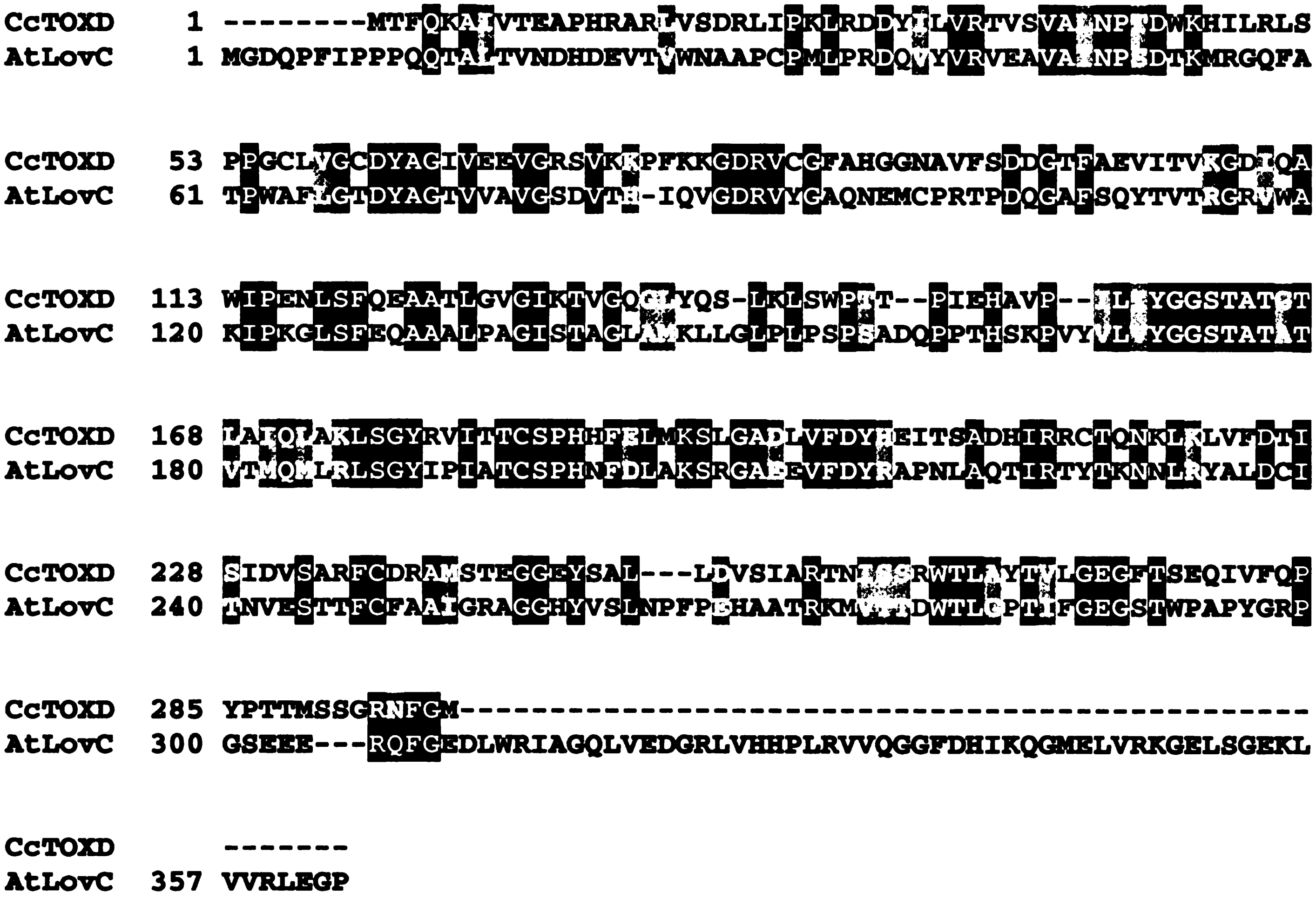


Figure 2-5. Protein sequence alignment of *TOXD* (CcTOXD) and *lovC* (AtLovC). Dark shading indicates identity; gray shading indicates similarity.

compared to the wild type 164R10. Together with the fact that *TOXD* is absent in all Tox2$^-$ isolates, we postulate that *TOXD* has no house-keeping function.

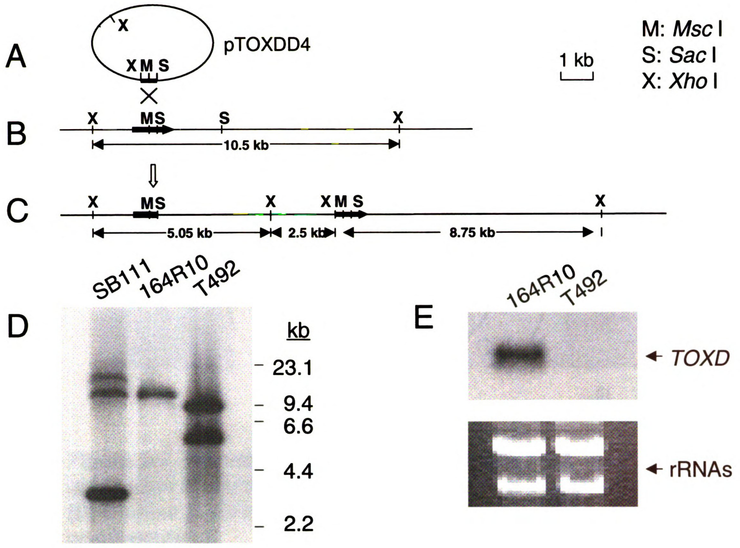
Analysis of Null Mutant: HC-toxin preparations from culture filtrates of the wild type and T492 were analyzed by TLC (Figure 2-7A). No reproducible difference was observed. Although the toxin spot in T492 appeared smaller than that of wild type, this was likely due to experimental variation. The disease phenotype of T492 was the same as wild type with regard to lesion morphology and disease development (However, no quantitative analysis was performed) (Figure 2-7B). This is consistent with the unaltered HC-toxin production in T492. Therefore, *TOXD* appears to have no, or at least no apparent, role in HC-toxin production or fungal pathogenicity.

DISCUSSION

TOXD was cloned based on its properties of being unique to Tox2$^+$ isolates of *C. carbonum*. *TOXD* is also within the *TOX2* locus region and is regulated by *TOXE*. However, the loss-of-function experiment described in this Chapter failed to establish a role for *TOXD* in HC-toxin production or fungal pathogenicity.

Although the sequence similarity between *TOXD* and *lovC* (Figure 2-5) suggests that the product of *TOXD* is likely an enzyme, no signature or motif has been detected in the deduced protein sequence. The protein encoded by *lovC*

Figure 2-6. Disruption of *TOXD* in *C. carbonum* isolate 164R10. (A) Disruption vector pTOXDD4. (B) Restriction map of *TOXD* and flanking region. (C) Map of *TOXD* with pTOXDD4 integrated. (D) DNA blot analysis of gene disruption. DNA from SB111, 164R10, and T492 was digested with *Xho* I. Blot was hybridized with a *TOXD* cDNA probe. (E) RNA blot verification of the disruption of *TOXD*. Twenty μg of total RNA was loaded in each lane. Blot was hybridized with a *TOXD* cDNA probe. Ethidium bromide-stained rRNA was used as loading control.


A
pTOXDD4
X
X M S
1 kb
M: *Msc* I
S: *Sac* I
X: *Xho* I
B
X
MS
S
X
10.5 kb
C
X
MS
X
X M S
X
5.05 kb
2.5 kb
8.75 kb
D
SB111
164R10
T492
kb
23.1
9.4
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4.4
2.2
E
164R10
T492
TOXD
rRNAs

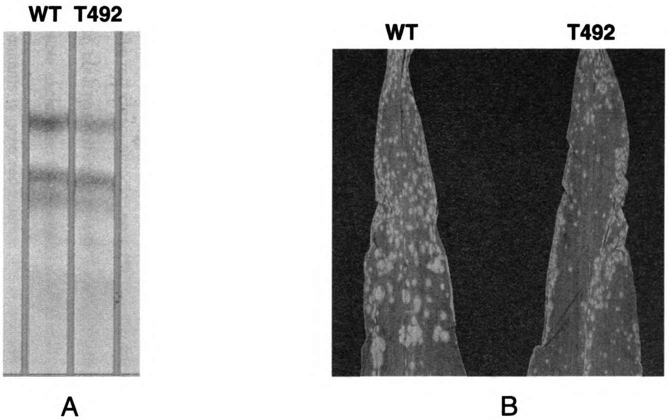


Figure 2-7. Phenotypes of *TOXD* null mutant T492. (A) TLC analysis of HC-toxin in wild type (164R10, as WT) and in *TOXD* null mutant (T492); (B) Disease phenotype of WT and T492. Leaves were photographed 4-d post-inoculation.

was proposed to have enoyl reductase activity, and to be an accessory component for the lovastatin polyketide synthase complex found in *Aspergillus terreus* (Kennedy et al., 1999). *lovC* mutant accumulated a lovastatin intermediate with double bonds in the polyketide backbone of a lovastatin precursor. Could the *TOXD* product possess a reductase activity that has a minor role in HC-toxin production? One possibility for this speculation is that the *TOXD* protein reduces the 8-carbonyl group of Aeo. HC-toxin with a reduced 8-carbonyl group (HC-toxin IV) has only 1% relative activity compared to HC-toxin I (Rasmussen, 1987). Maize lines that are resistant to HC-toxin produce a HC-toxin reductase (HCTR) encoded by *Hm1*. HCTR specifically reduces the same 8-carbonyl group in order to detoxify HC-toxin (Meeley and Walton, 1991; Johal and Briggs, 1992; Meeley et al., 1992). However, no protein sequence similarities were detected between that of *TOXD* and *Hm1*.

It is not unusual that a gene physically belongs to a gene cluster but has no apparent role in the metabolic pathway. In *C. heterostrophus* race T, a putative reductase- encoding gene (*RED1*) was cloned and shown to be linked to *PKS1*, but it has nothing to do with T-toxin biosynthesis (Kodama et al., 1999). A plausible explanation for the unalteration of toxin production in *TOXD* or *RED1* null mutants is gene redundancy. There are likely many enzymes possessing reductase activity in fungal cells. When *TOXD* or *RED1* is mutated, other reductases could process the normal toxin biosynthetic steps. Therefore no phenotype change would be observed. Otherwise, *TOXD* (and maybe also

RED1) could be regarded as remnant genes that happen to have similarities to known *TOX* genes, but no longer are needed for HC-toxin or T-toxin production, modification, or fungal pathogenicity. Nevertheless, *TOXD* has been useful as a genetic marker for mapping the *TOX2* locus in *C. carbonum* (Ahn and Walton, 1996) and for successfully cloning two more genes (*TOXF* and *TOXG*) that have proven roles in HC-toxin production (Chapters 3 and 4).

CHAPTER 3

A PUTATIVE BRANCHED-CHAIN AMINO ACID AMINOTRANSFERASE GENE REQUIRED FOR HC-TOXIN BIOSYNTHESIS AND PATHOGENICITY IN *COCHLIOBOLUS CARBONUM*

The gene sequence reported in this Chapter has been deposited in GenBank with accession number AF157629.

The content of this Chapter is a re-formatted version of a manuscript submitted to *Microbiology* (Cheng et al., 1999).

ABSTRACT

The cyclic tetrapeptide HC-toxin is required for pathogenicity of the filamentous fungus *Cochliobolus carbonum* on maize. HC-toxin production is controlled by a complex locus, *TOX2*. Here we report the isolation and characterization of a new gene in the *TOX2* locus, *TOXF*, and show that it is specifically required for HC-toxin production and pathogenicity. *TOXF* is present as two or three copies in all HC-toxin-producing (Tox2$^+$) isolates and is absent in toxin non-producing strains. The deduced amino acid sequence of *TOXF* has moderate homology to many known or putative branched-chain amino acid aminotransferases from various species. A strain of *C. carbonum* with all copies of *TOXF* disrupted grew normally but lost HC-toxin production and pathogenicity. We propose that *TOXF* has a biosynthetic role in HC-toxin synthesis, perhaps to aminate a precursor of Aeo (2-amino-9,10-epoxi-8-oxo-decanoic acid).

INTRODUCTION

Host specific toxins, which are produced exclusively by fungi, are generally low molecular weight secondary metabolites with diverse structures. They are critical determinants of virulence or pathogenicity in many plant disease interactions (Walton, 1996). The host-selective toxin made by race 1 isolates of *Cochliobolus carbonum*, called HC-toxin, selectively affects maize (*Zea mays* L.)

lines of genotype *hm1/hm1*. Structurally, HC-toxin is a cyclic tetrapeptide, cyclo(D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-9,10-epoxi-8-oxo-decanoic acid.

Previous studies on the Mendelian and molecular genetics of HC-toxin production have shown that HC-toxin production is controlled by a Mendelian locus, *TOX2*, but that this locus has a complex molecular structure (Nelson and Ullstrup, 1961; Scheffer et al., 1967; Ahn and Walton, 1996). All of the known genes necessary for HC-toxin production are present only in HC-toxin-producing (Tox2$^+$) isolates in multiple functional copies, are dedicated to HC-toxin production, and, with one exception, are linked over a ~540 kb region of one chromosome (Ahn and Walton, 1996, 1998). *TOX2* genes include *HTS1*, which encodes a 570-kDa tetrapartite non-ribosomal peptide synthetase (NRPS) called HC-toxin synthetase (HTS) (Panaccione et al., 1992; Scott-Craig et al., 1992); *TOXA*, encoding a putative HC-toxin transporter of the major facilitator superfamily (Pitkin et al., 1996); and *TOXC*, encoding a fatty acid synthase β subunit (Ahn and Walton, 1997). *TOXE* encodes a regulatory protein required for expression of *TOXA* and *TOXC* (Ahn and Walton, 1998). Another gene, *TOXD*, is also Tox2$^+$-unique, linked to the other *TOX2* genes, and co-regulated by *TOXE*, but *TOXD* has no defined role in HC-toxin biosynthesis (Chapter 2. Cheng and Walton, unpublished results). A homolog of *TOXD* was recently shown to be necessary for correct processing of the growing polyketide chain by the lovastatin nonaketide synthase in *Aspergillus terreus* (Kennedy et al., 1999).

The known genes of *TOX2* can account for the synthesis and assembly of the components of HC-toxin other than the unusual amino acid Aeo. Biogenetically, Aeo is a fatty acid or polyketide (Cheng et al., 1999. See Appendix), and the specific requirement of *TOXC* for toxin production argues that the decanoic acid backbone of Aeo is a fatty acid. Nothing is known about the other steps in Aeo biosynthesis, but at least two oxidations (to produce the 8-carbonyl and the 9,10-epoxide) and an amination (at the 2- position) must also occur.

We describe here a strategy using bacterial artificial chromosomes (BACs) to search for additional genes of *TOX2*. It is based on the assumption that new *TOX2* genes would be physically linked, but not clustered, to the known *TOX2* genes, and would also be restricted in their taxonomic distribution to Tox2$^+$ isolates of *C. carbonum*. Here we describe the identification of *TOXF*, a new *TOX2* gene.

METHODS

Fungal Strains. SB111 (ATCC 90305) and SB114 are standard Tox2$^+$ and Tox2$^-$ laboratory strains, respectively, and 164R10 is a Tox2$^+$ progeny of a cross between them (Ahn and Walton, 1997).

Bacterial Artificial Chromosome (BAC) Library Construction. The BAC vector pBACwich, a generous gift of the Clemson University Genomics Institute

(Zhu et al., 1997), was modified by the insertion of restriction sites for *Asc*I and *Pme*I (Figure 2-1). Two oligonucleotides, 5'-AGCTGTTTAAACTGGCGCGCC-3' and 5'-AGCTGGCGCGCCAGTTTAAAC-3', were mixed at equimolar concentration, heated at 95 ° C for 3 min, and annealed slowly to form a complementary DNA linker. This linker was subcloned in-frame into the unique *Hin*dIII site of pBACwich to make a new vector called pBACocta. Fungal chromosomal DNA was embedded and digested in low-melting agarose as described (Ahn and Walton, 1996). Proteinase K was removed from the agarose by washing with 50 mM EDTA, followed by 10 mM Tris/1 mM EDTA (TE) (pH 8.0), and then with the appropriate buffer for the particular restriction enzyme. DNA was digested with 10 U *Asc* I per gel block for 24 hr at 37 °C, and the gel blocks were then digested with β-agarase I (New England BioLabs, Boston, MA) following the manufacture's instructions. DNA fragments were gently precipitated with ethanol, dried under vacuum, and dissolved in TE. Ligation reactions contained 5.0 μg of pBACocta linearized with *Asc* I and dephosphorylated and 1 μg of digested DNA in a volume of 20 μl, and were incubated for 15 hr at 16 °C. Each aliquot (2 μl) of ligation mixture was transformed into 40 μl of ElectroMAX DH10B *E. coli* cells (Gibco-BRL, Bethesda, MD) by electroporation (2.5 kV, 25 F, 100 Ω, 0.1 cm cuvette) using a GenePulser apparatus (Bio-Rad, Richmond, CA). Cells were transferred immediately to a new tube, diluted with 1 ml SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose, pH 7.0), incubated at 37 °C for 1 hr, and spread on LB plates with 12.5 μg/ ml chloramphenicol, 0.75 μg/ml 5-bromo-4-

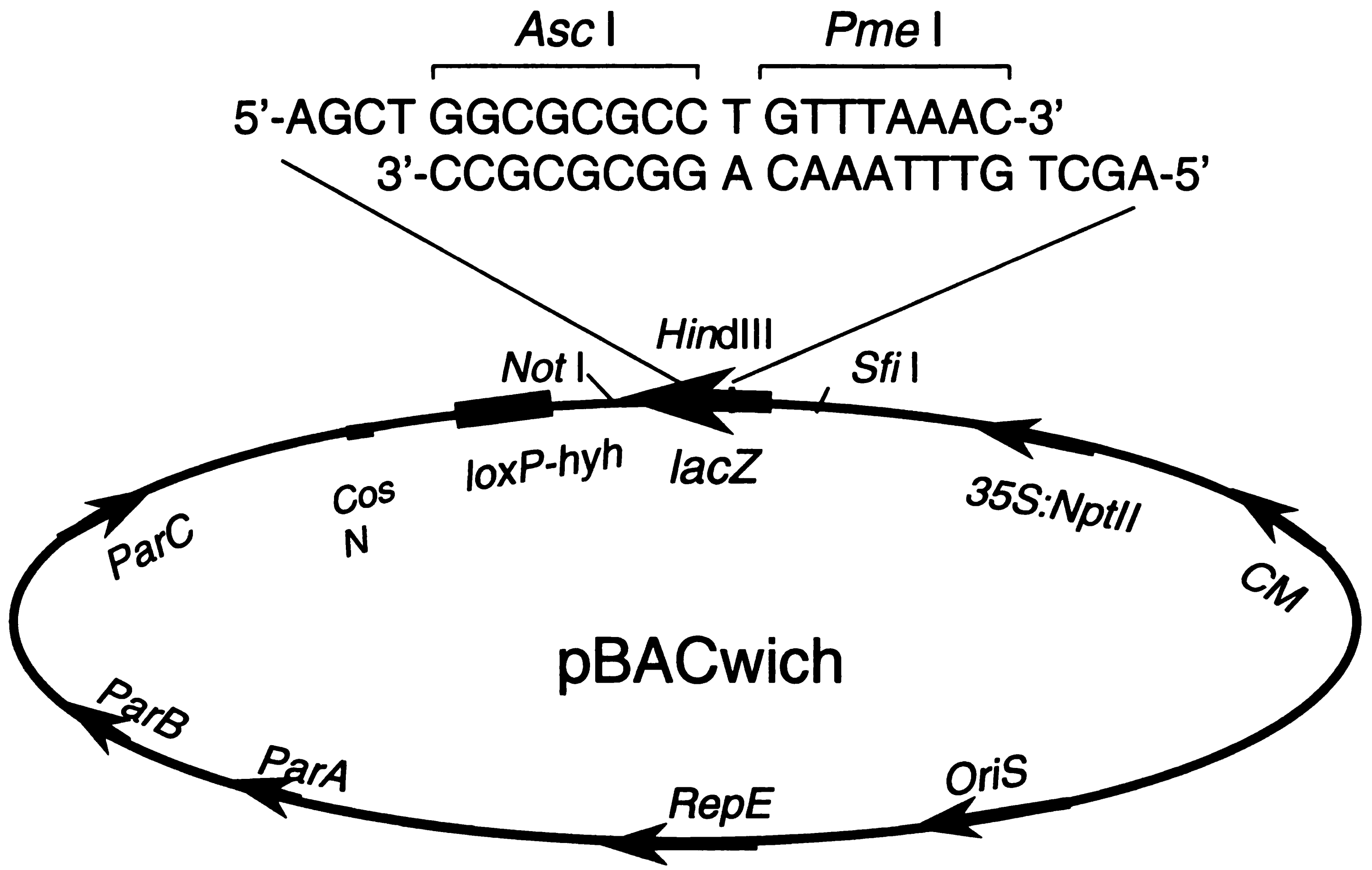


Figure 3-1. Construction of pBACocta. A 25-bp DNA linker containing *Asc* I and *Pme* I sites was inserted in-frame into the unique *Hind*III site of pBACwich. The *lacZ* gene retained normal function and was used as selection marker for recombinants.

chloro-3-indolyl-β-D-galactopyranoside (X-gal), and 100 μg/ml isopropyl-β-D-thiogalactopyranoside (IPTG). After 30 hr growth at 37 °C, white recombinant clones were transferred to 96-well microtiter plates. Each well contained 200 μl of LB medium with 12.5 μg/ml chloramphenicol. Plates were incubated for 24 hr at 37 °C, then stored at -80 °C.

Nucleic Acid Manipulations and Analysis. Fungal DNA and RNA isolation was previously described (Pitkin et al., 1996). DNA and RNA blotting, probe labeling, hybridization, cDNA and genomic library screening, and DNA subcloning were done following standard procedures (Sambrook et al., 1989). Oligonucleotides were synthesized by the Michigan State University Macromolecular Facility.

The method for BAC DNA extraction was adopted from Woo et al. (1994). BACs were analyzed by Contour-clamped homogenous electrical field (CHEF) electrophoresis (Bio-Rad) (Ahn and Walton, 1996). One μg of each BAC was digested with 2 U *Not* I and loaded on a gel of 1 % chromosome-grade agarose (Bio-Rad). The gels were run at 150 V for 15 hr in 0.5 x TBE buffer at 14 °C with a linearly varied 1- to 22-sec switching interval. Gels were subsequently stained with ethidium bromide for 20 min and destained in water before being photographed. Lambda concatemers (New England BioLabs) were used as molecular size standards.

DNA sequencing was done by automated fluorescence sequencing at the Yale University W. M. Keck Foundation Biotechnology Resource Laboratory and the Michigan State University DNA Sequencing Facility. Sequences were assembled and analyzed with the *DNAStar* Software package (DNAStar Inc., Madison, WI). Multiple sequence alignments were produced with the *ClustalW* Program (Thompson et al., 1994) and decorated with *BOXSHADE*.

The transcriptional start site of *TOXF* was determined by 5' RACE using a kit from Gibco-BRL (Frohman et al., 1988). The primer for reverse transcription (GSP1) was 5'-GGGGTTGAAGTCCAATTTGACAAC-3', and the nested primer for PCR amplification (GSP2) was 5'-GACGAAACAAGGGTCTGACCAGTA-3'. Two independent RACE products were sequenced.

Creation of Targeted Mutants. Fungal protoplast preparation, transformation, selection, and single-spore isolation of transformants were done as described (Panaccione et al., 1992; Pitkin et al., 1996). To make the disruption vector pTOXFD1, the central part of the coding region from a cDNA copy of *TOXF* (the fragment size was 531 bp; this corresponds to +644 bp to +1236 bp in the genomic sequence, taking into account the presence of one intron in the genomic sequence) was amplified by PCR and subcloned into the *Sal* I/*Xho* I sites of pAMD72 (Pitkin et al., 1996), which contains the *A. nidulans amdS* gene for acetamide utilization (Hynes et al., 1983), to make pTOXFD1 (7.0 kb). pTOXFD1 was linearized with *Bbr*PI before transformation. To make the replacement vector pTOXFR1, pAATG1, which contains a genomic copy of *TOXF*, was trimmed with *Sma*I and *Hpa* I to delete the 5' upstream region and re-ligated to make the intermediate construct pAATM3. The 383-bp internal *Msc* I/*Bbr*PI fragment of pAATM3 was then replaced by a hygromycin-resistance cassette (composed of the *E. coli hph* gene encoding hygromycin phosphotransferase driven by the *Aspergillus nidulans trpC* promoter) from plasmid pCB1003 (Carroll et al., 1994) to make the final replacement vector pTOXFR1 (6.1 kb). The fragment (3.1 kb) containing the *hph* cassette plus *TOXF* DNA was released from pTOXFR1 by digestion with *Bam*HI and *Pst* I and used for transformation.

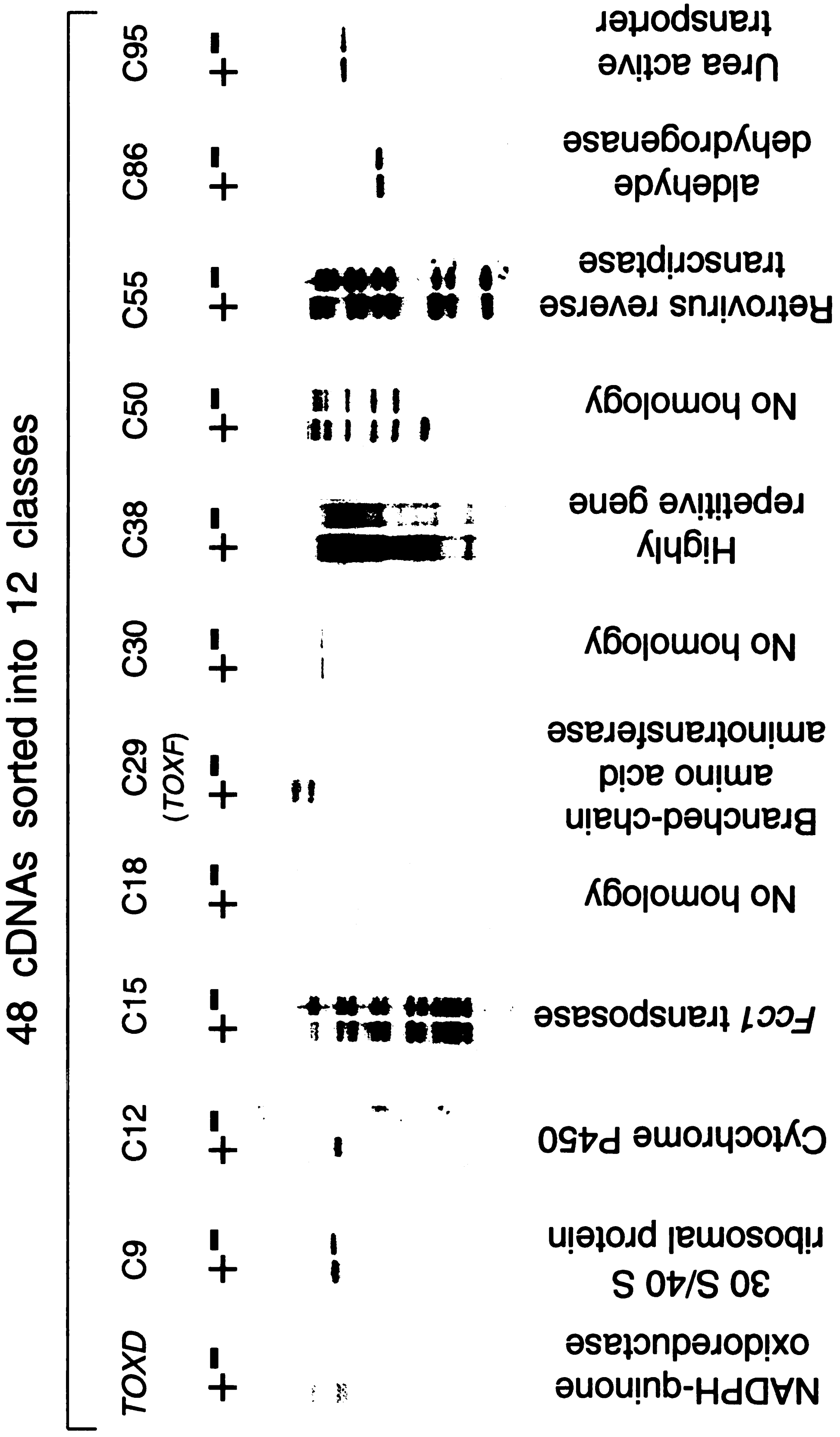
Analysis of Mutants. HC-toxin was extracted and analyzed by thin layer chromatography (TLC) as described (Walton et al., 1982; Meeley and Walton, 1991). Pathogenicity was assayed on maize inbred Pr (genotype *hm1/hm1*) by spraying with conidia (Panaccione et al., 1992; Ahn and Walton, 1997).

RESULTS

BAC Library Construction and Screening. The BAC library was constructed from genomic DNA of *C. carbonum* cut to completion with *Asc* I. A total of 3880 BAC recombinants were identified by blue/white color selection. Based on a sample of 20, the average insert size was ~55 kb. The BAC library was screened with a mixture of probes corresponding to the known *TOX2* genes (*HTS1*, *TOXA*, *TOXC*, *TOXD*, and *TOXE*). Four BACs with inserts totaling 220 kb were obtained. These BACs were mapped within the *TOX2* region by hybridizing with probes corresponding to the different copies of *HTS1*, *TOXA*, *TOXC*, *TOXD*, and *TOXE*, which can be distinguished from each other by restriction enzyme length polymorphisms (Ahn and Walton, 1996).

Analysis of *TOXF*. BAC A24C1, which harbors an insert of ~70 kb and contains copy 3 of *TOXD*, was used as a probe to screen a *C. carbonum* cDNA library. The hybridizing cDNAs were sorted into 12 classes (Figure 3-2). One representative of each class was tested by Southern hybridization to determine its distribution in Tox2$^+$ vs. Tox2$^-$ isolates. Three of the cDNAs (*TOXD* itself, C12, and C29) were present in SB111 (Tox2$^+$) but not in SB114 (Tox2$^-$). Five of the cDNAs (C9, C18, C30, C86, C95) were apparently single-copy genes common to both SB111 and SB114. The presence of single-copy common genes within the *TOX2* locus would have interesting implications for the evolution of *TOX2*; however, the chromosomal locations of these common genes have not yet been confirmed by further studies. The other four cDNAs represent moderate to highly repetitive genes. All of the cDNAs shown in Figure 3-2 were sequenced for ~500 bp from the 5' end and the sequences compared against the non-redundant databases. Only those sequences with BLAST scores higher than 80 and E < 0.001 are indicated with homology notations. The novel Tox2$^+$-unique genes C12 and C29 were further investigated. The sequence of C12 showed moderate similarity to several cytochrome P450 genes from other fungi (GenBank accession numbers X82490 of *Fusarium oxysporum*, S09643 of *Neurospora crassa*, and Y17243 of *Gibberella fujikuroi*). The predicted product of C12 contains a conserved cytochrome P450 cysteine heme-iron ligand signature. Gene C12 is present in most Tox2$^+$ isolates, including SB111, and is absent in all tested Tox2$^-$ isolates. However, C12 is absent in isolate 164R10, which produces

Figure 3-2. Genes identified on BAC clone A24C1. The entire BAC clone was radiolabeled and used to screen a *C. carbonum* cDNA library. Positive cDNAs were sorted by cross-hybridization into 12 classes. One representative of each class was tested by DNA hybridization for presence in fungal isolates SB111 ($Tox2^+$, indicated by +) and SB114 ($Tox2^-$, indicated by -). Genomic DNA was digested with *Bam*HI, separated on a 0.9 % agarose gel, blotted to a nylon membrane, and probed individually. Putative functions based on partial sequencing of each cDNA are indicated. The gene corresponding to C29 was later named *TOXF*.


48 cDNAs sorted into 12 classes
TOXD
C9
C12
C15
C18
C29
(TOXF)
C30
C38
C50
C55
C86
C95
+
-
NADPH-quinone oxidoreductase
30 S/40 S ribosomal protein
Cytochrome P450
Fcc1 transposase
No homology
Branched-chain amino acid aminotransferase
No homology
Highly repetitive gene
No homology
Retrovirus reverse transcriptase
aldehyde dehydrogenase
Urea active transporter

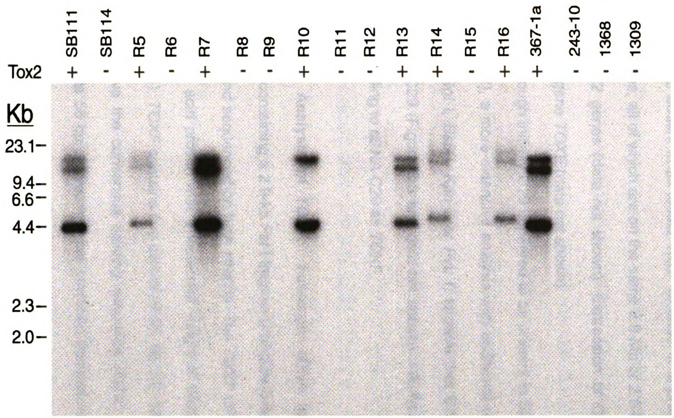


Figure 3-3. Presence of *TOXF* only in HC-toxin-producing (Tox2$^+$) isolates of *C. carbonum*. Genomic DNA was digested with *Bgl* I and the blot was probed with the full-length *TOXF* cDNA insert of pAATC1. "**+**" and "**-**" indicate whether a particular isolate makes HC-toxin or not. Isolates SB111 and 367-1a are Tox2$^+$; SB114, 243-10, 1368, and 1309 are Tox2$^-$. R5 though R16 are the random ascospore progeny of a cross between SB111 and SB114; their full strain designations are 164R5 though 164R16.

HC-toxin (Ahn and Walton, 1997). This fact excludes it having an essential role in HC-toxin biosynthesis.

cDNA C29 is present in all Tox2$^+$ isolates tested (Figure 3-3 and data not shown) and absent in all tested Tox2$^-$ isolates. It is present in most isolates in three copies, all of which are on the same 3.5-Mb or 2.5-Mb chromosome as the other *TOX2* genes (data not shown). Expression of C29 requires the *TOX2* regulatory gene *TOXE* (data not shown).

Although initially C29 appeared to be present in SB111 in only two copies (Figure 3-2), a more extensive analysis with additional restriction enzymes (*Aat* II, *Avr* II, *Bgl* I, *Bst*XI, *Kpn* I, or *Pvu* I) showed that SB111 actually has three copies of C29 (Figure 3-3 and data not shown). We have designated the gene corresponding to cDNA C29 as *TOXF*.

Sequence Analysis of *TOXF*. Full-length cDNA (pAATC1) and genomic (pAATG1, containing a 2.9-kb *Pst* I fragment) (Figure 3-6a) copies of *TOXF* were obtained and sequenced on both strands. The *TOXF* ORF encodes a predicted 357-amino acid protein with a molecular weight of 39.6 kDa and a pI of 6.60 (Figure 3-4). *TOXF* contains five introns of 58, 93, 52, 126, and 56 bp. All of the introns have the consensus splicing sequence (GT/C...AG). Transcription of *TOXF* starts 58 bp upstream of the predicted translation start site. *TOXF* is not predicted to have a signal peptide and to be located in the cytosol (Nakai and Kanehisa, 1992; Nielsen et al., 1997). A cytosolic location is consistent with the predicted pI of *TOXF* gene product (designated TOXFp), because mitochondrial branched-chain amino acid aminotransferases (BCATs; E.C.2.6.1.42) have basic pI's whereas cytosolic BCATs have acidic pI's (Eden et al., 1996).

BLAST analysis (Altschul et al., 1997) of *TOXF* indicated a significant similarity between TOXFp and BCATs of bacteria, fungi, nematodes, plants, and mammals. The best match was to a putative BCAT of *Haemophilus influenzae* (32% amino acid identity; GenBank U32798). Of those sequences correponding to biochemically defined BCATs, TOXFp was most closely related to the cytosolic BCAT of *Saccharomyces cerevisiae* (26% amino acid identity; GenBank X78961; Eden et al., 1996; Kispal et al., 1996) (Figure 3-5) and to the human cytosolic BCAT (25% identity; GenBank U35774; Hutson et al, 1995). TOXFp is 22% identical to the BCAT of *E.coli* (encoded by *ilvE*) (Figure 3-5). Weaker amino acid similarity was found between TOXFp and bacterial D-amino acid aminotransferases, which are structurally related to BCAT (Tanizawa et al., 1989).

From the crystal structure of *E. coli* BCAT, nine residues at the active site have been identified (Okada et al., 1997). These amino acids are strongly conserved in all known BCATs. All but one of these amino acids are also conserved in the same relative positions in TOXFp (Figure 3-5). The single exception is a conservative substitution of Leu for Val at amino acid 144 (corresponding to amino acid 110 in *E. coli* BCAT). Conserved amino acids in TOXFp include the two critical amino acids that bind pyridoxal-5'-phosphate (PLP), Glu227 and Lys188 (Glu 194 and Lys160, respectively, in *E. coli* BCAT) (Okada et al., 1997).

Figure 3-4. DNA and deduced protein sequence of *TOXF*. The experimentally determined transcriptional start site is indicated by an arrow. The five introns are typed in lower -case italic letters. The polyadenylation site is indicated by "^" under the appropriate nucleotide.

```
AATCTTACGTAATCTGAGTTCATCTCGCGTATCCAATCACGTAAGCGATGACTCTATCTA  -115
                                                        ➦
TCTTCGACAAGGAGCTGTTCTTCTATAACTTATGGTAAACCCTCTTATACTTAATTCAAG   -55
AACAAATCAGCGAAAAGGTTAACTAATCTCTAAAGGTTCTCAGCCATCGACATCATGGCG     6
                                                       M  A      2
ATACCCTTGCCTGCATTTTACAgtatgttagtggaagttcgaattgttaactagcatttg    66
 I  P  L  P  A  F  Y                                             9
atcttaattttcccatttagAATGGGATGTTTACGATAGTAAATTAAACAATGgtatgta   126
                    K  W  D  V  Y  D  S  K  L  N  N             20
ctggcaatcacaccgccatagacttctattactacttttgtttacacctatcaacaagcc   186
atgtgtgctgaatttgctgtctccagTACACGGACACGTAGAATGTCGATACACAGCCCA   246
                          V  H  G  H  V  E  C  R  Y  T  A  Q    32
AACGGGCTACTGGTCAGACCCTTGTTTCGTCCAAAGCCCTTTTCTGAGCGTCCATGGTCT   306
  T  G  Y  W  S  D  P  C  F  V  Q  S  P  F  L  S  V  H  G  L    52
TGCACCGGGGCTAAATTATGgtaaatgtagctagtatcgtcgcatcagaacactgcttat   366
  A  P  G  L  N  Y                                              58
atataactttagGGCAGCAAGTATACGAAGGAATTCAAGgcaagtaagctatgttttgaa   426
            G  Q  Q  V  Y  E  G  I  Q                           67
acaagtctcctaggtagcatggtgtaggtcttgtctgatacgtttaatcagctggatatg   486
agaacggtcttaagatgagtaaatgcaatgctgattgtgtgttagCTCGGCGAACCGCTC   546
                                             A  R  R  T  A      72
GTAACGAAATCTTGATATTCCGTCCCGGCGCGAGTGCCGATCGTATGGCCAAGTCAGCAA   606
R  N  E  I  L  I  F  R  P  G  A  S  A  D  R  M  A  K  S  A      92
CGGCTGTCTCCATGCCCCCGGTGCCCTACGAATTGTTCGTGAGATCGGTTCACATGGCTG   666
T  A  V  S  M  P  P  V  P  Y  E  L  F  V  R  S  V  H  M  A     112
TTGCTTTGAACGCCGATTATGTTCCTCCACATGATTTTCATGGGAGCATGTATATTCGTC   726
V  A  L  N  A  D  Y  V  P  P  H  D  F  H  G  S  M  Y  I  R     132
CGTGCCAATTTGGATCGAGTTGTCAAATTGGACTTCAACCCCCCGACGAGTTTATCTTCT   786
P  C  Q  F  G  S  S  C  Q  I  G  L  Q  P  P  D  E  F  I  F     152
GCGTCTTCGTACAGCCACACATTGCGTTGCACGGGCACGGTTCCCTGCGGGCGCTGATAG   846
C  V  F  V  Q  P  H  I  A  L  H  G  H  G  S  L  R  A  L  I     172
CAGAGGACTTTGACCGGGCCGCTACGAGGGGCACTGGTCATGTCAAAATAGGCGGTAATT   906
A  E  D  F  D  R  A  A  T  R  G  T  G  H  V  K  I  G  G  N     192
ATGCCCCCGTGATAAGGTGGACACAGTCAGCGAAGAAGGAGGAAAATGGTGGGTGGGACG   966
Y  A  P  V  I  R  W  T  Q  S  A  K  K  E  E  N  G  G  W  D     212
TTTTGCTCCACGTGGACAGCAAAACGCAAACGCGGATTGACGAATTCAGTACTTCAGCAT  1026
V  L  L  H  V  D  S  K  T  Q  T  R  I  D  E  F  S  T  S  A     232
TTATTGGGACAAAATATGCAGAGGAACAGAATGAGCCACCACAAATCATCTTGCCAGAAA  1086
F  I  G  T  K  Y  A  E  E  Q  N  E  P  P  Q  I  I  L  P  E     252
GTGCAGCGGCTATTCAAAGCATCACTTCGGATTCCGTCGCATGGTTAGCGAAGTCATTCG  1146
S  A  A  A  I  Q  S  I  T  S  D  S  V  A  W  L  A  K  S  F     272
GCTGGAATATTGTCAAACAGCCTgtaagcctgtgtttcttagatgttattttacgcgggc  1206
G  W  N  I  V  K  Q  P                                         280
atcgtctaacacatcaaagGTCACCATTGATGAACTTGCTTCCTTATCAGAAGTAATGGC  1266
                    V  T  I  D  E  L  A  S  L  S  E  V  M  A   294
TGTGGGCACCGCTGCCGGTCTTGTGCCTGTTAGTTGTATACGTCATAACTCCACCAATCG  1326
  V  G  T  A  A  G  L  V  P  V  S  C  I  R  H  N  S  T  N  R   314
GACGTTTGAATTCCCTTCTGCGGGACCTATGTACAGACAGCTCAAGGAAACGCTAGATAA  1386
  T  F  E  F  P  S  A  G  P  M  Y  R  Q  L  K  E  T  L  D  N   334
TATACAACGGGGCAGGTCAAGTGATTCGTTTGGATGGTGTGAGAAACTTCGATACGCCGA  1446
  I  Q  R  G  R  S  S  D  S  F  G  W  C  E  K  L  R  Y  A  E   354
ATTCGTACAATAGCATAGCAGTTTAAAATTTTCTGTTTAACTAATTACCTGTACACCTGT  1506
  F  V  Q  *                                                   357
CAGATTTGACGGGTCTTCAGTCATGGGGTCAAAAACAGGTGTTCATTGTTTCTATCTCCT  1566

                  ^
GAGGTGTCGCCTTTTTGTAGCTTTTTCTTTTCTTTTGTTTCCTTGGCCTCATGCATGGGC  1626
CTCAACTAAAGCCTTACTTCTGACACTTATTCATGTACATATGTATGTATAGGCAGAACT  1686
ACTCCCATTTCTTGTGTTCATTGCACCACATTTCCTCTTTCTGCAG                1733
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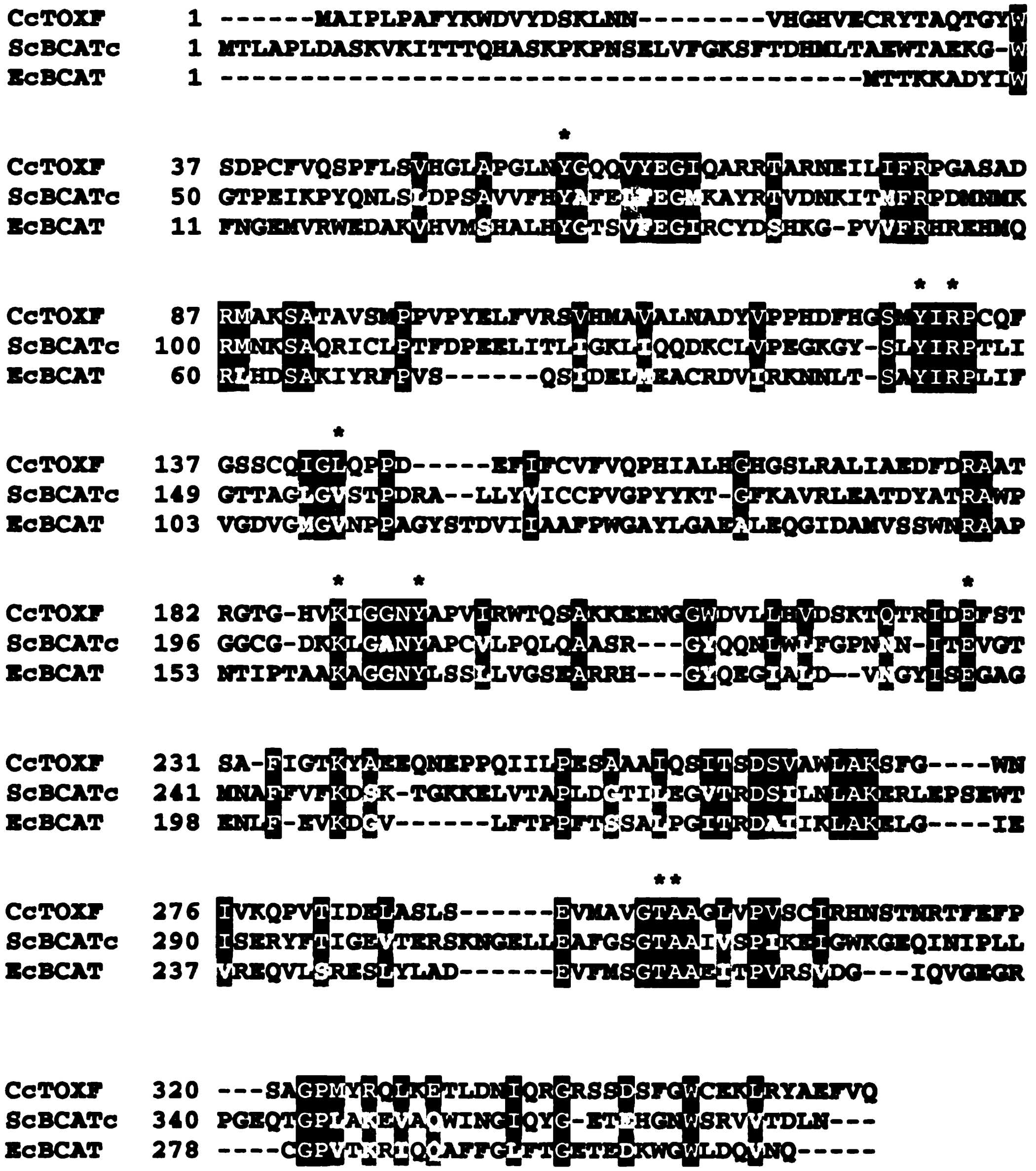
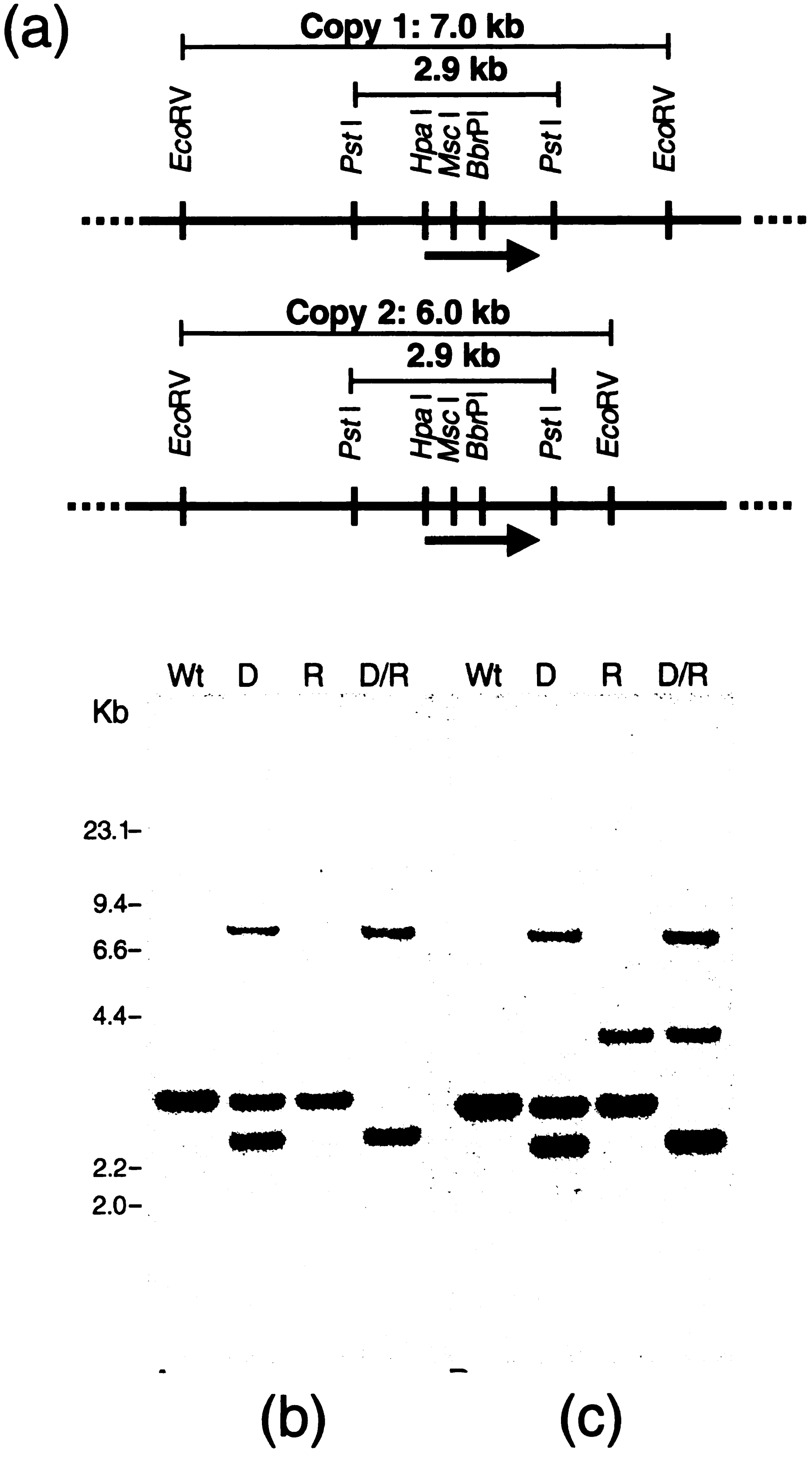


Figure 3-5. Sequence alignment of TOXFp with BCATs from other organisms. Alignment was performed with *ClustalW* and decorated with *BOXSHADE*. Black shading indicates identical amino acids between TOXFp and at least one of the others; light shading indicates similar amino acids. The origin of amino acid sequences is: CcTOXF from *C. carbonum* (GenBank AF157629), EcBCAT (GenBank P00510) from *E. coli*, and ScBCATc (GenBank P47176), the cytosolic form from *S. cerevisiae*. Conserved residues of all BCATs are indicated by asterisks.

Gene Disruption and Replacement of *TOXF*. To test the role of *TOXF* in HC-toxin biosynthesis, both copies of *TOXF* in isolate 164R10, which is a fully pathogenic Tox2$^+$ isolate with two copies of *TOXF* (Figure 3-3 lane 8), were mutated by homologous recombination-mediated disruption (that is, single-crossover) or replacement (double-crossover) (Figure 3-6). Copy 1 of *TOXF* was disrupted using vector pTOXFD1 to create strain D. Transformants were selected for growth on acetamide and genetically purified by two rounds of single-spore isolation. Transformants were tested by probing DNA blots with a 383-bp fragment of *TOXF*, which is completely contained within the 531-bp *TOXF* fragment present in pTOXFD1 (7.0 kb). Homologous integration of pTOXFD1 was predicted to result in the disappearance of one copy of the 2.9-kb bands (*Pst* I does not distinguish between the two copies of *TOXF*) and appearance of two bands of 6.9 kb and 2.5 kb, which was observed (Figure 3-6b). (The *amdS* gene contains three *Pst* I sites within 0.6 kb of each other).

Strain D as well as 164R10 were transformed with the 3.1-kb fragment from the replacement vector pTOXFR1 (6.1 kb). A double-crossover homologous integration event was predicted to result in the disappearance of one of the 2.9-kb *Pst* I hybridizing bands in 164R10 when probed with the 383-bp internal fragment of *TOXF* that had been replaced by the 1.4-kb *hyg* gene in pTOXFR1. This was observed in strain R. Homologous integration of pTOXFR1 into the remaining copy (copy 2) of *TOXF* in strain D was predicted to result in the complete disappearance of the 2.9-kb *Pst* I fragment, as was found in strain D/R (Figure 3-6b).

Figure 3-6. Targeted mutation of *TOXF*. (a) Restriction maps of the two copies of *TOXF* in 164R10. (b) and (c): DNA blots of wild type 164R10, transformant D in which copy 1 of *TOXF* has been disrupted by homologous integration of pTOXFD1, transformant R in which an internal portion of copy 2 of *TOXF* has been replaced by pTOXFR1, and transformant D/R in which transformant D was subsequently transformed with pTOXFR1.
(b) probed with the 383-bp internal fragment of *TOXF*.
(c) probed with the *TOXF* cDNA (pAATC1).


(a)
Copy 1: 7.0 kb
2.9 kb
EcoRV
Pst I
Hpa I
Msc I
BbrPI
Pst I
EcoRV
Copy 2: 6.0 kb
2.9 kb
EcoRV
Pst I
Hpa I
Msc I
BbrPI
Pst I
EcoRV
Wt D R D/R Wt D R D/R
Kb
23.1
9.4
6.6
4.4
2.2
2.0
(b)
(c)

The conclusions drawn from probing the transformants with the 383-bp internal fragment were confirmed by re-probing with the entire *TOXF* cDNA. In the replacement strain R and the double mutant D/R, a new band of 3.9 kb was visible. This 3.9-kb signal corresponds to the original 2.9-kb *Pst* I fragment plus the 1.4-kb *hyg* gene cassette minus the 383-bp fragment of *TOXF* that had been deleted (Figure 3-6c).

Phenotypes of *TOXF* Mutants. The single (D and R) and double *TOXF* mutants (D/R) showed normal growth and development on V8-agar or in modified Fries' liquid medium (Walton et al., 1982). As expected, the *TOXF* mRNA was absent in the D/R strain (Figure 3-7a). Strain D/R also failed to make detectable HC-toxin in culture (Figure 3-7b). Mutation of one or the other copy of *TOXF* (D or R strains) did not affect pathogenicity of *C. carbonum* on maize of genotype *hm1/hm1*, but mutation of both copies completely abolished pathogenicity (Figure 3-8). Therefore, both copies of *TOXF* are functional. These experiments establish that *TOXF* is not required for normal growth and development but is specifically required for HC-toxin biosynthesis and hence pathogenicity.

DISCUSSION

TOX2 is a complex locus consisting of multiple genes each present in multiple functional copies (Ahn and Walton, 1997). Unlike the genes for other secondary metabolite pathways in fungi, such as those for aflatoxins

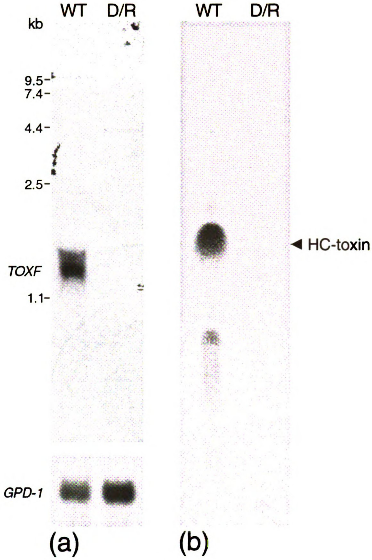


Figure 3-7. Analyses of *TOXF* mutants. (a) RNA blot showing the *TOXF* mRNA in 164R10 (WT) and its disappearance in the double mutant (D/R). Thirty µg of total RNA were loaded per lane, and the blot was probed with the *TOXF* cDNA (pAATC1). The blot was subsequently stripped and re-probed with *C. carbonum GPD-1* encoding glyceraldehyde-3-phosphate dehydrogenase as loading control. (b) Thin layer chromatography (TLC) of extracts of culture filtrates of *C. carbonum* 164R10 (WT) and the double mutant (D/R) showing that the double mutant does not make HC-toxin. HC-toxin was visualized with an epoxide-specific reagent.

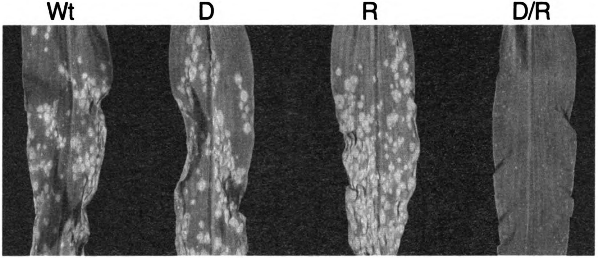


Figure 3-8. Pathogenicity assay of *TOXF* mutants. Wt, wild type 164R10; D, copy one of *TOXF* mutated; R, copy two of *TOXF* mutated; D/R, both copies mutated. Leaves were photographed 4-d post-inoculation.

/sterigmatocystins, trichothecenes, and gibberellins, those involved in HC-toxin production spread over more than 540 kb and show only limited clustering (Yu et al., 1995; Ahn and Walton, 1996; Brown et al., 1996; Trapp et al., 1998; Tudzynski and Hölter, 1998). Therefore, the molecular genetic analysis of HC-toxin biosynthesis cannot rely on sequencing and short-range chromosome walking. The functional redundancy of the HC-toxin biosynthetic genes excludes the use of mutagenesis approaches such as restriction enzyme mediated integration (REMI) (Lu et al., 1994; Sweigard et al., 1998). Here we have described the use of an alternate strategy, based on bacterial artificial chromosomes (BACs), to find HC-toxin biosynthetic genes. This approach assumes that new HC-toxin biosynthetic genes will be within 50-150 kb of each other and present only in Tox2$^+$ isolates. The BAC method is being extended by analysis of regions adjacent to other known *TOX2* genes (e.g., *HTS1*, *TOXC*, and *TOXE*) and by constructing a BAC library with larger inserts (up to 400 kb) (Monaco and Larin, 1994).

The *TOXF* product, TOXFp, shares moderate homology to a large group of branched-chain amino acid aminotransferases (BCATs) (Figure 3-5). All of the essential amino acid residues necessary for transaminase function are conserved in TOXFp. Considering that three of the four amino acids of HC-toxin (D-Pro, L-Ala, and D-Ala) are directly derived from primary metabolism, a role for *TOXF* in the biosynthesis of Aeo (2-amino-8-oxo-9,10-epoxidecanoic acid) seems most reasonable. A plausible reaction catalyzed by TOXFp would be to aminate a precursor of Aeo. Insofar as the decanoic acid backbone of Aeo is biogenically a fatty acid (Ahn and Walton, 1997; Cheng et al., 1999), a transamination reaction seems essential. The amino-acceptor substrate for the reaction catalyzed by TOXFp could be 2-oxo-decanoic acid, which itself could be produced by oxidation of decanoic acid by an unidentified enzyme. Alternative substrates for TOXFp could be derivatives of 2-oxo-decanoic acid that already contained the 8-carbonyl and/or the 9,10-epoxide.

All known BCATs are involved in primary catabolism of branched-chain amino acids. Typically, they catalyze the transfer of an amino group from Leu, Val, or Ile to α-ketoglutaric acid. TOXFp appears to be the first BCAT dedicated to a non-essential secondary metabolic pathway. The failure of *TOXF* to cross-hybridize with any other genes in *C. carbonum* at low stringency (data not shown) suggests that *TOXF* and the sequences of the house-keeping BCATs of *C. carbonum* are not closely related. It may be significant that BCATs are structurally and functionally related to bacterial D-amino acid aminotransferases and that TOXFp also shows limited sequence similarity to this sub-family of enzymes (Tanizawa et al., 1989; Mehta et al., 1993; Alexander et al., 1994; Sugio et al., 1995). D-amino acids are common in nonribosomal peptides, and HC-toxin contains two D-amino acids. From its similarity to BCATs, TOXFp probably uses a branched-chain amino acid as amino donor, but the possibility that the donor is a D-amino acid should also be considered.

CHAPTER 4

A NOVEL EUKARYOTIC ALANINE RACEMASE GENE INVOLVED IN CYCLIC PEPTIDE BIOSYNTHESIS IN THE FUNGUS *COCHLIOBOLUS CARBONUM*

The gene sequence reported in this Chapter has been deposited in GenBank with accession number AF169478.

The content of this Chapter is a re-formatted version of a manuscript submitted to *J. Biol. Chem.* (Cheng and Walton, 1999).

ABSTRACT

Non-ribosomally synthesized peptides and other secondary metabolites mediate many interactions between organisms and also have major importance in human medicine. HC-toxin, a cyclic tetrapeptide containing D-Ala and D-Pro, is an essential virulence determinant for the plant pathogenic fungus *Cochliobolus carbonum*. HC-toxin biosynthesis is controlled by a complex genetic locus, *TOX2*, that contains multiple functional copies of genes involved in the biosynthesis, export, and regulation of HC-toxin. The central enzyme in HC-toxin biosynthesis is HC-toxin synthetase (HTS), a 570-kDa non-ribosomal peptide synthetase that activates all four amino acids and epimerizes L-proline. We present here the cloning and characterization of a new gene of the *TOX2* locus, *TOXG*. *TOXG* is present as two or three copies in all HC-toxin-producing (Tox2$^+$) isolates and is absent from toxin-non-producing (Tox2$^-$) isolates. The deduced amino acid sequence of *TOXG* has significant similarity to that of an alanine racemase involved in cyclosporin biosynthesis by the fungus *Tolypocladium inflatum*. Despite no significant similarity of its deduced amino acid sequence to bacterial alanine racemases, *TOXG* was able to complement a bacterial strain defective in D-alanine synthesis. *C. carbonum* with all copies of *TOXG* mutated by homologous recombination grew normally but had an altered HC-toxin profile. Specifically, it could no longer make the three forms of HC-toxin that contain D-alanine but could

still make a minor form of HC-toxin that contains glycine in place of D-alanine. The mutant has reduced virulence, as manifested by delayed disease development, probably due to its inability to make the most predominant and most biologically active form of HC-toxin. Feeding D-alanine to the mutant in culture restored production of the forms of HC-toxin containing D-alanine. We conclude that *TOXG* encodes a novel alanine racemase that converts L-alanine to D-alanine specifically for incorporation into HC-toxin by HTS.

INTRODUCTION

Cyclic peptides are a large class of medically and biologically important secondary metabolites produced by bacteria and fungi. Cyclic and some linear peptides are synthesized by a special class of enzyme called non-ribosomal peptide synthetases (NRPS) (Kleinkauf and von Döhren, 1996). NRPS enzymes are organized into domains, each of which activates and binds one amino acid. Some NRPS domains can catalyze amino acid modifications, especially N-methylation and epimerization of L to D amino acids.

The filamentous fungus *Cochliobolus carbonum* (anamorph *Helminthosporium carbonum*) makes a cyclic tetrapeptide known as HC-toxin. The major form of HC-toxin (HC-toxin I) has the structure cyclo(D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-9,10-epoxi-8-oxo-decanoic acid. Three minor forms (HC-toxin II, III and IV) slightly differ in amino acid composition (Rasmussen, 1987; Rasmussen and Scheffer, 1988). HC-toxin is required for *C. carbonum* to pathogenize maize of genotype *hm1/hm1*. Most maize is resistant because it contains the dominant allele of *Hm1*, which encodes an enzyme, HC-toxin reductase, that detoxifies HC-toxin by reducing the 8-carbonyl of the Aeo side chain (Meeley and Walton, 1991; Meeley et al., 1992; Johal and Briggs, 1992). Like other Aeo-containing cyclic tetrapeptides, HC-toxin inhibits histone deacetylase in maize and other organisms (Brosch et al., 1995; Ransom and Walton, 1997).

Previous studies on the Mendelian and molecular genetics of HC-toxin production have shown that HC-toxin production is controlled by a single Mendelian locus, *TOX2*, but that this locus has a complex molecular structure (Scheffer et al., 1967; Ahn and Walton, 1996). All of the known genes (*HTS1*, *TOXA*, *TOXC*, *TOXE*, *TOXF*) that are specifically required for HC-toxin production are present only in HC-toxin-producing ($Tox2^+$) isolates in multiple functional copies, have no housekeeping roles, and, with one exception in some isolates, are linked over a ~540 kb region on one chromosome (Panaccione et al., 1992; Scott-Craig et al., 1992; Pitkin et al., 1996; Ahn and Walton, 1996, 1997, 1998; Chapter 3, Cheng et al., submitted).

The central enzyme in HC-toxin synthesis is HC-toxin synthetase (HTS), a 570-kDa NRPS with four amino acid activating domains. HTS is encoded by a 15.7-kb gene called *HTS1* (Panaccione et al., 1992; Scott-Craig et al., 1992). Biochemical assays of partially purified HTS indicated that it activates by ATP/PP_i-exchange L-proline, L-alanine, and D-alanine, but not D-proline, and epimerizes L-proline and L-alanine to the corresponding D-amino acids (Walton and Holden, 1988). Subsequent analysis of the sequence of HTS revealed a motif in domain A of HTS that was also present only in other epimerizing domains, very few of which were known at that time (i.e., the single-domain enzymes gramicidin synthetase I and tyrocidine synthetase I, and domain C of the three-domain enzyme ACV synthetase) (Scott-Craig et al., 1992). However, the putative epimerase signature motif was found only once in HTS, in domain A, whereas there should have been another copy in domain C, on the basis of the ability of partially purified HTS to epimerize L-alanine as well as L-proline. The subsequent cloning and sequencing of numerous additional NRPS genes, including many more domains that have epimerizing capacity, has resulted in the conclusion that there are characteristic signature motifs for epimerization domains, and that HTS clearly has only one, in domain A (Marahiel et al., 1997). This left in doubt the actual source of D-alanine for HC-toxin.

Cyclosporin is an undecapeptide containing D-alanine. Analysis of the primary sequence of cyclosporin synthetase also indicated a lack of an epimerization signature motif for domain A, which activates D-alanine (Marahiel et al., 1997). This led to the discovery of a separate enzyme, alanine racemase, that is responsible for the synthesis of D-alanine for incorporation into cyclosporin by cyclosporin synthetase (Hoffmann et al., 1994). The gene from *Tolypocladium inflatum* that encodes the alanine racemase, *cssB*, has been submitted to GenBank (accession number A40406) as a patent submission (patent number WO9425606) but not otherwise published.

Our search for new genes involved in HC-toxin biosynthesis has proceeded on the assumption that they will be linked, but not tightly clustered, to the known *TOX2* genes, and that they will be restricted in distribution to Tox2$^+$ isolates. Using a bacterial artificial chromosome (BAC) library, a new gene involved in HC-toxin biosynthesis, *TOXF*, was recently cloned and described (Chapter 4. Cheng et al., submitted). Immediately adjacent to *TOXF* is another gene, which we call *TOXG*. This paper describes the characterization of *TOXG* and biochemical and genetic evidence that it encodes an alanine racemase dedicated to HC-toxin production.

MATERIALS AND METHODS

Fungal Strains and Growth Conditions. SB111 (ATCC 90305) and SB114 are standard Tox2$^+$ (HC-toxin-producing) and Tox2$^-$ (HC-toxin-non-producing) laboratory strains of *C. carbonum*, respectively, and 164R10 is a Tox2$^+$ progeny of a cross between them (Ahn and Walton, 1997). Growth conditions for fungal culture on solid V8-plate or in modified Fries' liquid medium were previously described (Van Hoof et al., 1991).

Nucleic Acid Manipulations and Sequence Analysis. *C. carbonum* DNA and RNA isolation was previously described (Pitkin et al., 1996). DNA and RNA blotting, probe labeling, hybridization, cDNA and genomic library screening, and DNA subcloning were done following standard procedures (Sambrook et al., 1989). Oligonucleotides were synthesized by the Michigan State University (MSU) Macromolecular Facility. Automated DNA sequencing was done at the Yale University Keck Foundation Biotechnology Resource Laboratory and at the MSU DNA Sequencing Facility. Sequences were assembled and analyzed with the *DNAStar* Software package (DNAStar Inc., Madison, WI). Protein sequence alignment was generated with the *ClustalW* Program (Thompson et al., 1994). The transcriptional start site of *TOXG* was determined by 5' RACE using a kit from Gibco-BRL (Frohman et al., 1988). The primer for reverse transcription (GSP1) was 5'-CGATTCATTTTAGGGTGTGCCAGAT-3' and the nested primer for PCR amplification (GSP2) was 5'-TGTTTCGACTAACCGGTAGCAGGG-3'.

Bacterial Complementation and Enzyme Assay. *E. coli* strain TKL10 (*dadX-alr-ts*, CGSC#5466) (Wijsman, 1972) was obtained from the *E. coli* Genetic Stock Center at Yale University and maintained in Luria-Bertani (LB) medium supplemented with 200 μg/ml D-alanine. The *TOXG* expression vector pARE was constructed as follows: a 1.2 kb *TOXG* coding region (lacks 14 amino acids at the 5'-end of ORF) was released from pGC1 by *Bam*HI/*Kpn* I digestion and subcloned into pQE31 (Qiagen, Valencia, CA). pARE was verified by sequencing and transformed into TKL10 by electroporation (2.5 kV, 25 μF, 200 Ω, 0.1 cm cuvette) using a Bio-Rad Gene Pulser (Bio-Rad, Richmond, CA). Transformants were selected on LB plates with 100 μg/ml ampicillin at 42 °C. Two independent transformants were designated as TKL10T1 and TKL10T2.

Bacterial crude protein extract was prepared as follows. Cells were allowed to grow in LB medium with 100 μg/ml ampicillin at 37 °C for 12 hr and were collected by centrifugation (20 min, 5000 g) at 4 °C. The pellet was resuspended in extraction buffer of 50 mM Tris, pH 8.7 with 10% glycerol, 4 mM EDTA, 20 mM DTT, and 30 μM pyridoxal-5'-phosphate (PLP). Lysozyme (Sigma, St. Louis, MO) was added to 1 mg/ml and incubated on ice for 30 min. Cells were broken by sonication (3 min, 50% cycle, maximum output for microtip on a Model 450 Sonifier, Branson, CT). Cellular suspension was centrifuged at 20,000 g for 30 min at 4 °C. The supernatant was then saved as crude protein extract. The alanine racemase assay was adopted from Hoffmann et al. (1994). Step 1 reactions contained 50 mM Tris (pH 8.7), 50 mM L-alanine, 20 mM DTT, 30 μM PLP and an appropriate amount of protein extract in a total volume of 1 ml. Reactions were allowed to proceed for up to 4 hr at 42 °C, then terminated by heating at 95 °C for 10 min. The mixture was centrifuged for 10 min and 500 μl of supernatant was transferred to a new tube. Five hundred μl of reaction 2 mixture (50 mM Tris, pH 8.7 with 0.5 U of D-amino acid oxidase, 25 U of lactate dehydrogenase, 2 mM FAD, 0.2 mM NADH) (enzymes from Sigma, St. Louis, MO) was added to the tube and incubated at 37 °C for 2 hr. The decrease of absorbance at 340 nm was used to calculate the relative racemase activity. For each sample, a control with boiled protein extract was used as blank.

Creation of *TOXG* Null Mutant. Fungal protoplast preparation, transformation, selection, and single-spore isolation of transformants were done as described

(Panaccione et al., 1992; Pitkin et al., 1996). The two copies of *TOXG* in strain 164R10 were sequentially mutated by targeted gene inactivation. One was mutated by gene replacement mediated by double-crossover homologous recombination, and the other by gene disruption mediated by single-crossover integration.

To make the replacement vector pTOXGR1, a genomic clone pAATG1 was trimmed with *Eco*RV and *Hpa* I to eliminate the *TOXF* coding region and re-ligated to make the intermediate construct pARM2. A 420-bp *Apa* I/*Pst* I fragment (containing the 3' end of the *TOXG* cDNA) from pGC1 was subcloned into the *Bam*HI/*Pst* I sites of pARM2 to make another intermediate construct pARM3. The 512-bp internal *Sph* I/*Pst* I fragment (corresponding to +362 bp to +874 bp in the genomic sequence) of pARM3 was replaced by a hygromycin-resistance cassette (composed of the *E. coli hph* gene encoding hygromycin phosphotransferase driven by the *Aspergillus nidulans trpC* promoter from plasmid pCB1003) (Carroll et al., 1994) to make the final replacement vector pTOXGR1 (5.9 kb). The fragment (2.5 kb) containing the *hph* cassette plus flanking *TOXG* DNA was released from pTOXGR1 by digestion with *Not* I and *Hin*dIII and used for transformation.

To make the disruption vector pTOXGD1, an 8-bp *Bam*HI linker (Boehringer, Colone, Germany) was inserted into the unique *Sac* I site of pARM3 to make pARM4. The central part of the coding region from pARM4 (a 512-bp *Sph* I/*Pst* I fragment plus 8-bp linker) was then subcloned into the *Hin*dIII and *Xba* I sites of the pBC-phleo vector (CAYLA, Toulouse, France), which contains the *A. nidulans ZEO* gene cassette for phleomycin resistance, to obtain the final construct pTOXGD1 (6.6 kb). Vector pTOXGD1 was linearized with *Bam*HI before transformation.

Strain 164R10 was first transformed with the 2.5-kb fragment from pTOXGR1. Strain T697 with one copy of *TOXG* replaced with the *hph* cassette was selected in medium containing 120 μg/ml hygromycin. Strain T697 was subsequently transformed with linearized pTOXGD1. Strain T698 with the second copy of *TOXG* disrupted was selected in medium containing 50 μg/ml phleomycin.

Analysis of Mutants. HC-toxin was extracted from fungal culture filtrates, separated by silica-gel thin layer chromatography (TLC) (Si250-PA, J. T. Baker, NJ) with a solvent of acetone:dichloromethane (50:50 by volume), and detected with an epoxide-specific spray as described (Meeley and Walton, 1991). Compounds of interest were scraped from the TLC plate, suspended in ethanol, and briefly centrifuged. The supernatant was dried under vacuum and redissolved in ethanol for mass spectrometry analysis by the MSU Mass Spectrometry Laboratory. Fungal pathogenicity was assayed on maize inbred Pr (genotype *hm1/hm1*) by spraying with conidia (Panaccione et al., 1992; Pitkin et al., 1996). Infected maize leaves were photographed every day from 2- through 6-d post-inoculation.

Feeding Studies. Modified Fries' liquid medium (Van Hoof et al., 1991) was inoculated with conidia and grown at room temperature (25 °C) in still culture for 3 d before addition of D-alanine to a final concentration of 1, 10, 25, or 50 mM. Cultures were allowed to grow for an additional 12 d before collecting the culture filtrates for HC-toxin extraction and analysis.

RESULTS

Characterization of *TOXG*. We previously reported the cloning of *TOXF* on the basis of its linkage to known *TOX2* genes using bacterial artificial chromosomes (Chapter 3; Cheng et al., submitted). Within a 2.9-kb genomic DNA fragment (cloned in pAATG1) containing *TOXF*, we detected a partial open reading frame that starts immediately in the 5' direction of *TOXF* and is transcribed in the opposite direction (Figure 4-1). The deduced partial protein sequence of this new open reading frame, whose corresponding gene has been named *TOXG*, showed strong similarity to many threonine aldolases (e.g., GLY1p of *Saccharomyces cerevisiae*, GenBank P30831) and to an unnamed patent sequence in the data base (GenBank A40406) from the fungus *Tolypocladium inflatum*. A40406 is annotated as encoding an alanine racemase (EC 5.1.1.1) involved in cyclosporin biosynthesis. This gene has now been named *cssB* (K. Schörgendorfer, Novartis-Biochemie, Austria, personal communication).

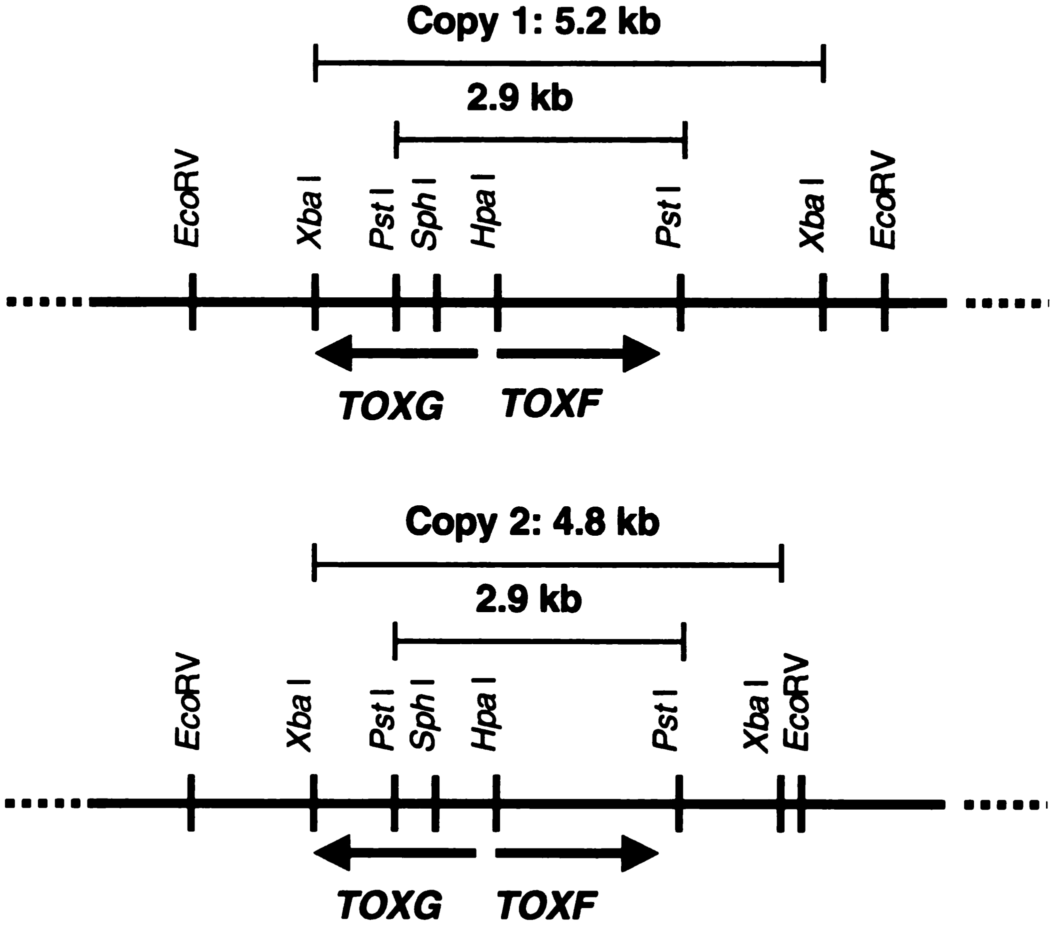


Figure 4-1. Restriction map of the two copies of the *TOXF/G* region in 164R10. The 2.9-kb *Pst* I fragment (cloned in pAATG1) was sequenced.

Full-length cDNA and genomic sequences of *TOXG* were obtained. A 1.1-kb *Pst I*/*Hpa* I fragment from pAATG1 was used as probe to obtain a full-length cDNA clone of *TOXG* (pGC1). The insert of pGC1 was used to obtain the 3'-end of a genomic copy of *TOXG* (pGG2). The inserts of plasmids pGC1 and pGG2 were sequenced on both strands. *TOXG* encodes a protein (TOXGp) of 389 amino acids with a calculated molecular weight of 42.7 kDa and a pI of 6.5. TOXGp contains no predicted signal peptide or glycosylation sites. *TOXG* has one intron of 52 bp. The transcriptional start site is at –46 bp. The distance between the translational and transcriptional start sites of *TOXF* and *TOXG* is 299 bp and 195 bp, respectively (Figure 4-2). The deduced protein sequence of *TOXG* has an overall 42% identity to the product of *cssB* and 32% identity to GLY1p (Figure 4-3). A conserved lysine residue for binding of the cofactor pyridoxal-5'-phosphate (PLP) was identified at position 235 in TOXGp (Liu et al., 1997; Monschau et al, 1997).

TOXG is present in three copies in all tested Tox2$^+$ isolates except 164R10, which has only two copies, and is completely absent in all Tox2$^-$ strains tested (data not shown). Like *TOXF*, *TOXG* expression requires the regulatory gene *TOXE* (Figure 4-4) (Ahn and Walton, 1998).

Complementation of Bacterial Mutant. To test the putative function of TOXGp as an alanine racemase, we constructed a *TOXG* expression vector pARE and transformed it into bacterial strain TKL10, which is defective in D-alanine biosynthesis and therefore requires D-alanine in the medium for survival

Figure 4-2. Sequence of *TOXG* and its relationship to *TOXF*. Transcriptional and translational start sites are indicated above the DNA sequence. Deduced amino acid sequence and the polyadenylation site (†) are placed under the DNA sequence. The sole intron is typed in italic lower case.

```
   TOXF translational start site ←|
TAAAATGCAGGCAAGGGTATCGCCATGATGTCGATGGCTGAGAACCTTTAG   -275
       TOXF transcriptional start site ←|
AGATTAGTTAACCTTTTCGCTGATTTGTTCTTGAATTAAGTATAAGAGGGT   -224
TTACCATAAGTTATAGAAGAACAGCTCCTTGTCGAAGATAGATAGAGTCAT   -173
CGCTTACGTGATTGGATACGCGAGATGAACTCAGATTACGTAAGATTGTTG   -122
TGGTCTTCCATCCTGCACTATTACTTAATCTACGTGTTATTCCTTCACCCG    -71
                     |→ TOXG transcriptional start site
TAGCCTTAAAGCATGTGCTTCCCAATTTGAAAAATCCATCTGTTGCAACGG    -20
                 |→ TOXG translational start site
ATATTTTCGAAATTTCGAGATGTCGAACATGGTTTTGAATGGAAACATTGA     32
                    M  S  N  M  V  L  N  G  N  I  D     11
CAAATCCGATAGGAATTCCATACTAGACATACTTCAATCGCTTGAGAATAT     83
  K  S  D  R  N  S  I  L  D  I  L  Q  S  L  E  N  I     28
TGCATGGGGACAACCAGGCTCTGCAAGGTGCGATTTTAGGAgtaattaaca    134
  A  W  G  Q  P  G  S  A  R  C  D  F  R                 41
tgctgaatcgtacaaagaagtccgatactaactattgtgcagGTGATGTTA    185
                                           S  D  V      44
TTACACGCCCTAGCCTTAGGATGTTGTCCGCGGTCCTCAAGACTACACTCG    236
I  T  R  P  S  L  R  M  L  S  A  V  L  K  T  T  L       61
GCGATGACGTATTTCGAGAAGACCTAACGACTGCCCACTTCGAAGCGCATG    287
G  D  D  V  F  R  E  D  L  T  T  A  H  F  E  A  H       78
TGGCTGAAATCAGCGGCCGAGAAGAAGGCATGTTCGTGATCACAGGGACGA    338
V  A  E  I  S  G  R  E  E  G  M  F  V  I  T  G  T       95
TGGCCAATCAGCTTTGTTTGCATGCTCTAGTCTCAACTAGACCGTGTGGGA    389
M  A  N  Q  L  C  L  H  A  L  V  S  T  R  P  C  G      112
TTTTACTTAGCTCAGAGTCCCATGCCATACACTATGAGGCAGGTGGCTCTT    440
I  L  L  S  S  E  S  H  A  I  H  Y  E  A  G  G  S      129
CGATGCTTAGCGGCGCCATGCTTCAGCCTGTGCAGCCGTCGAATGGTAAAT    491
S  M  L  S  G  A  M  L  Q  P  V  Q  P  S  N  G  K      146
ATCTGAGAGTGGAAGATTTGGAGGAGCACGCAATTCTAACCGACGATGTCC    542
Y  L  R  V  E  D  L  E  E  H  A  I  L  T  D  D  V      163
ACAAATGCCCCACAAGCATTGTTTCTATGGAAAATACAGCTGGTGGAGCAG    593
H  K  C  P  T  S  I  V  S  M  E  N  T  A  G  G  A      180
TCGTTCCTGTCCATGAGCTCCGCCGCATCCGGGATTGGGCAAAGCAAAATA    644
V  V  P  V  H  E  L  R  R  I  R  D  W  A  K  Q  N      197
ACGTGAGGACTCACCTGGACGGTGCTAGACTTTTCGAGGCTGTTGCTACCG    695
N  V  R  T  H  L  D  G  A  R  L  F  E  A  V  A  T      214
GTGCTGGGACTCTCAAAGAATATTGCAGCCTTATTGACCTAGTCTCCGTGG    746
G  A  G  T  L  K  E  Y  C  S  L  I  D  L  V  S  V      231
ATTTTAGTAAGAATCTTGGAGCCCCAATGGGCGCAATGATTCTTGGTGACA    797
D  F  S  K  N  L  G  A  P  M  G  A  M  I  L  G  D      248
AAAAATTGATTCAGCAGATGCGAAGAACTCGAAAAGGGATTGGAGGAGGAA    848
K  K  L  I  Q  Q  M  R  R  T  R  K  G  I  G  G  G      265
TGAGACAAGGAGGGGTAATCACTGCAGCTGCACGGGAGGCACTGTTTGAAA    899
M  R  Q  G  G  V  I  T  A  A  A  R  E  A  L  F  E      282
ACTTTGGACTGGGAGCGGAAATCGAAAGTCAAACCCTATTGCAAGTGCACA    950
N  F  G  L  G  A  E  I  E  S  Q  T  L  L  Q  V  H      299
AAGTCGCAAAGCGTCTAGGGGAAGAATGGACTAGAAAAGGTGGGAAGTTGA   1001
K  V  A  K  R  L  G  E  E  W  T  R  K  G  G  K  L      316
GCAAAGAGATCGAGACAAATATCATATGGCTCGATCTTGATGCGGTTGGAA   1052
S  K  E  I  E  T  N  I  I  W  L  D  L  D  A  V  G      333
TTAAGAAAAGTCAATTTATTGATAAGGGAAGGGAATATGGGGTGATTCTAG   1103
I  K  K  S  Q  F  I  D  K  G  R  E  Y  G  V  I  L      350
ACGGCTGTCGCATTGTTTGCCATCACCAGATTGATATATATGCAGTAGAGG   1154
D  G  C  R  I  V  C  H  H  Q  I  D  I  Y  A  V  E      367
CTCTTATCGACGTCTTCCATGATATACTAAAAGCAGATCCTATCAAAAACA   1205
A  L  I  D  V  F  H  D  I  L  K  A  D  P  I  K  N      384
AGAATAGCGATAGATAGTCATGAATATACACACCGGGATAAATATATAAGT   1256
K  N  S  D  R  *                                       389
TTTAACTACTAGTTAAGTCTAGATAACTAGTTGTCTAGGTAGTTGGCATCC   1307
            †
AATATACTACGGCTGGAAAGTTAGGTGTCTACCTAGTGCTGAGAAGGTGTT   1358
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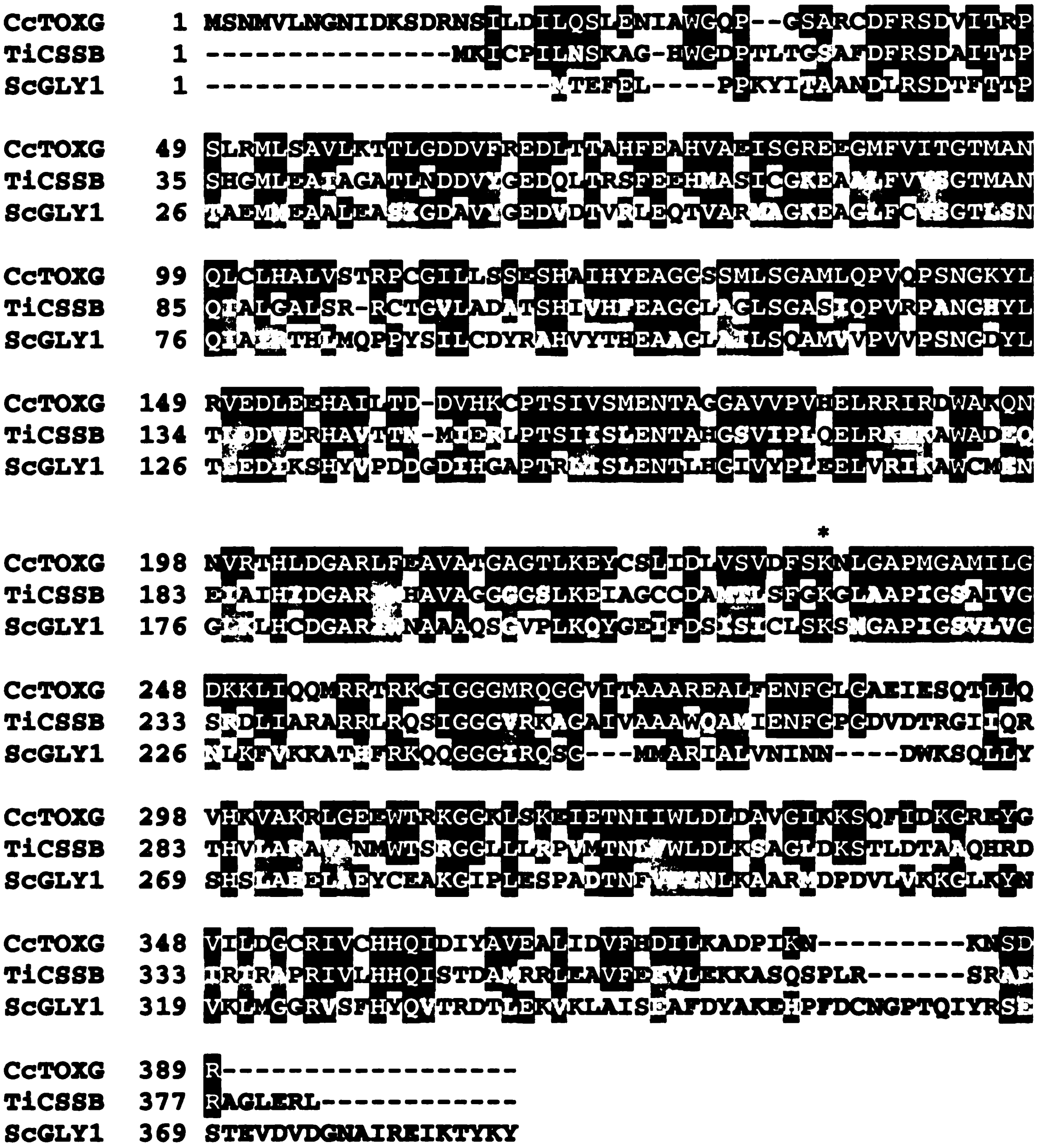

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CcTOXG     1 MSNMVLNGNIDKSDRNSILDILQSLENIAWGQP--GSARCDFRSDVITRP
TiCSSB     1 ---------------MKICPILNSKAG-HWGDPTLTGSAFDFRSDAITTP
ScGLY1     1 ---------------------MTEFEL----PPKYITAANDLRSDTFTTP

CcTOXG    49 SLRMLSAVLKTTLGDDVFREDLTTAHFEAHVAEISGREEGMFVITGTMAN
TiCSSB    35 SHGMLEAIAGATLNDDVYGEDQLTRSFEEHMASICGKEAMLFVVSGTMAN
ScGLY1    26 TAEMMEAALEASKGDAVYGEDVDTVRLEQTVARMAGKEAGLFCVSGTLSN

CcTOXG    99 QLCLHALVSTRPCGILLSSESHAIHYEAGGSSMLSGAMLQPVQPSNGKYL
TiCSSB    85 QIALGALSR-RCTGVLADATSHIVHFEAGGLAGLSGASIQPVRPANGHYL
ScGLY1    76 QIAIRTHLMQPPYSILCDYRAHVYTHEAAGLAILSQAMVVPVVPSNGDYL

CcTOXG   149 RVEDLEEHAILTD-DVHKCPTSIVSMENTAGGAVVPVHELRRIRDWAKQN
TiCSSB   134 TKDDVERHAVTTN-MIERLPTSIISLENTAHGSVIPLQELRKLKAWADEQ
ScGLY1   126 TLEDIKSHYVPDDGDIHGAPTRLISLENTLHGIVYPLEELVRIKAWCMEN

                                                   *
CcTOXG   198 NVRTHLDGARLFEAVATGAGTLKEYCSLIDLVSVDFSKNLGAPMGAMILG
TiCSSB   183 EIAIHIDGARLFHAVAGGGSLKEIAGCCDAMTILSFGKGLAAPIGSAIVG
ScGLY1   176 GLKLHCDGARIWNAAAQSGVPLKQYGEIFDSISICLSKSMGAPIGSVLVG

CcTOXG   248 DKKLIQQMRRTRKGIGGGMRQGGVITAAAREALFENFGLGAEIESQTLLQ
TiCSSB   233 SRDLIARARRLRQSIGGGVRKAGAIVAAAWQAMIENFGPGDVDTRGIIQR
ScGLY1   226 NLKFVKKATHFRKQQGGGIRQSG---MMARIALVNINN----DWKSQLLY

CcTOXG   298 VHKVAKRLGEEWTRKGGKLSKEIETNIIWLDLDAVGIKKSQFIDKGREYG
TiCSSB   283 THVLARAVANMWTSRGGLLLRPVMTNLIWLDLKSAGLDKSTLDTAAQHRD
ScGLY1   269 SHSLAHELAEYCEAKGIPLESPADTNFVFINLKAARMDPDVLVKKGLKYN

CcTOXG   348 VILDGCRIVCHHQIDIYAVEALIDVFHDILKADPIKN---------KNSD
TiCSSB   333 IRIRAPRIVLHHQISTDAMRRLEAVFEEVLEKKASQSPLR------SRAE
ScGLY1   319 VKLMGGRVSFHYQVTRDTLEKVKLAISEAFDYAKEHPFDCNGPTQIYRSE

CcTOXG   389 R------------------
TiCSSB   377 RAGLERL------------
ScGLY1   369 STEVDVDGNAIREIKTYKY
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Figure 4-3. Multiple sequence alignment of TOXGp and homologs. Alignment was performed with *ClustalW* and decorated with *BOXSHADE*. Black shading indicates identical amino acids between TOXGp and at least one of the others; light shading indicates similar amino acids. The origin of amino acid sequences is: CcTOXG from *C. carbonum* (GenBank AF169478), TiCSSB (GenBank A40406) from *T. inflatum* and ScGLY1 (GenBank P30831) from *S. cerevisiae*. Asterisk indicates the cofactor pyridoxal-5'-phosphate binding site.

(Wijsman, 1972). Two random transformants, TKL10T1 and TKL10T2, selected on LB plates with 100 μg/ml ampicillin at 42°C, survived without D-alanine supplementation (Figure 4-5). The alanine racemase activity of TKL10T1 and TKL10T2 was two to three-fold above background (data not shown). These experiments indicated that *TOXG* encodes a functional alanine racemase.

Creation of a *TOXG* Null Mutant and Analysis of its Phenotype. To investigate the role of *TOXG* in HC-toxin biosynthesis, both copies of *TOXG* in isolate 164R10 were mutated by homologous recombination-mediated gene replacement or disruption. The correct integration of constructs into strains T697 and T698 was confirmed by Southern analysis (Figure 4-6). The 5.2-kb *Xba* I band, corresponding to copy 1 of *TOXG*, was gone in both T697 and T698 (Figure 4-6A). Integration of pTOXGD1 into the 4.8-kb *Xba* I band (corresponding to copy 2 of *TOXG*) in strain T697 resulted in the disappearance of this band and appearance of a new band of 18.0 kb in strain T698, presumably due to tandem integration of two copies of pTOXGD1 (Panaccione et al., 1992) (Figure 4-6A). When the same blot was re-probed with the full-length cDNA clone pGC1, a 6.1-kb band was visible in both T697 and T698, resulting from the gene replacement event (Figure 4-6B).

Creation of a *TOXG* null mutant was tested by RNA blotting. Because *TOXF* and *TOXG* are tightly clustered, *TOXF* was included in the analysis to be sure that mutation of one gene did not affect the expression of the other. Total RNA from 164R10 (wild type), the *TOXF* null mutant D/R (renamed as T696;

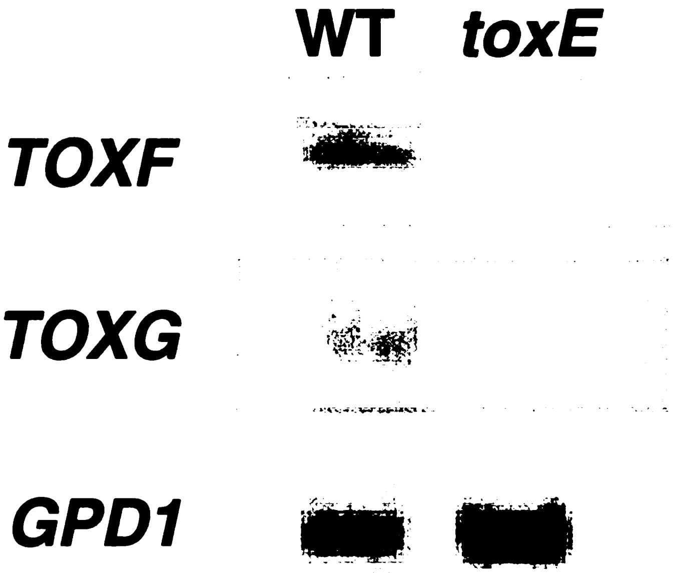

Figure 4-4. *TOXF* and *TOXG* expression requires *TOXE*. Twenty μg of total RNA from wild type (WT, 164R10) and a *TOXE* null mutant (*toxE*) was separated in a denaturing formaldehyde gel and blotted to nylon membrane. Three identical blots were probed separately with *TOXF*, *TOXG* or *GPD1*.

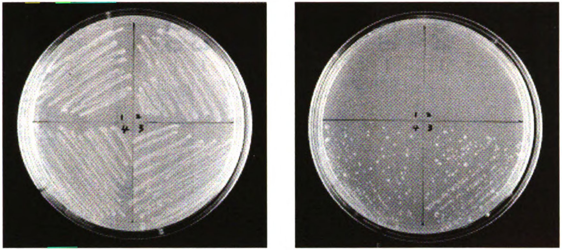

Plate A. LB + D-Ala **Plate B. LB + Amp**

Figure 4-5. Complementation of *E. coli* alanine racemase mutant by *TOXG*. *TOXG* expression vector pARE was introduced into TKL10 by electroporation. Transformants were selected for ampicillin resistance and D-alanine independence. 1. Bacterial mutant TKL10; 2. Control transformant with empty vector pQE31; 3 and 4, Two independent transformants TKL10T1 and TKL10T2 with pARE.

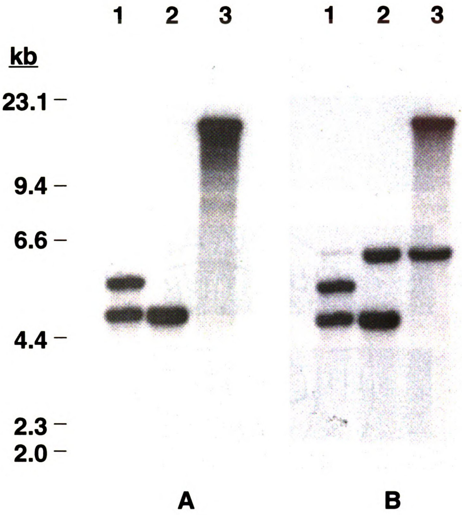

Figure 4-6. Southern blot analysis of *TOXG* mutants. Genomic DNA of (1) wild type 164R10, (2) single mutant T697, and (3) double mutant T698 was digested with *Xba* I, separated in a 0.9% agarose gel, blotted to nylon membrane, and probed with (A) the 512-bp *Sph* I/*Pst* I fragment that was replaced, (B) the full-length cDNA clone (pGC1).

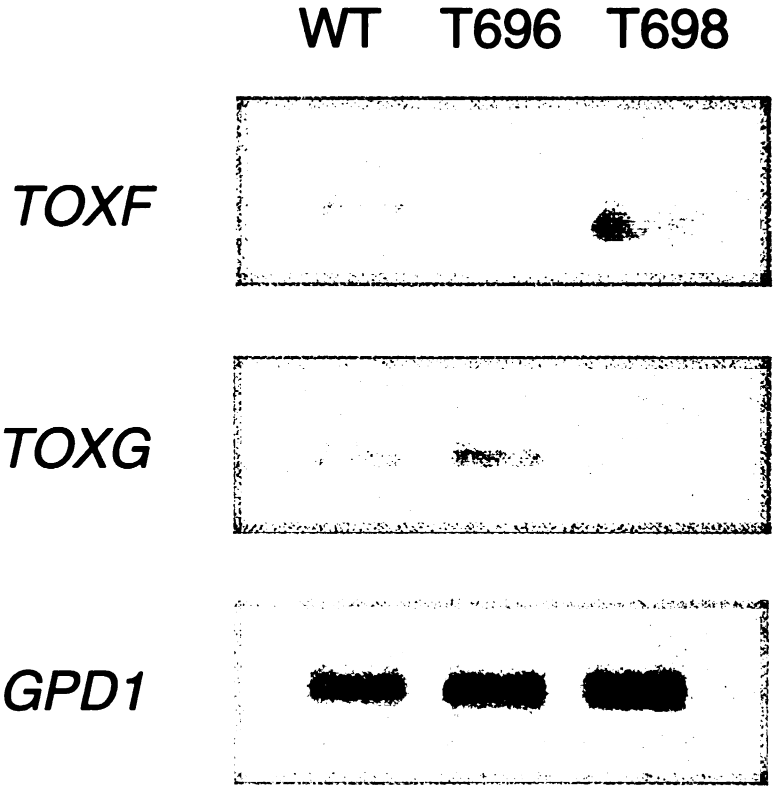

Figure 4-7. Northern blot verification of the independent mutation of *TOXF* and *TOXG*. Twenty μg of total RNA from wild type 164R10 (WT), a *TOXF* null mutant (T696), and a *TOXG* null mutant (T698) was separated in a denaturing formaldehyde gel and blotted to nylon membrane. Three identical blots were probed separately with *TOXF*, *TOXG* or *GPD1*.

Cheng et al., submitted), and the *TOXG* null mutant T698 was analyzed on three identical blots and probed with *TOXF*, *TOXG*, or *GPD1* (encoding glyceraldehyde-3-phosphate dehydrogenase, as loading control). The results indicate that the *TOXF* and *TOXG* mutants had no detectable *TOXF* or *TOXG* mRNA, respectively, and that disruption of one gene did not influence expression of the other (Figure 4-7).

Both the single (T697) and double (T698) *TOXG* mutants showed normal growth and development on agar plates or in liquid culture. Along with the restricted distribution of *TOXG* to Tox2$^+$ isolates, this indicates that *TOXG* has no essential housekeeping functions.

HC-toxin was extracted from culture filtrates of the wild type and T698 null mutant with or without D-alanine supplementation. Wild type isolates of Tox2$^+$ *C. carbonum* produce four forms of HC-toxin that can be detected on TLC plates using an epoxide-specific reagent (Figure 4-8) (Meeley and Walton, 1991). The three minor forms, HC-toxin II, III, and IV, contain glycine in place of D-alanine, D-*trans*-3-hydroxyproline in place of D-proline, and 8-hydroxy-Aeo in place of Aeo, respectively (Kim et al., 1985; Tanis et al., 1986; Rasmussen, 1987; Rasmussen and Scheffer, 1988). HC-toxin forms I, II, and III are active at 0.2, 0.4, and 2.0 µg/ml, respectively, whereas form IV is active only at ~20 µg/ml (Rasmussen and Scheffer, 1988).

The wild type isolate 164R10 produced abundant HC-toxin forms I, II, and IV but HC-toxin III could not be reliably detected. The *TOXG* null strain did not produce forms I or IV but still produced form II. Therefore, disrupting *TOXG* did not affect production of all forms of HC-toxin. Form II differs from the others in having glycine in place of D-alanine (Kim et al., 1985). Supplementing the medium with D-alanine restored production of HC-toxins I and IV (Figure 4-8). The TLC results are consistent with *TOXG* having a specific role in the synthesis of forms of HC-toxin that contain D-alanine.

Pathogenicity of the *toxG* Mutant. To test the affect of the *TOXG* mutation on fungal virulence, conidia of *C. carbonum* wild type 164R10, single-mutant T697 and double-mutant T698 were spray-inoculated onto the leaves of susceptible maize. On plants inoculated with the wild type, lesions became visible in 2 d and after 6 d the entire infected leaf became totally brown and dessicated. Mutation of one copy of *TOXG* had no obvious affect on virulence, as seen in T697. Mutation of both copies of *TOXG* in T698 caused a noticeable slowing of disease development (Figure 4-9). A large portion of initial infection sites of T698 failed to develop into full lesions. The lesions that developed were consistently smaller than those caused by the wild type within the first 6 d of disease development. This modest decrease in virulence of the *TOXG* mutant differs from the results obtained with mutants of other *TOX2* genes such as *HTS1*, *TOXC*, *TOXE*, and *TOXF*, which are completely avirulent (Panaccione et al., 1992; Ahn and Walton, 1997, 1998; Cheng et al., submitted). The apparent explanation for this is that the *TOXG* mutant can still make HC-toxin II, which is about half as biologically active as HC-toxin I (Rasmussen and Scheffer, 1988), but sufficient enough to cause disease symptoms.

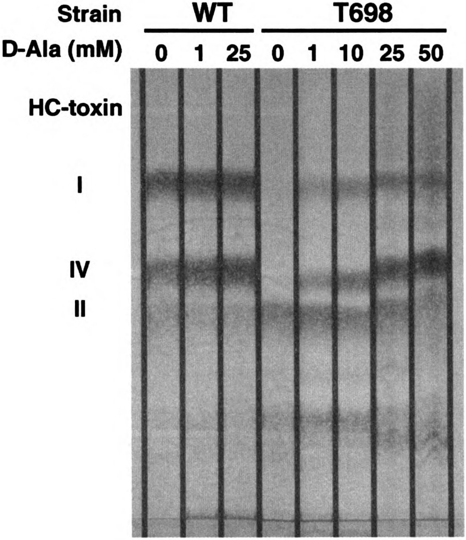

Figure 4-8. TLC Analysis of HC-toxin in Wild type (WT, 164R10) and in *TOXG* null Mutant (T698).

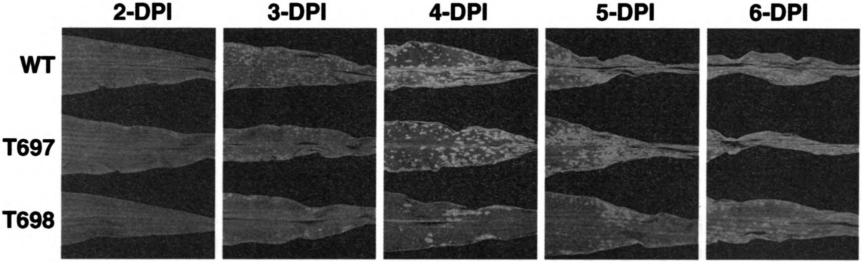

Figure 4-9. Pathogenicity assay of 164R10 (WT), single mutant (T697) and double mutant (T698). Representative leaves were photographed daily at 2, 3, 4, 5, and 6-d post-inoculation.

DISCUSSION

HC-toxin is the agent of compatibility in the interaction between the fungal pathogen *C. carbonum* and its host, maize (Walton, 1996). The complete biosynthesis pathway of HC-toxin has not yet been deciphered. HC-toxin, a cyclic tetrapeptide, contains two D-amino acids and two L-amino acids. In contract to the essential role of D-alanine in bacterial cell wall biogenesis (Faraci and Walsh, 1988), the existence of D-amino acids in eukaryotic cells is not common. Other than the occurrence of D-aspartate and D-serine as neuromodulators in mammalian tissues (Nagata et al., 1989; Hashimoto and Oka, 1997; Wolosker et al., 1999), D-amino acids or derivatives are restricted to secondary metabolites in fungi (Kleinkauf and von Döhren, 1996).

Hoffmann et al. (1994) reported the purification and characterization of a eukaryotic alanine racemase as a key enzyme in cyclosporin biosynthesis in *Tolypocladium niveum* and demonstrated that the enzyme catalyzes the interconversion of L- and D-alanine. In this paper, we present the molecular cloning of a gene encoding an alanine racemase from a plant fungal pathogen *C. carbonum* and the genetic confirmation of its specific role in HC-toxin biosynthesis. Like five other genes proven to be involved in HC-toxin production, *TOXG* is present only in Tox2$^+$ isolates of *C. carbonum*. Null mutants of *TOXG* failed to synthesize three forms of HC-toxin that contain D-alanine. D-ala supplementation restored the wild type toxin profile (Figure 4-8).

TOXGp shares strong sequence homology with CSSBp of *T. inflatum*, as well as threonine aldolases from many species (Figure 4-3). However, there is no obvious homology between the sequences of eukaryotic alanine racemases including TOXGp and CSSBp, and their bacterial counterparts (e.g. GenBank AB021683, AF038438, AF081283), despite the fact that they are functionally compatible, as demonstrated in the TKL10 complementation experiment (Figure 4-5). Based on recent structural and evolutionary studies (Alexander et al., 1994; Jansonius, 1998), most PLP-dependent enzymes can be classified into three families (α,β, and γ) by sequence analysis. Bacterial alanine racemase is among the few PLP enzymes that does not fall into the above classification system. These exceptions may represent another family of PLP enzymes. With the cloning of *TOXG* and *cssB* as eukaryotic alanine racemases that have no evolutionary relationship with their bacterial counterparts, a novel family of PLP-dependent enzymes emerges.

Biochemically, alanine racemase activity has been previously detected specifically in Tox2$^+$ isolates of *C. carbonum*. Initial purification of HC-toxin biosynthetic activities resulted in the identification of two enzymes, HTS-1 and HTS-2 (Walton, 1987). HTS-1, with a molecular weight of ~220 kDa, epimerizes L-proline to D-proline and catalyzes ATP/PP$_i$ exchange in the presence of L-proline; HTS-1, with a molecular weight of ~160 kDa, appeared to epimerize L-alanine to D-alanine and catalyze both L-alanine-depedent and D-alanine-dependent ATP/PP$_i$ exchange. Later studies identified and cloned a single 15.7-kb ORF that encodes the entire HTS protein; the appearance of two separate proteins as HTS-1 and HTS-2 was, in fact, a purification artifact (Panaccione et al., 1992; Scott-Craig et al., 1992). Sequence analysis indeed found epimerization signatures in the region between domain A and B of HTS and is believed to be responsible for D-proline formation, but no similar signatures were found after domain C which is assumed to activate D-alanine (Scott-Craig et al., 1992; Kleinkauf and von Döhren, 1996). Together with the purification of a separate alanine racemase protein from the cyclosporin-producing fungus *Tolypocladium niveum* (Hoffmann et al., 1994), we speculated that the epimerization activity detected in previously named HTS-2 enzyme might result from co-purification of an independent alanine racemase. The cloning of *TOXG* together with genetic evidence presented in this paper proved this speculation.

The clustering of *TOXG* with the recently reported *TOXF* bears both similarity and contrast with that of *HTS1* and *TOXA*. There is a 386-bp space between the transcriptional start sites of *HTS1* and *TOXA* (Pitkin et al., 1996), and 195 bp between *TOXF* and *TOXG* (Figure 4-2). Both pairs of genes are transcribed divergently. However, the evidence showed that intergenic regions of these clusters contain different elements with regard to response to the pathway-specific transcription factor encoded by *TOXE*. Both *TOXF* and *TOXG* are regulated by *TOXE*. In a *TOXE* null mutant, both *TOXF* and *TOXG* are silent (Figure 4-4). *TOXA* is also silent in the *TOXE* null mutant, but the expression of *HTS1* remains unaffected (Ahn and Walton, 1998). A similar multifaceted regulation mechanism has been found in the penicillin biosynthesis pathway (Brakhage, 1998).

In the broad view, the clustering of genes involved in fungal secondary metabolism pathways is common, if not the rule. Gene clusters have been discovered in *Penicillium* for penicillin biosynthesis, in *Aspergillus* for aflatoxins/sterigmatocystins, in *Fusarium* for trichothecenes, and in *Gibberella* for gibberellins (Yu et al., 1995; Brown et al., 1996; Trapp et al., 1998; Tudzynski and Hölter, 1998). In contrast, genes involved in HC-toxin production are spread over more than 540 kb and show only limited clustering (Ahn and Walton, 1996, 1998). The distance between *TOX2* genes can be as short as a few hundred bp (between *HTS1* and *TOXA*, and *TOXF* and *TOXG*), or can be as far as 220 kb between *HTS1* copy 1 and *TOXC* copy 2.

The molecular basis of interactions between *C. carbonum* and its host, maize, is one of the most thoroughly studied cases of plant fungal diseases. Together with *TOXG*, four genes (*HTS1*, *TOXC*, *TOXF* and *TOXG*) encode enzymes involved in HC-toxin biosynthesis, one gene (*TOXA*) encodes an efflux pump for toxin export, and *TOXE* encodes a transcription factor that regulates the expression of all known *TOX2* genes other than *HTS1*. Taking the complexity of Aeo into account, at least four more biochemical activities are required for its biosynthesis. One is a putative fatty acid α subunit, which would work together with the fatty acid synthase β subunit encoded by *TOXC*, to synthesize decanoic acid from acetyl-CoA and malonyl-CoA; one enzyme is needed to oxidize decanoic acid to 2-oxo-decanoic acid; and two other enzymes are postulated to catalyze the formation of the 8-carbonyl group and the 9,10-epoxide group of Aeo. Whether the genes encoding these putative proteins are clustered with known *TOX2* genes, and whether they are regulated by *TOXE*, awaits future studies.

CHAPTER 5

SUMMARY AND PERSPECTIVE

The studies presented in this dissertation focused on the molecular genetics of HC-toxin biosynthesis. The striking features shared by the known *TOX2* genes (*HTS1*, *TOXA*, *TOXC* and *TOXE*) are: (1) Each has two or three functional copies per genome; (2) they are present only in HC-toxin-producing isolates (Tox2$^+$) of *C. carbonum*; (3) they are scattered over ~540 kb on a special chromosome; (4) when all copies of an individual gene are mutated (except *TOXA*, which was unsuccessful due to lethality), the mutants lost the capability to produce HC-toxin and also fungal pathogenicity. These features guided my studies toward cloning additional genes that are involved in HC-toxin production.

By taking a bacterial artificial chromosomal clone-mapping, transcript-screening and sequencing approach, I have identified two additional genes, *TOXF* and *TOXG*, that participate in HC-toxin biosynthesis. *TOXF* and *TOXG* are two tightly linked genes. Both are present in three copies in the standard lab strain SB111, and two functional copies in isolate 164R10; both are exclusively present in Tox2$^+$ isolates of *C. carbonum*; both map to the *TOX2* locus, and the expression of both is regulated by *TOXE*.

TOXF encodes a branched-chain amino acid aminotransferase that is believed to aminate an α-keto acid on the Aeo biosynthesis pathway. A null mutant of *TOXF* no longer produces HC-toxin and causes severe leaf spot disease on maize leaves. Therefore, *TOXF* can be regraded as a pathogenicity gene.

TOXG encodes a eukaryotic alanine racemase that catalyzes the interconversion of L-alanine and D-alanine. D-alanine is a critical constituent in

HC-toxin I, III, and IV. A null mutant of *TOXG* fails to make HC-toxin I, III and IV, but the biosynthesis of HC-toxin II is unaffected because HC-toxin II has a glycine in place of D-alanine. Compared to wild type, the *TOXG* mutant causes a delayed disease phenotype that eventually results in full symptoms due to the presence of HC-toxin II. Therefore, *TOXG* can only be classified as a virulence gene.

The biochemical function of *TOXF* is unproven due to the lack of substrate and assay methodology. In contrast, genetic and biochemical experiments were successfully adopted to confirm *TOXG* function. *TOXG* complemented a bacterial mutant defective in D-alanine biosynthesis. Alanine racemase activity (L to D) was also detected in a bacterial crude extract that expresses *TOXG* protein (TOXGp) under a constitutive promoter. Attempts to study the enzyme kinetics were not successful due to the insolubility of overexpressed *TOXG* gene product in an *E. coli* expression system (data not shown).

Another *TOX2* candidate gene, *TOXD*, was cloned many years ago, based on its indirect linkage to *TOXC* and its unique presence in Tox2$^+$ isolates of *C. carbonum*. *TOXD* shares the first three features (previous page) of known *TOX2* genes. However, HC-toxin production and fungal pathogenicity were unchanged in a *TOXD* null mutant. It appeared that *TOXD* has no obvious role in HC-toxin production. Recent reports showed that *TOXD* has strong similarity to the *lovC* gene of the lovastatin gene cluster in *Aspergillus terreus*. Knowledge from other systems may ultimately assist us in understanding the function(s) of *TOXD* in the future.

TOXF and *TOXG* along with the previously identified *TOX2* genes (including *TOXD*) have been mapped to the *TOX2* locus on the 3.5-Mb chromosome in strain SB111 (Figure 5-1) (Ahn and Walton, 1996; J. -H. Ahn and J. D. Walton, unpublished data). In essence, *Pac* I sites were introduced into each copy of *TOXF/G* by transformation-mediated homologous recombination. Chromosomes or chromosomal fragments were then separated by pulsed-field gel electrophoresis (CHEF), and Southern hybridizations were performed to decide the locations of the three copies of *TOXF/G* relative to other mapped genes. *TOXF/G* copy 1 is located about 20 kb away from *HTS1* copy 1 toward *HTS1* copy 2. *TOXF/G* copy 2 is about 20 kb away from *TOXD* copy 1 toward *TOXD* copy 3 and *TOXF/G* copy 3 is about 40 kb away from *TOXC* copy 1 (Figure 5-1).

Up to now, a total of six genes (*HTS1*, *TOXA, TOXC, TOXE, TOXF*, and *TOXG*) have been cloned and shown to be involved in HC-toxin production. Among them, four genes (*HTS1*, *TOXC, TOXF*, and *TOXG*) encode biosynthetic enzymes, one gene (*TOXE*) encodes a pathway trancriptional factor that regulates the expression of *TOXA, TOXC, TOXF* and *TOXG* but not *HTS1*, and one (*TOXA*) encodes a membrane protein to export HC-toxin. The biosynthesis pathway for HC-toxin is tentatively summarized (Figure 5-2). As for the four amino acid constituents of HC-toxin, L-alanine comes from the cellular amino acid pool; D-proline is from L-proline by the action of an epimerization domain in HC-toxin synthetase (HTS) encoded by *HTS1*; the *TOXG* protein converts L-alanine into D-alanine, and L-Aeo comes from a long route. Aeo has a backbone

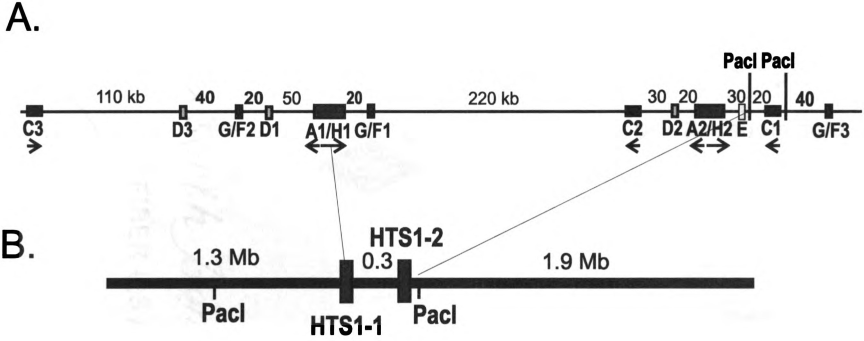

Figure 5-1. Chromosomal map of *TOX2* genes (A) and the 3.5-Mb chromosome (B). H, A, C, D, F, and G represent *HTS1*, *TOXA*, *TOXC*, *TOXD*, *TOXF*, and *TOXG*, respectively. Arabic number following gene symbol indicateds gene-copy number. Arrows indicate the transcription orientations (Adapted from Ahn and Walton, 1996; J. -H. Ahn and J. D. Walton, unpublished data).

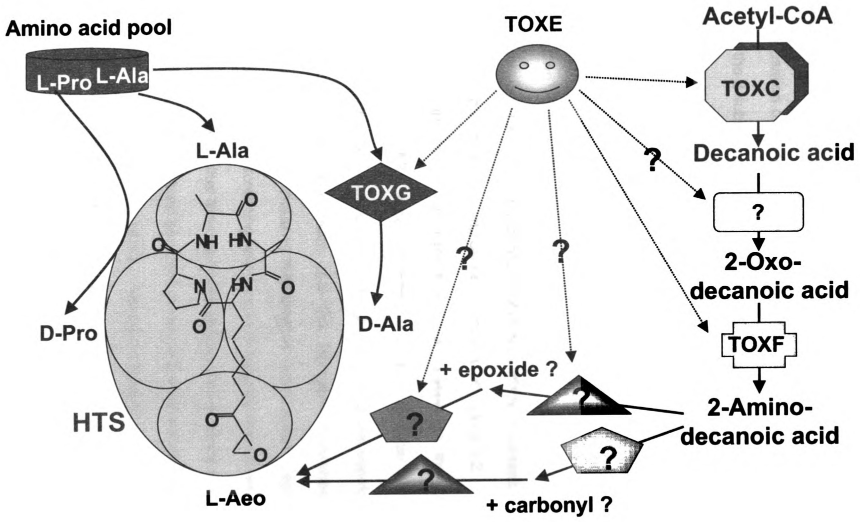

Figure 5-2. Diagram of the HC-toxin (I) biosynthesis machinery.

of a fatty acid nature that has been confirmed by *in vivo* ^{13}C labeling and NMR analysis (Cheng et al, 1999; See Appendix). Its biosynthesis starts with the condensation of acetyl-CoA by a putative fatty acid synthase (FAS) complex in which the β subunit is encoded by *TOXC*. The product of FAS is likely decanoic acid. From decanoic acid to Aeo, there are at least 4 biochemical steps: oxidation of decanoic acid to generate α-keto decanoic acid, amination by *TOXF* protein of the α-keto group to make 2-amino decanoic acid, and the addition of an 8-carbonyl group and a 9,10-epoxide group. The order of the final two reactions is not known. Finally, HTS activates the four components and cyclizes them into the final product. The *TOXE* product coordinates most of the processes. The function of *TOXA* product as efflux pump cannot be included in Figure 5-2.

There are still a few black boxes, all existing on the route to Aeo biosynthesis. The FAS α subunit is unknown. It could be either a dedicated one existing only in Tox2^{+} isolates of the fungus, or the housekeeping one that is shared by primary metabolism and the HC-toxin biosynthesis pathway. Numerous approaches, including heterologous hybridization, degenerate PCR amplification, immunoscreening, and functional tagging, did not reveal a second FAS α subunit gene other than the housekeeping one. Attempts to knock out the housekeeping FAS α subunit gene and to rescue the mutant with exogenous C_{18}, C_{16}, or C_{14} fatty acids also failed. Nothing is known about the dehydration, oxidation and epoxidation steps, though the putative enzymes needed for these biochemical reactions are postulated to be a dehydrase, an oxidase, and a cytochrome P450, respectively.

Future studies in deciphering the complete biosynthesis pathway of HC-toxin may require novel approaches. Since the *TOX2* genes are not tightly clustered, and there is much repetitive DNA in the *TOX2* locus region, chromosome walking would be practically difficult. A reverse genetics approach can be very challenging because of the functional redundancy of dehydrases, oxidases, and cytochrome P450s. The most promising way is to sequence the whole genome, or the specific "Tox2" 3.5-Mb chromosome, or more narrowly, the *TOX2* locus and flanking regions. But potential problems still exist. Whole genome sequencing requires a large investment, and chromosome- or locus-specific sequencing requires sophisticated cloning steps as a prerequisite.

APPENDIX

^{13}C LABELLING INDICATES THAT THE EPOXIDE-CONTAINING AMINO ACID OF HC-TOXIN IS BIOSYNTHESIZED BY HEAD-TO-TAIL CONDENSATION OF ACETATE

The content of this Appendix has been published on the *J. Nat. Prod.* (Cheng et al. 1999. **62,** 143-145.). It has been re-formatted to keep the consistence of this dissertation.

J. Nat. Prod. **1999,** 62, 143-145

^{13}C Labelling Indicates that the Epoxide-containing Amino Acid of HC-toxin is Biosynthesized by Head-to-Tail Condensation of Acetate

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HC-toxin, cyclo(D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-9,10-epoxi-8-oxodecanoic acid, is a potent inhibitor of histone deacetylase. Previous molecular genetic studies indicated that HC-toxin biosynthesis requires a dedicated fatty acid synthase. The incorporation of $[^{13}C]$acetate into HC-toxin was studied using NMR. The pattern of incorporation of ^{13}C was consistent with the carbons of Aeo being derived from head-to-tail condensation of acetate.

HC-toxin, a cyclic tetrapeptide with the structure cyclo(D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-9,10-epoxi-8-oxodecanoic acid (Figure 1), is a virulence determinant for the producing fungus, *Cochliobolus carbonum*, on its host, maize (*Zea mays*).[1] HC-toxin and other Aeo-containing cyclic tetrapeptides cause detransformation of oncogene-transformed mammalian cells and have cytostatic activity against a variety of cell types, including several important protozoal parasites.[2] The site of action of Aeo-containing peptides is histone deacetylase, an enzyme that is emerging as a critical regulator of chromatin structure and gene regulation.[3-7]

There are five known Aeo-containing cyclic tetrapeptides, as well as a number of synthetic derivatives.[8,9] The 8-carbonyl and the intact 9,10-epoxide of Aeo are critical for biological activity. Reduction of the 8-carbonyl is particularly important because this reaction is the basis of resistance of maize to HC-toxin and hence *C. carbonum*.[10] The absolute necessity of the terminal epoxide, however, has been recently challenged by the discovery of two biologically active compounds related to the Aeo-containing peptides but lacking the terminal epoxide. One, apicidin, has 2-amino-8-oxodecanoic acid in place of Aeo yet is comparable to HC-toxin in its ability to inhibit histone deacetylase.[2] The other compound is identical to chlamydocin (cyclo[α-aminoisobutyrate-L-Phe-D-Pro-L-Aeo]) except that the epoxide is replaced by a 9-hydroxy group.[11]

HC-toxin production in *C. carbonum* is controlled by a single Mendelian locus, *TOX2*.[9] At the molecular level, *TOX2* is complex, containing multiple copies of multiple genes. Genes of *TOX2* that have been identified to date include *HTS1*, encoding a 570-kDa tetrapartite peptide synthetase; *TOXA*, encoding an HC-toxin efflux carrier of the major facilitator superfamily; and *TOXC*, encoding a fatty acid synthase beta subunit.[12-16] The product of *HTS1* is the central enzyme in HC-toxin biosynthesis, but nothing in its sequence suggests that it could have any role in Aeo biosynthesis.

The backbone of Aeo is aminodecanoic acid, *TOXC* encodes a fatty acid synthase beta subunit, and *TOXC* is required only for HC-toxin biosynthesis.[16] *TOXC* is not involved in primary metabolism because *TOXC* is present only in Tox2$^+$ isolates of *C. carbonum*, and *TOXC* mutants have no phenotype other than loss of HC-toxin production.[16] Therefore, a plausible role for the product of *TOXC* is in the biosynthesis of Aeo. If the product of *TOXC* contributes to the synthesis of the backbone of Aeo, then Aeo is predicted to be synthesized by head-to-tail condensation of acetate. Wessel et al.[17] showed that [^{14}C]acetate is incorporated into HC-toxin in short-term feeding studies, but the pattern of labeling was not analyzed. Here we use an alternative method, feeding

[^{13}C]acetate to fungal cultures and analyzing the labeled HC-toxin by NMR, to study the biogenic origin of Aeo.

The complete assignments of the ^{1}H and ^{13}C NMR signals of HC-toxin have not previously been made.[1,18-21] HMQC combined with 2-D TOCSY provided nearly complete proton and carbon resonance assignments for HC-toxin (Tables 1 and 2). The only ambiguities that persist are the assignments of signals to protons on the same methylene carbons of Aeo and proline. However, for this study these assignments are not critical since it is the identities of the carbons that are the most important biogenically.

Based on the assignments of the carbon atoms from these studies, HC-toxin labeled by feeding fungal cultures with 1-[^{13}C]acetate or 2-[^{13}C]acetate was purified and analyzed by solution-state high resolution 1-D ^{13}C NMR (Figure 2). There was a clear difference in the enrichment pattern when the fungus was incubated with 1-[^{13}C]acetate as compared to 2-[^{13}C]acetate (Figures 2B, C). 1-[^{13}C]Acetate enriched the ^{13}C signals of carbons 3, 5, 7 and 9 of Aeo (Figure 2B) whereas 2-[^{13}C]acetate enriched the signals of carbons 2, 4, 6, 8, and 10 (Figure 2C). The relative ^{13}C signal intensities of the native, 1-[^{13}C], and 2- [^{13}C] labeled HC-toxins were compared and analyzed in order to determine the degree of isotopic enrichment. These values were derived by first normalizing all of the ^{13}C resonance intensities to the intensity of the ^{13}C signal from carbon 2 of L-alanine within each spectrum. The degree of enrichment was then determined by calculating the ratio of each normalized resonance intensity in the labeled samples with its counterpart in the intensities from the native HC-toxin.

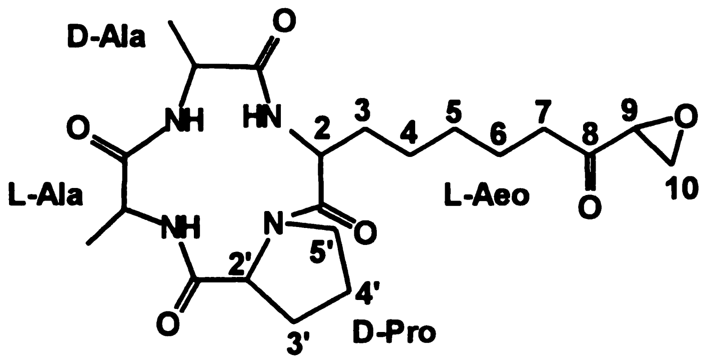

Figure 1. Structure of HC-toxin. The chirality of carbon #9 of Aeo is S.[29]

Table 1. Proton NMR Assignments for HC-toxin[a]

	Aeo	Pro	D-Ala	L-Ala
N	6.25		6.14	7.07
2	4.73	4.67	4.55	4.42
3	1.77, 1.61	2.37, 1.82	1.22	1.28
4	1.22, 1.28	2.25, 1.93		
5	1.25, 1.25	3.97, 3.48		
6	1.51, 1.52			
7	2.39, 2.20			
8				
9	2.97, 2.83			
10	3.38			

[a] Chemical shifts are indicated in ppm.

Table 2. Carbon NMR assignments for HC-toxin[a]

	Aeo	Pro	D-Ala	L-Ala
1	171.6	173.5	173.8	173.8
2	51.7	57.6	45.5	47.8
3	28.4	24.5	12.9	13.4
4	25.2	24.4		
5	27.8	46.8		
6	22.4			
7	35.9			
8	207.5			
9	53.4			
10	45.9			

[a] Chemical shifts are indicated in ppm.

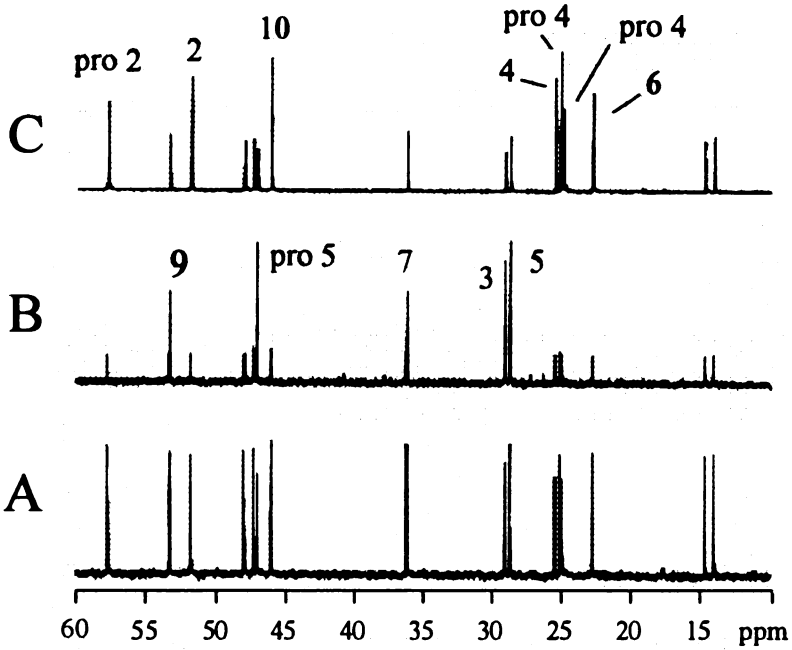

Figure 2. ^{13}C NMR spectra of (A) native HC-toxin and HC-toxin from culture filtrates of *C. carbonum* grown in the presence of either (B) 1-[^{13}C]acetate or (C) 2-[^{13}C]acetate.

As seen in Table 3, there is an alternating pattern of isotopic enrichment in the Aeo decanoic acid backbone, as expected if the Aeo side chain is formed by head-to-tail condensation of acetate. Determining the relative enrichment of the carbonyl resonances was more difficult due to their longer relaxation times. To acquire the data in a reasonable amount of time, the pulse delay had to be shorter than what would permit complete relaxation of the resonances with long T_1 values such as the carbonyl resonances. The intensities of these resonances are reduced more than those with shorter T_1 values, and the resulting errors are higher. Therefore, the carbonyl resonance values must be considered only semi-quantitative.

Acetate condensation to produce unsaturated linear alkanoic acids can be catalyzed by either polyketide synthases or fatty acid synthases. Although these two classes of enzymes catalyze many of the same reactions, their domain organizations are distinct.[22] Earlier, we had described a gene, *TOXC*, that is dedicated to HC-toxin biosynthesis and whose predicted product is a fatty acid synthase β subunit.[16] Considering that (a) the other amino acids present in HC-toxin, alanine and proline, are primary metabolites, (b) the product of *HTS1* has no domains other than those typical of other cyclic peptide synthetases, and hence probably activates, but does not synthesize, Aeo[13], and (c) the studies described here showing that the Aeo backbone is derived from head-to-tail condensation of acetate, the most reasonable function of the product of *TOXC* is to participate in the biosynthesis of the Aeo backbone.

Table 3. Quantitative analysis of enrichment of relevant carbon atoms in HC-toxin after labelling with 1-[^{13}C]acetate or 2-[^{13}C]acetate[a]

residue	2-[^{13}C]	1-[^{13}C]
Aeo CO	1.40	***2.52***
Aeo 2	***2.30***	0.80
Aeo 3	1.03	***4.32***
Aeo 4	***2.57***	0.80
Aeo 5	1.05	***4.25***
Aeo 6	***2.67***	0.87
Aeo 7	1.04	***3.84***
Aeo 8	***2.19***	1.13
Aeo 9	1.13	***6.10***
Aeo 10	***2.49***	0.68
L&D-Ala CO	0.90	1.46
L-Ala 2	1.03	0.88
L-Ala 3	1.00	1.00
D-Ala 2	0.99	0.84
D-Ala 3	1.09	1.08
Pro CO	1.16	1.88
Pro 2'	***2.87***	0.61
Pro 3'	***3.85***	0.84
Pro 4'	***2.45***	1.15
Pro 5'	1.03	***4.00***

[a] Sites of isotopic enrichment are shown in bold italic type.

Our studies indicate that all 10 carbons of Aeo are derived from acetate. The product of *TOXC* is a likely candidate enzyme for catalysis of its condensation. However, we cannot conclude that the *TOXC* protein catalyzes the complete synthesis of the Aeo backbone, because it might make a smaller acetate polymer that is then extended by another enzyme such as a polyketide synthase. Sterigmatocystin biosynthesis in *Aspergillus nidulans* involves genes encoding α and β fatty acid synthase subunits that are distinct from the genes involved in primary fatty acid biosynthesis. The product of the sterigmatocystin-dedicated fatty acid synthase (FAS) is hexanoic acid, which is then elongated by condensation with acetyl-CoA catalyzed by a dedicated polyketide synthase.[23]

The importance in HC-toxin biosynthesis of *TOXC*, encoding a fatty acid synthase β subunit, and the studies reported here indicating that Aeo is biogenically a fatty acid together indicate that Aeo biosynthesis must also require the participation of a fungal fatty acid synthase α subunit. This α subunit could be the one involved in primary fatty acid biosynthesis or could be dedicated to HC-toxin biosynthesis.

In the [^{13}C]acetate labeling studies, the ^{13}C label was also incorporated into the proline residue of HC-toxin, but not into the alanine residues (Figure 2). Whereas alanine is directly synthesized from pyruvate, proline is synthesized from α-ketoglutarate, an intermediate of the citric acid cycle. When exogenous acetate is metabolized, it is incorporated into the citric acid cycle after derivatization with CoA, and therefore it is not unexpected that amino acids biosynthesized from citric acid cycle intermediates would also become labeled.

Experimental Section

Culture and Isolation of HC-toxin. *Cochliobolus carbonum* Nelson (anamorph *Bipolaris zeicola* [Nisak. and Miyake] or *Helminthosporium carbonum* Ullstrup) race 1 strain SB111 (ATCC 90305) was grown for 10 days in still culture on modified Fries' medium at ambient temperature.[1] The culture filtrate was filtered through a layer of Miracloth (Calbiochem, CA). Proteins and other high molecular weight compounds were precipitated overnight with an equal volume of MeOH at 4°C and removed by filtration. After evaporation of the MeOH under reduced pressure, the remaining H_2O phase was extracted three times with equal volumes of $CHCl_3$. The $CHCl_3$ was evaporated under reduced pressure and the final reddish oil was redissolved in H_2O. Further purification was done by reverse-phase (C18) HPLC eluted with a linear gradient of 100% H_2O to 60% MeCN in 30 min, at a flow rate of 1 mL/min.[1] Fractions containing HC-toxin were pooled and rechromatographed under the same conditions. Finally, the toxin samples were lyophilized and redissolved in $CDCl_3$.

Biosynthetic Conditions. The optimal conditions for [^{13}C]acetate acid incorporation into HC-toxin were determined by preliminary [^{14}C]acetate acid labeling experiments (data not shown). Two days after initial inoculation of flasks of medium with fungal spores, [^{13}C]acetic acid (sodium salt, 1-[^{13}C] or 2-[^{13}C], Sigma) was added to the fungal cultures at a final concentration of 12 mM. Cultures were allowed to grow for a further 8 days, and HC-toxin was purified from the culture filtrates by $CHCl_3$ extraction and HPLC as described above.

NMR Methods. HC-toxin (ca. 2 mg) was dissolved in 600 μL $CDCl_3$ in a 5 mm Wilmad 528pp NMR tube. The NMR data were acquired on a 500-MHz Varian VXR500 NMR spectrometer at 25°C. The spectra were processed using Varian's VNMR software and NMRPipe software[24] on a Silicon Graphics Indigo 2XZ computer. A combination of homonuclear 1-D and 2-D (^1H, ^{13}C) and heteronuclear 2-D (^1H-^{13}C) experiments were used in the analysis.[25-28] A sweep width of 4189 Hz in each dimension was used, and 2048 acquired points in t_2, 512 complex t_1 increments and a total of 32 scans per t_1 with a pulse delay of 2.0 s were collected. The processing parameters of the TOCSY spectrum were as follows: t_2, first point multiplication by 0.5, apodization with a cosine squared shifted by a factor of 0.35, zero order baseline correction; t_1, apodization with a cosine squared shifted by a factor of 0.35, zero filling to 2048 points, and zero order baseline correction.

The heteronuclear multiple-quantum coherence (HMQC, also called ^1H-detected ^1H-^{13}C correlated spectroscopy) data were acquired with a sweep width of 4189 Hz in t_2 (proton dimension) and 31422 Hz in t_1 (carbon dimension). A total of 32 scans per t_1 increment were acquired. All spectra were referenced to the $CHCl_3$ peak at 7.24 ppm for ^1H and 77 ppm for ^{13}C. 1D and C NMR spectra were acquired with the same parameters.

Supporting Information Available: Copies of ^1H and ^{13}C NMR spectra of HC-toxin, upfield portion of the 2D-TOCSY spectrum of HC-toxin, and upfield portion of the 2D-HMQC spectrum of HC-toxin (3 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from ACS; see any current masthead page for ordering information.

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