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A Fatty Acid Synthase Gene in *Cochliobolus carbonum* Required for Production of HC-Toxin, Cyclo(D-Prolyl-L-Alanyl- D-Alanyl- L-2-Amino-9,10-Epoxi-8-Oxodecanoyl)

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The fungal maize pathogen *Cochliobolus carbonum* produces a phytotoxic and cytostatic cyclic peptide, HC-toxin, of structure cyclo(D-prolyl-L-alanyl-D-alanyl-L-Aeo), in which Aeo stands for 2-amino-9,10-epoxi-8-oxodecanoic acid. Here we report the isolation of a gene, *TOXC*, that is present only in HC-toxin-producing (Tox2$^+$) fungal strains. *TOXC* is present in most Tox2$^+$ strains in three functional copies, all of which are on the same chromosome as the gene encoding HC-toxin synthetase. When all copies of *TOXC* are mutated by targeted gene disruption, the fungus grows and sporulates normally in vitro but no longer makes HC-toxin and is not pathogenic, indicating that *TOXC* has a specific role in HC-toxin production and hence virulence. The *TOXC* mRNA is 6.5 kb and the predicted product has 2,080 amino acids and a molecular weight of 233,000. The primary amino acid sequence is highly similar (45 to 47% identity) to the β subunit of fatty acid synthase from several lower eukaryotes, and contains, in the same order as in other β subunits, domains predicted to encode acetyl transferase, enoyl reductase, dehydratase, and malonyl-palmityl transferase. The most plausible function of *TOXC* is to contribute to the synthesis of the decanoic acid backbone of Aeo.

Additional keyword: Helminthosporium.

Cyclic and linear peptides made by the non-ribosomal "thiotemplate" mechanism are a diverse and biologically important group of secondary metabolites from bacteria and lower eukaryotes. The enzymes involved in the biosynthesis of such peptides act by a common mechanism involving activation of the individual amino acids as aminoacyl adenylates and thioesterification to enzyme-bound panthothenic acid (Kleinkauf and von Döhren 1996). Many non-ribosomal peptides contain unusual amino acids and non-amino acid constituents, which are frequently aliphatic acids, e.g., β-hydroxytetradecanoic acid in surfactin, 3-hydroxydodecanoic acid in syringomycin and related compounds, 6-methylheptanoic or hexanoic acid in the polymyxins, and 2-amino-3-hydroxy-4-methyl-6-octenoic acid in cyclosporin (Fliri and Wegner 1990; Segre et al. 1989; von Döhren 1990).

Five cyclic tetrapeptides are known that contain the aliphatic amino acid L-2-amino-9,10-epoxi-8-oxodecanoic acid (Aeo) (Walton 1990; Itazaki et al. 1990). These compounds are cytostatic against mammalian cells at nanomolar concentrations and inhibit histone deacetylases of mammals, plants, and other organisms in vitro and in vivo (Kijima et al. 1993; Brosch et al. 1995). Both the 8-carbonyl and the 9,10-epoxy groups of Aeo are necessary for the biological activities of Aeo-containing cyclic peptides (Walton and Earle 1983; Kim et al. 1987). HC-toxin, of structure cyclo(D-Prol-L-Ala-D-Ala-L-Aeo), in which Aeo stands for 2-amino-9,10-epoxi-8-oxodecanoic acid, is selectively active against maize that is homozygous recessive at the *Hm* locus, and the producing fungus, *Cochliobolus carbonum* (formerly called *Helminthosporium carbonum*) race 1, is selectively pathogenic on the same genotype (Walton 1996). The *Hm* gene encodes an enzyme, HC-toxin reductase, that confers insensitivity to HC-toxin and resistance to *C. carbonum* by reducing the carbonyl group of Aeo (Johal and Briggs 1992; Meeley et al. 1992).

The central enzyme in HC-toxin biosynthesis, HC-toxin synthetase (HTS), has been purified and partially characterized. HTS is a 570-kDa polypeptide encoded by the 15.7-kb open reading frame of *HTS1* (Panaccione et al. 1992; Scott-Craig et al. 1992). HTS has been shown to catalyze ATP/PP_i exchange dependent on three of the four amino acids in HC-toxin (L-Pro, D-Ala, and L-Ala) and to epimerize L-Pro and L-Ala (Walton 1987; Walton and Holden 1988), and is presumed also to activate Aeo or an Aeo precursor. Based on its sequence, however, it is unlikely that HTS itself synthesizes Aeo, although it could conceivably catalyze its modification (Scott-Craig et al. 1992).

Little is known about the biosynthesis of Aeo in any of the organisms that make it. Wessel et al. (1988) showed that ^{14}C-acetate is incorporated specifically into the Aeo moiety of HC-toxin, and since cerulenin did not inhibit this process it was proposed that Aeo is synthesized by a polyketide synthase or cerulenin-resistant fatty acid synthase.

Since *HTS1* is absent from natural HC-toxin–nonproducing (Tox2$^-$) strains of *C. carbonum* (Panaccione et al. 1992; Ahn

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Nucleotide and/or amino acid sequence data are to be found at GenBank as accession number U73650.

and Walton 1996), other genes encoding enzymes involved in HC-toxin biosynthesis, such as a putative Aeo synthase, might also be unique to HC-toxin-producing ($Tox2^+$) strains. Here we report the characterization of a new region of $Tox2^+$-unique DNA and show that it contains a new gene called *TOXC*. *TOXC* encodes a fatty acid synthase and is required for HC-toxin production and pathogenicity by *C. carbonum*.

RESULTS

Discovery of *TOXC*.

HTS1, which encodes a tetrapartite cyclic peptide synthetase required for HC-toxin production, is present only in $Tox2^+$ strains (Panaccione et al. 1992). Most $Tox2^+$ strains of *C. carbonum* have two copies of *HTS1* and in strain SB111 they are 270 kb apart on the same chromosome (Panaccione et al. 1992; Ahn and Walton 1996). Flanking the 3′ end of *HTS1* is a region, contained in the *Eco*RI/*Sal*I genomic fragment CC62, that is present in the genome of strain SB111 in at least three copies, two of which directly flank the two copies of *HTS1* (see Figure 1 in Panaccione et al. 1992). A 5-kb *Bam*HI fragment that hybridizes to CC62 (Fig. 1), however, represents DNA that is also present only in $Tox2^+$ strains but that is not flanking either copy of *HTS1*, and therefore represents novel $Tox2^+$-unique DNA elsewhere in the genome. A genomic library of *C. carbonum* SB111 DNA in EMBL3 was screened with CC62, and λ inserts containing 5-kb *Bam*HI fragments that hybridize with CC62 (fragment A3B2; Fig. 1) were isolated and mapped. One such λ insert (λA3) was subcloned into pBluescript KSII to create pA3 (Fig. 1). Approximately 10 kb of the 11-kb insert in pA3 is absent in $Tox2^-$ strains of *C. carbonum* (data not shown). Sub-fragments of this DNA were used to screen a cDNA library made from mRNA from *C. carbonum* SB111. One 1.8-kb polyadenylated cDNA, called pAJ8, mapped to a 4.5-kb *Bam*HI genomic fragment (A3B3) from λA3 that lies adjacent to fragment A3B2 (Fig. 1). pAJ8 is polyadenylated and contains a single stop codon. Fragment A3B3 was used as a probe to isolate a λEMBL3 genomic clone, λA4, that overlaps λA3 (Fig. 1). Within λA4, the 2.5-kb *Bam*HI/*Eco*RI fragment AJ17 is also present in $Tox2^+$ but not $Tox2^-$ strains (data not shown). pAJ8 was the only cDNA found when genomic fragments from this region were used as probes to screen the cDNA library. The gene corresponding to pAJ8 is called *TOXC*.

Nucleotide and amino acid sequences of *TOXC*.

A 7-kb genomic *Xba*I fragment (Fig. 1) containing pAJ8 and upstream sequence was sequenced on both strands (Fig. 2). This region contains one large open reading frame of 6.4 kb except for one intron of 59 bp identified by comparison of the genomic sequence to that of pAJ8 (Fig. 2). The size of this open reading frame is consistent with the estimated size, 6.5 kb, of the *TOXC* mRNA (Fig. 3). There appear to be no other introns because the rest of the sequence of *TOXC* has only one stop codon and no frame shifts and also because it closely matches the sequences of related genes throughout its length. We cannot exclude the possibility of introns in the 5′ untranslated region.

The predicted product of *TOXC*, ToxC, has 2,080 amino acids and a molecular mass of 233 kDa. Its sequence is strongly similar in size and sequence to fatty acid synthase subunits from *Yarrowia lipolytica* (2,057 amino acids, 66% similarity, and 47% identity; Köttig et al. 1991), *Candida albicans* (2,076 amino acids, 65% similarity, and 47% identity; Zhao and Cihlar 1994), and *Saccharomyces cerevisiae* (encoded by *FAS1*; 2,037 amino acids, 64% similarity, and 45% identity; Schweizer et al. 1986). ToxC has 58% similarity and 38% identity to the fatty acid synthase subunit (1,900 amino acids) found in the sterigmatocystin cluster of *Aspergillus nidulans* (Brown et al. 1996). Domains corresponding to acetyl transferase, enoyl reductase, dehydratase, and malonyl-palmitate transferase are present in ToxC as are the two conserved serine residues in the two transferase domains (amino acids 276 and 1828; Fig. 2). The corresponding ToxC domains are 34, 50, 38, and 49% identical to the acetyl transferase, enoyl reductase, dehydratase, and malonyl-palmityl transferase domains, respectively, of the product of the yeast *FAS1*

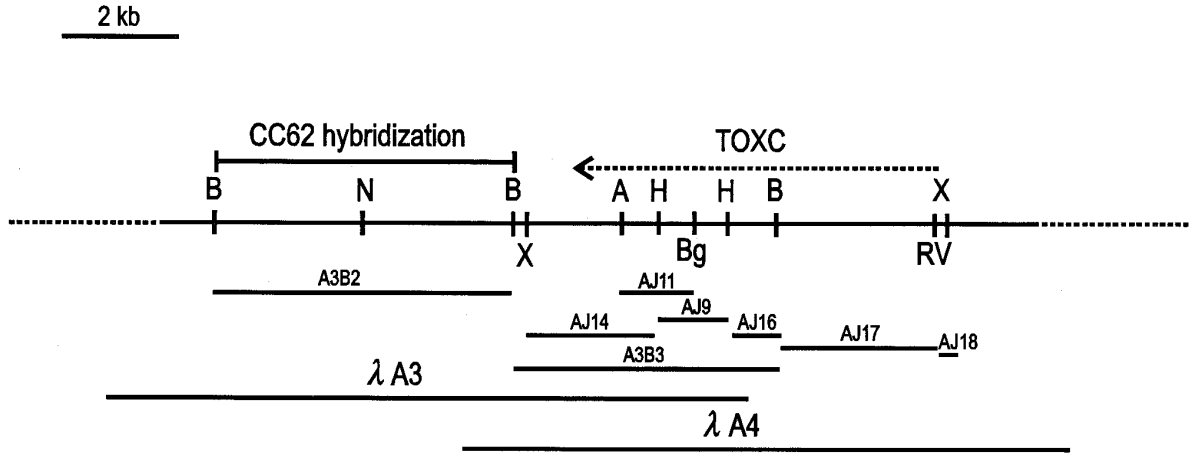

Fig. 1. Restriction map of *TOXC* and flanking DNA. *Cochliobolus carbonum* strain SB111 has three copies of *TOXC*; the map of copy 1 is shown. Two overlapping lambda genomic clones (A3 and A4) are indicated. DNA from the left-most *Bam*HI site to the right-most *Xba*I site is unique to $Tox2^+$ fungal strains. The *TOXC* transcribed region and direction of transcription are indicated by the dashed arrow. AJ8 is a partial *TOXC* cDNA. A3B2, A3B3, AJ14, AJ16, AJ17, and AJ18 are subcloned fragments used as hybridization probes. AJ9 and AJ11 were used for the gene disruption experiments. A, *Acc*I; B, *Bam*HI, Bg, *Bgl*II; H, *Hin*dIII; N, *Not*I; RV, *Eco*RV; X, *Xba*I.

gene (Schweizer et al. 1986). By BLAST (Altschul et al. 1990), ToxC is only weakly related to polyketide synthases. The polyketide synthase most closely related to ToxC is the sterigmatocystin polyketide synthase of *A. nidulans* (Yu and Leonard 1995), which gives a score of only 71, compared with 1273 for the fatty acid synthase subunit of *Y. lipolytica*.

Characteristics of *TOXC*.

TOXC is found only in isolates of *Cochliobolus* that make HC-toxin (Fig. 4). In a cross between SB111 ($Tox2^+$) and SB114 ($Tox2^-$), *TOXC* segregates with HC-toxin production (Fig. 4).

Most $Tox2^+$ strains of *C. carbonum* have two copies of *HTS1* (Ahn and Walton 1996) and three copies of *TOXC* (Fig.

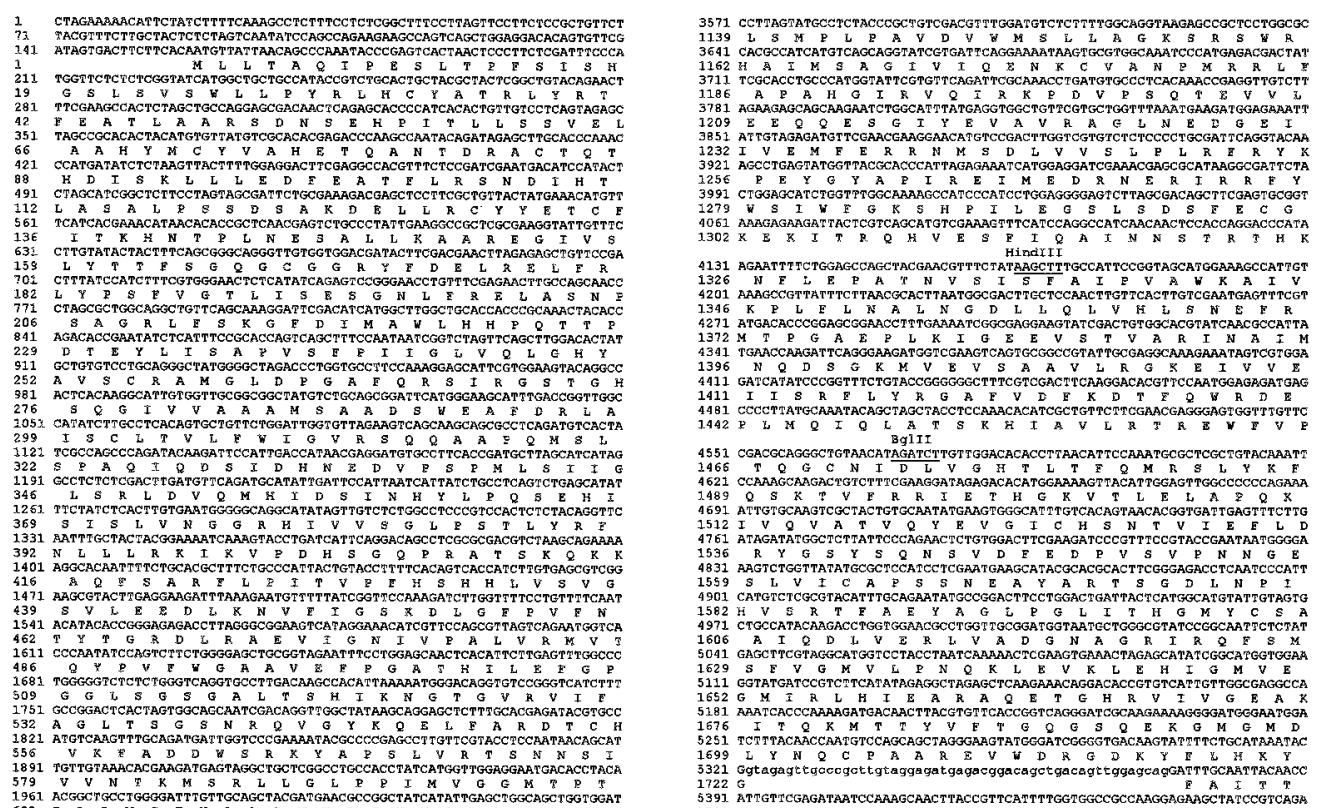

Fig. 2. DNA and deduced amino acid sequences of *TOXC*. Amino acids are placed underneath the encoding nucleotides. The single known intron is shown in lowercase letters. The stop codon is indicated by * and the polyadenylation site by #. The restriction sites used for construction and linearization of the two disruption vectors are indicated. Sequence data for *TOXC* are to be found at GenBank as accession number U73650.

5A). Strain 171 has four copies of *TOXC* (Fig. 5A); this same strain also has three instead of the normal two copies of at least part of *HTS1* (Ahn and Walton 1996). In SB111, the three copies of *TOXC* have the same restriction enzyme pattern from the *Not*I site on the left to the right-most *Xba*I site as drawn in Figure 1. However, the three copies can be distinguished by digestion with *Eco*RI, *Xho*I, *Sal*I, or *Apa*I. *Apa*I digestion gives a number of fragment length polymorphisms in independent Tox2$^+$ strains with *TOXC* as probe (Fig. 5A). *ApaI* also gives polymorphisms when blots are hybridized with *HTS1* (Ahn and Walton 1996). Strain 164R10, which produces HC-toxin and is fully pathogenic, has only two copies of *TOXC* (Fig. 5B). In strain SB111, all three copies of *TOXC* are on the same 3.5-MB chromosome as the two copies of *HTS1* but are not tightly linked (Ahn and Walton 1996).

Creation of a *TOXC* null mutant.

To test the involvement of *TOXC* in HC-toxin biosynthesis, the two copies of *TOXC* in strain 164R10 were mutated by transformation-mediated gene disruption (Panaccione et al. 1992). Alternate copies of *TOXC* were disrupted with an internal *TOXC* fragment (AJ11; see Figure 1) and acetamide selection. The two copies of *TOXC* can be distinguished with *Sal*I, which generates *TOXC*-containing fragments of 8.5 kb and 12 kb (Fig. 6A, lanes 1, 4). Since the acetamide disruption vector pAJ11 contains no *Sal*I sites, homologous recombination of pAJ11 at *TOXC* should result in disappearance of either the 8.5 kb or 12 kb *Sal*I band and appearance of a new band that is 8.0 kb larger. As shown in Figure 6A, the pattern of hybridization is consistent with transformant T491-1 having pAJ11 integrated into the 8.5-kb copy of *TOXC* (lane 2) and transformant T491-2 having pAJ11 integrated into the 12-kb copy of *TOXC* (lane 3). In both transformants, one intact copy of *TOXC* remains (Fig. 6A, lanes 2, 3).

Transformant T491-2 was re-transformed with a vector, pAJ9, containing a different internal fragment of *TOXC* (AJ9;

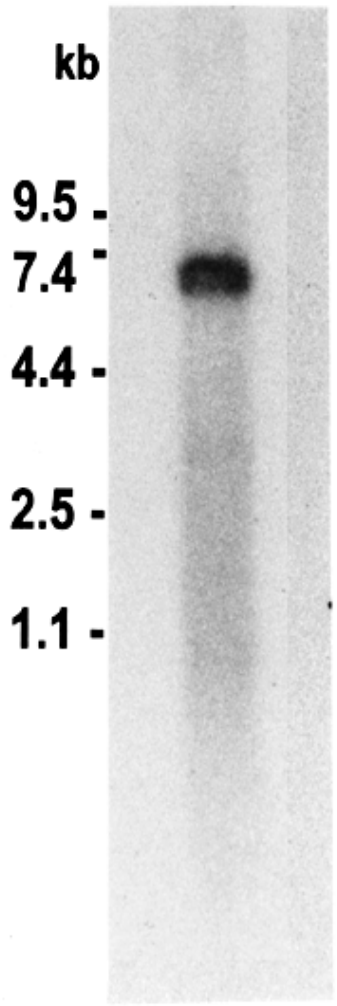

Fig. 3. RNA gel blot analysis of *TOXC*. Approximately 2.5 µg of polyA$^+$-RNA was fractionated on a 1.0% agarose gel containing formaldehyde and the gel blotted. The blot was probed with pJA8, a partial *TOXC* cDNA.

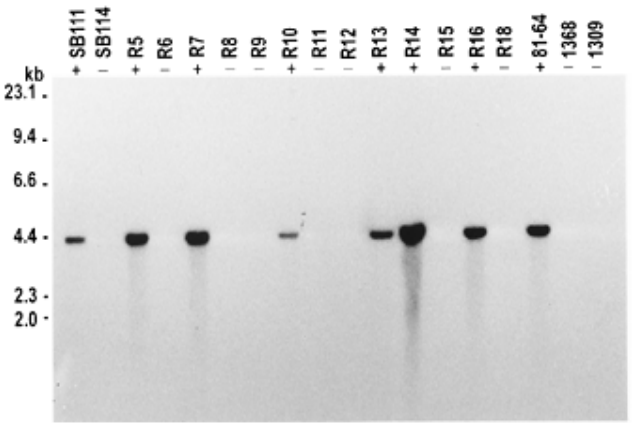

Fig. 4. Presence of *TOXC* only in HC-toxin-producing strains of *Cochliobolus carbonum*. DNA was digested with *Bam*HI and the blot probed with AJ9 (see Figure 1). + and – indicate whether a particular strain makes HC-toxin. SB111 and 81-64 are race 1 (toxin-producing), SB114 and 1309 are race 2 (toxin-non-producing), and 1368 is race 3 (toxin-non-producing). Races 2 and 3 are distinguished from each other by their differential virulence on different maize lines and the appearance of their lesions (Panaccione et al. 1992; Walton 1990). R5 through R18 are the random ascospore progeny of a cross between SB111 and SB114; their full strain designations are 164R5 through 164R18.

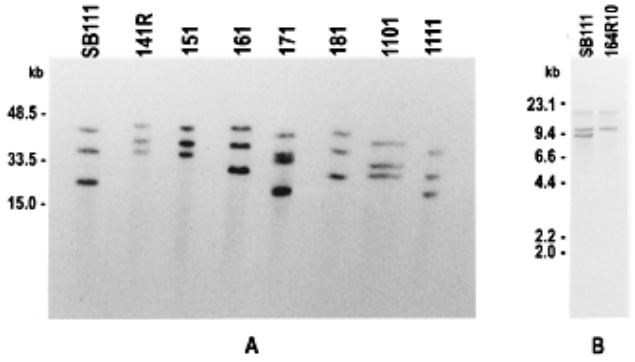

Fig. 5. *TOXC* copy number in different isolates of *Cochliobolus carbonum*. **(A)** Independent field isolates of *C. carbonum*. Isolate numbers are given above the lanes (Ahn and Walton 1996; Jones and Dunkle 1993). DNA was cut with *Apa*I, separated by contour-clamped homogeneous electric field (CHEF) electrophoresis (0.5 to 2.5 s switching time, 150 V, 20 h) and hybridized with probe AJ11 (see Figure 1). **(B)** 164R10 has only two copies of *TOXC*. DNA was cut with *Xho*I and hybridized with probe AJ11 (see Figure 1).

see Figure 1) and the *hph* gene for selection on hygromycin. pAJ9 has a single *Bam*HI site. Fragment AJ9 hybridizes to only one *Bam*HI fragment in 164R10 because this 4.5-kb *Bam*HI fragment (A3B3; see Figure 1) is the same size in both copies of *TOXC* (Fig. 6B, lane 1). In transformants T491-1 and T491-2, which have alternate copies of *TOXC* disrupted, homologous integration is evidenced by the appearance of a new band of 6 kb (Fig. 6B, lanes 2, 3). The pattern of hybridization of A3B3 to transformant T511 is consistent with integration of pAJ9 into the remaining wild-type copy: the 4.5-kb *Bam*HI fragment is gone and a band of 2.3 kb has appeared (Fig. 6B, lane 4). Disruption of both copies of *TOXC* in T511 was confirmed by cutting with *Xba*I; both wild-type A3B3-hybridizing fragments of 7 kb are gone and fragments of 8 kb and 5 kb have appeared (Fig. 6B, lanes 5 to 8).

Phenotype of the *TOXC* null mutant.

All *TOXC* disruptant strains, including the *TOXC* null T511, grow and sporulate as well as wild type on V8 agar plates. Therefore, *TOXC* is not required for normal growth and development of *C. carbonum*. HC-toxin in culture filtrates of strains 164R10, T491-1, T491-2, and T511 was analyzed by thin-layer chromatography (Meeley and Walton 1991). *TOXC* single disruptants make wild-type levels of HC-toxin, but the *TOXC* null mutant T511 makes no detectable epoxide-containing compound with the same R_f as native HC-toxin. T511 also has no detectable HC-toxin by reverse-phase high-pressure liquid chromatography with detection at 230 nm (Fig. 7A). Since T491-1 and T491-2 both make HC-toxin but T511 does not, both copies of *TOXC* in 164R10 are functional. T511 apparently does not make any novel epoxide-containing compounds; no new peaks of absorbance at 215, 230, 254, or 280 nm that might represent shunt metabolites related to HC-toxin were consistently observed in culture filtrates of T511 or in chloroform extracts of the mycelial mats.

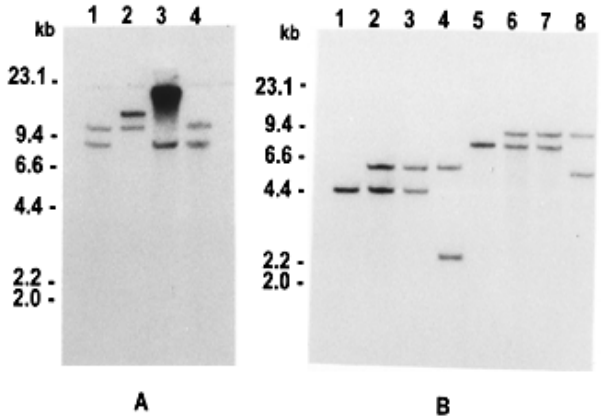

Fig. 6. Targeted disruption of *TOXC*. **(A)** DNA blot showing disruption of one or the other copy of *TOXC* with plasmid pAJ11. DNA was cut with *Sal*I and the blot probed with AJ11. Lanes 1 and 4: 164R10 (wild type); lane 2: T491-1; lane 3: T491-2. Strains T491-1 and T491-2 have alternate copies of *TOXC* disrupted. The pattern of hybridization of T491-2 is consistent with homologous integration of multiple tandem copies of the plasmid. **(B)** DNA blot showing double disruptions of *TOXC*. Genomic DNA from each strain was digested with *Bam*HI (lanes 1 to 4) or *Xba*I (lanes 5 to 8). Strains T491-1 and T491-2 have single copies of *TOXC* disrupted. Strain T511 has both copies disrupted. Lanes 1 and 5: 164R10 (wild type); lanes 2 and 6: T491-1; lanes 3 and 7: T491-2; lanes 4 and 8: T511.

^{14}C-D-alanine was used to label HC-toxin in vivo (Meeley and Walton 1991). Strain 164R10 incorporates D-alanine into a chloroform-extractable metabolite with the same high-pressure liquid chromatography retention time as HC-toxin, but the *TOXC* null mutant T511 does not (Fig. 7B). The smaller peak co-eluting just before HC-toxin is either HC-toxin with a hydrolyzed epoxide (9,10-diol-HC-toxin) (Meeley and Walton 1991) or one of the minor forms of HC-toxin (Walton 1996).

Although it cannot produce HC-toxin, T511 retains HTS activity comparable to that of the wild-type strain 164R10 (Table 1). Thus, the lack of HC-toxin production in T511 is not due to inadvertent disruption of *HTS1*, nor is *TOXC* required for expression of *HTS1* (Panaccione et al. 1992).

Strains T491-1, T491-2, and T511 were tested for pathogenicity on maize of genotype *hm/hm*. The single *TOXC* mutants are still pathogenic but the pathogenicity of T511 is indistinguishable from that of SB114, a wild-type Tox2$^-$ strain (Fig. 8). Thus, at least one functional copy of *TOXC* is required for pathogenicity of *C. carbonum* on susceptible maize.

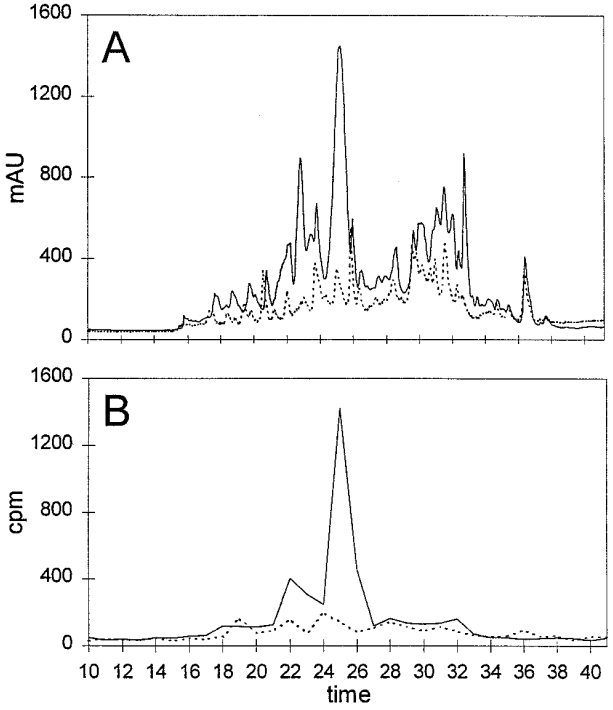

Fig. 7. Reverse-phase high-pressure liquid chromatography analysis of toxin production by wild type and *TOXC*-null mutant T511. HC-toxin elutes at 25 min. **(A)** Absorbance at 230 nm. **(B)** ^{14}C-D-alanine incorporation. Solid lines: wild-type strain 164R10. Dashed lines: *TOXC*-null strain T511.

Table 1. ATP/PP_i exchange activity in extracts of wild-type (164R10) and *TOXC* null mutant (T511) strains of *Cochliobolus carbonum* (HC-toxin synthetase activity was purified through ammonium sulfate precipitation [Walton 1987])

	Substrate (cpm per µg of protein)			
Fungal strain	**H_2O**	**D-alanine**	**L-alanine**	**L-proline**
164R10	420	1,360	1,550	1,730
T511	480	1,170	1,200	1,600

T511 causes small flecks indistinguishable from those caused by the wild type on resistant (*Hm*/–) maize.

To test that the Tox2$^-$ phenotype of T511 is not due to a mutation unrelated to *TOXC*, T5111 was crossed to wild-type strain 367-1 and hygromycin resistance, acetamide utilization, and HC-toxin production analyzed in 52 random ascospore progeny. Twenty-eight progeny have both selectable markers and are nonpathogenic; 24 progeny have neither marker and are pathogenic. Since the two markers co-segregate absolutely with the nonpathogenic phenotype, the loss of pathogenicity is not due to inadvertent mutation at a site unlinked to *TOXC*.

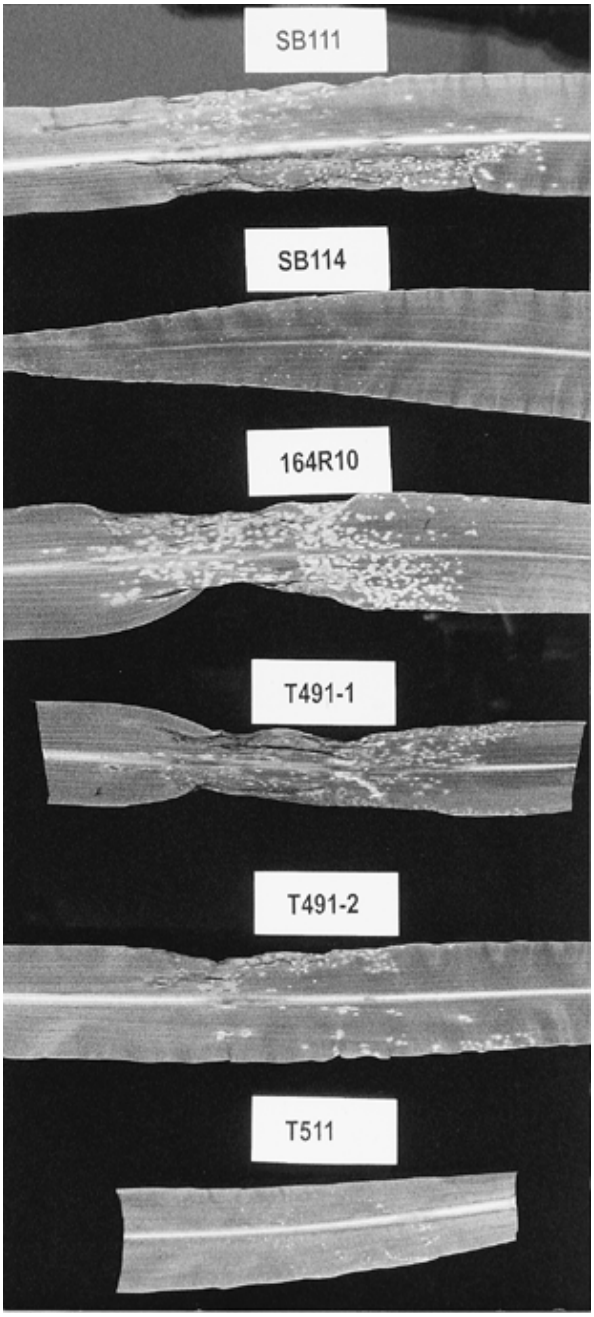

Fig. 8. Pathogenicity phenotypes of wild type and *TOXC* disruption mutant T511. SB111: wild-type Tox2$^+$ strain that has three copies of *TOXC*; SB114, wild-type Tox2 strain; 164R10: wild-type Tox2$^+$ strain that has two copies of *TOXC*; T491-1 and T491-2: strain 164R10 with alternate copies of *TOXC* disrupted; T511: 164R10 with both copies of *TOXC* disrupted.

Enzyme activity of HTS with putative products of the *TOXC* gene.

Based on the comparative enzymology and gene structure of other cyclc peptide synthetases, it is presumed that one of the four domains of HTS activates Aeo or an Aeo precursor (Scott-Craig et al. 1992). ToxC is predicted to synthesize a fatty acid and this fatty acid might be a substrate for HTS. We tested HTS for its ability to activate potential products of ToxC with the ATP/PP_i exchange reaction. The HTS enzyme preparation was prepared by ammonium sulfate precipitation from cell extracts of *C. carbonum* 164R10 and was shown to catalyze L-proline, D-alanine, and L-alanine-dependent ATP/PP_i exchange. Hexanoic (caproic) acid, octanoic (caprylic) acid, 2-aminodecanoic (2-aminocapric) acid, decanoic (capric) acid, and 2-aminohexanoic (2-aminocaproic) acid do not stimulate the ATP/PP_i exchange reaction above the activity seen with water alone. 2-Amino-octanoic (2-aminocaprylic) acid stimulates ATP/PP_i exchange slightly (data not shown).

DISCUSSION

The filamentous fungus *C. carbonum* has a gene, *TOXC*, that encodes a fatty acid synthase. This gene is present only in natural isolates of the fungus that make HC-toxin and is on the same chromosome as *HTS1*, a cyclic peptide synthetase gene required for toxin biosynthesis (Ahn and Walton 1996). Because a strain lacking a functional copy of *TOXC* has unaltered growth and sporulation in culture but no longer makes HC-toxin and is not pathogenic on maize of genotype *hm*/*hm*, we conclude that *TOXC* does not have an essential metabolic function and is necessary only for HC-toxin production.

The most plausible function of *TOXC* is to contribute to the biosynthesis of the decanoic acid backbone of Aeo. The product of *TOXC*, ToxC, might make decanoic acid but might also make a shorter alkanoic acid or an alkenoic acid. Several potential ToxC products are ineffective at stimulating ATP/PP_i exchange by HTS. 2-Amino-octanoic acid is somewhat active at stimulating ATP/PP_i exchange, but the stimulation is too low to allow any conclusions to be drawn.

Incorporation of ^{14}C-acetate into HC-toxin was earlier found to be only weakly inhibited by cerulenin, suggesting that Aeo is more likely synthesized by a polyketide synthase rather than by a fatty acid synthase (Wessel et al. 1988). However, at least some short-chain fatty acid synthases are not inhibited by cerulenin (Mohamed et al. 1988; Schweizer et al. 1987), so there is apparently no contradiction between the earlier results (Wessel et al. 1988) and the conclusions of the current work.

Since all five known Aeo-containing cyclic peptides (Walton 1990; Itazaki et al. 1990) are from filamentous fungi, Aeo is probably made by an enzyme similar to *TOXC* in all five fungi. Because fatty acid synthesis in yeast and other fungi requires the cooperation of α and β subunits, the existence in *C. carbonum* of a β subunit gene that is specific for HC-toxin biosynthesis implies that there is also an α subunit, encoded by a homolog of *FAS2*, that biosynthetically cooperates with ToxC (Mohamed et al. 1988). This gene could be a gene that, like *HTS1* and *TOXC*, is dedicated to HC-toxin bio-

synthesis and is present only in Tox2$^+$ strains. Alternatively, the α subunit that interacts with ToxC could be the same α subunit that participates in primary fatty acid synthesis. As part of its 60-kb gene cluster dedicated to sterigmatocystin biosynthesis, *Aspergillus nidulans* has homologs of both the α and β fatty acid synthase subunits. The *A. nidulans* fatty acid synthase probably synthesizes hexanoic acid, which is then the substrate for the sterigmatocystin polyketide synthase (Brown et al. 1996; Yu and Leonard 1995). In *A. parasiticus*, biosynthesis of aflatoxin, which is related to sterigmatocystin, also requires a homolog of *FAS1*, called *fas-1*, that is distinct from the housekeeping fatty acid synthase (Mahanti et al. 1996). On the basis of these two precedents, and the fact that both *HTS1* and *TOXC* are found only in Tox2$^+$ isolates of *C. carbonum*, it seems more likely that the putative α subunit that interacts with ToxC is not the same one that contributes to the synthesis of primary fatty acids but is, instead, dedicated to HC-toxin biosynthesis.

MATERIALS AND METHODS

Fungal strains, culture, and pathogenicity testing.

Strains of *Cochliobolus carbonum* used in this study were maintained as glycerol stocks and grown on V8 juice agar plates (Walton 1987; Pitkin et al. 1996). SB111 (ATCC 90305), a standard laboratory strain, produces HC-toxin (Tox2$^+$). SB114 is a standard non-toxin-producing (Tox2$^-$) strain, and 164R10 is a progeny of a cross between SB111 and SB114 (Walton 1987).

Pathogenicity was analyzed by spraying 2-week-old maize (*Zea mays* L.) inbred Pr or K61 (genotype *hm*/*hm*, susceptible to race 1 of *C. carbonum* and sensitive to HC-toxin) with spore suspensions (10^4 spores/ml) in 0.1% Tween 20. Plants were observed daily for 2 weeks.

Nucleic acid manipulations and sequencing.

The genomic and cDNA libraries have been described (Scott-Craig et al. 1992; Pitkin et al. 1996). Isolation of fungal DNA and RNA was done as described (Pitkin et al. 1996; Apel et al. 1993). DNA electrophoresis, blotting, and hybridization were done as described (Panaccione et al. 1992). RNA electrophoresis was done in formaldehyde (Sambrook et al. 1989), RNA was transferred to Nytran (Schleicher and Schuell, Keene, NH), and RNA blots were hybridized overnight at 42°C in 5× SSPE (1× SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 0.1% sodium dodecyl sulfate [SDS], 2× Denhardt's solution, 0.1 mg of denatured salmon sperm DNA per ml, and 50% (vol/vol) formamide, according to the manufacturer's recommendations. Probes for DNA and RNA blots were labeled with α-^{32}P-dCTP by random priming and were present in the hybridization solution at 100 to 1,000 kcpm/ml. DNA and RNA blots were washed twice for 10 min in 2× SSPE plus 0.1% SDS at 22°C and twice in 0.1× SSPE plus 0.1% SDS for 30 min at 65°C.

For sequencing, DNA was subcloned into Bluescript SKII (Stratagene, La Jolla, CA) or pGEM-7Zf(+) (Promega, Madison, WI). Custom oligonucleotides as well as T7, T3, and SP6 primers were used as sequencing primers. Automated fluorescent sequencing was done at the MSU-DOE-PRL Plant Biochemistry Facility. Sequences were analyzed with BLAST (Altschul et al. 1990), DNASIS (Hitachi Software Engineering Co., San Bruno, CA), and the Wisconsin GCG Package (Program Manual 1994).

Fungal transformation constructs and analysis of transformants.

The vector for gene disruption based on the *hph* gene encoding hygromycin phosphotransferase was pHYG1 (Sposato et al. 1995). The gene disruption vector pAJ9 (6.5 kb) was constructed by subcloning fragment AJ9 (see Figure 1) into the *Hin*dIII site of pHYG1 and linearizing with *Bgl*II prior to transformation.

The vector for gene disruption based on the *amdS* gene for acetamide utilization (Hynes et al. 1983) was constructed by first subcloning AJ11 (an *Acc*I/*Bgl*II fragment from A3B3 with the *Bgl*II site blunt-ended; see Figure 1) into pBluescript SKII cut with *Eco*RV and *Acc*I. This plasmid was digested with *Eco*RI and *Sma*I and ligated with an *Eco*RI/*Sal*I fragment (in which the *Sal*I site had been blunt-ended) containing the *amdS* gene (Panaccione et al. 1992). The resulting plasmid, pAJ11 (8.0 kb), was linearized with *Hin*dIII prior to transformation. Transformation and selection of transformants with either hygromycin or acetamide was as described (Panaccione et al. 1992).

Analysis of HC-toxin and HC-toxin synthetase.

HC-toxin was extracted with chloroform from culture filtrates of *C. carbonum* strains grown for 18 to 25 days in still culture on modified Fries's medium (Walton et al. 1982). The extracts were analyzed by thin-layer chromatography with detection by a specific epoxide detection spray (Meeley and Walton 1991), and by reverse-phase high-pressure liquid chromatography (Walton et al. 1982). In vivo radiolabeling of HC-toxin was done by adding 15 μCi ^{14}C-D-alanine to a flask at the time of inoculation and harvesting the culture filtrate after 4 days (Meeley and Walton 1991). Amino-acid dependent ATP/PP_i exchange was measured in extracts of *C. carbonum* mycelium as described (Walton 1987; Walton and Holden 1988). Briefly, 4-day-old mycelia were lyophilized, ground in buffer, and centrifuged to remove cell wall debris. HC-toxin synthetase (HTS) was precipitated with 40% saturation (0.226 gm/ml) ammonium sulfate. Reactions were done at 37°C for 30 min with amino acid substrates at 2 mM. Substrates were from Sigma except for D,L-2-aminodecanoic acid, which was purchased from Chemica Alta Ltd., Edmonton, Canada.

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