Reduced Virulence Caused by Meiotic Instability of the *TOX2* Chromosome of the Maize Pathogen *Cochliobolus carbonum*

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The mechanisms by which pathogenic fungi evolve are poorly understood. Production of the host-selective cyclic peptide HC-toxin is controlled by a complex locus, *TOX2*, in the plant pathogen *Cochliobolus carbonum*. Crosses between toxin-producing (*Tox2*⁺) and toxin-nonproducing (*Tox2*[−]) isolates, as well as crosses between isolates in which the *TOX2* genes were on chromosomes of different size, yielded progeny that had lost one or more copies of one or more of the *TOX2* genes. Of approximately 200 progeny analyzed, eight (4%) had lost at least one *TOX2* gene. All of them still had at least one functional copy of all of the known genes required for HC-toxin production (*HTS1*, *TOXA*, *TOXC*, and *TOXE*). Most deletion strains could be explained by simple chromosome breaks resulting in the loss of major contiguous portions (0.8 to 1.4 Mb) of the 3.5-Mb *TOX2* chromosome, whereas others had more complicated patterns. All deletion strains had normal growth and were fertile, indicating that the 1.4 Mb of DNA contained no essential housekeeping genes. Most strains were also still virulent (*Tox2*⁺), but two had a novel phenotype of reduced virulence (RV), characterized by smaller lesions that expanded at a reduced rate and an inability to colonize plants systemically. Although the RV strains made no detectable HC-toxin in culture, the RV phenotype was dependent on the presence of a functional copy of *HTS1*, which encodes the central enzyme in HC-toxin biosynthesis. We propose that the RV strains still make a low level of HC-toxin, at least in planta, and that this is due to the loss of one or more genes that contribute to, but are not absolutely required for, HC-toxin synthesis.

It is well documented that the host range and virulence of plant-pathogenic fungi can change rapidly, but little is known about the underlying molecular mechanisms. In a few cases, karyotype polymorphisms due to inherent chromosomal instabilities have been associated with changes in virulence (Covert 1998; Kistler and Miao 1992; Tzeng et al 1992; Zolan et al. 1995). For example, genes for phytoalexin detoxification as well as other virulence determinants are on supernumerary (dispensable) chromosomes in the pea pathogen *Nectria haematococca*, and altered host range is associated with the presence of a particular 2-Mb chromosome in *Colletotrichum gloeosporioides* (Covert et al. 1996; He et al. 1998; Masel et al. 1993; Miao et al. 1991).

Isolates of the Ascomycete *Cochliobolus carbonum* that make the cyclic tetrapeptide HC-toxin are exceptionally

Fig. 1. Maps of the *TOX2* chromosome in wild-type and deletion strains. **A**, Whole 3.5-Mb *TOX2* chromosome of SB111 (wild type) (adapted from Ahn and Walton 1996). **B**, Map of the region of the 3.5-Mb chromosome containing the known *TOX2* genes. Distances are in kb. A1/H1 and A2/H2 are copies 1 and 2 of *TOXA* and *HTS1*; C1, C2, and C3 are copies 1, 2, and 3 of *TOXC*; D1, D2, and D3 are copies 1, 2, and 3 of *TOXD*; and E indicates *TOXE*. *TOXD* has no apparent role in HC-toxin production; it is used in this study as a mapping marker. **C**, Maps of deleted DNA in deletion strains. Dark shading, region of the chromosome remaining; light shading, region of the chromosome missing; medium shading, region of the chromosome where the break point maps.

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virulent on maize of genotype *hm1/hm1*. HC-toxin production is controlled by a complex locus, *TOX2*, that extends over more than 540 kb and contains several multicopy genes (Walton 1996) (Fig. 1). The known genes of the *TOX2* locus include the following: *HTS1*, encoding a 570-kDa, nonribosomal, peptide synthetase; *TOXA*, encoding an HC-toxin efflux carrier; *TOXC*, encoding a fatty acid synthase β subunit; and *TOXE*, encoding a regulatory protein (Ahn and Walton 1997, 1998; Panaccione et al. 1992; Pitkin et al. 1996; Scott-Craig et al. 1992). All of the genes of *TOX2* are present in multiple, functional copies (Fig. 1). (In strain SB111, a second functional copy of *TOXE* is on a 0.7-Mb chromosome; Ahn and Walton 1996, 1998). For example, both copies of *HTS1* must be disrupted in isolate SB111 to eliminate HTS activity and HC-toxin production (Panaccione et al. 1992). The genes of *TOX2* are completely absent from toxin-nonproducing (*Tox2*⁻) isolates.

A progeny of a cross between SB111 (*Tox2*⁺) and SB114 (*Tox2*⁻), called 243-7, had a truncated *TOX2* chromosome and the 1-Mb *PacI* fragment that contains both copies of *HTS1* in SB111 was reduced to 0.28 Mb (Ahn and Walton 1996). The

map of the remaining *TOX2* genes in 243-7 is consistent with the original 3.5-Mb *TOX2* chromosome of SB111 having undergone a simple chromosome break that resulted in the loss of up to 1.4 Mb of contiguous DNA (Fig. 1). Isolate 243-7 still has at least one functional copy of each of the known *TOX2* genes, including the regulatory gene *TOXE* (Ahn and Walton 1998). A detailed analysis of 243-7, as well as of additional isolates with major deletions of the *TOX2* chromosome, documents instability of the *TOX2* chromosome that, in some cases, results in a novel pathogenic phenotype.

RESULTS

Disease phenotype of 243-7.

Pathogenicity of strain 243-7 was evaluated on plants of maize inbred Pr in several ways. On plants with a height of 10 (two true leaves), 16, or 25 (four true leaves) cm sprayed with a conidial suspension at 5×10^4 spores per ml, no obvious differences in lesion morphology or size were observed 2 days post inoculation, but at 4 days the development of lesions on the leaves infected with 243-7 was

Fig. 2. Pathogenicity of *Cochliobolus carbonum* wild-type and deletion strains. Maize leaves of genotype *hm1/hm1* were inoculated 14 days after planting with conidia of strains 367-2 (*Tox2*⁺), 243-7 (RV), or 243-10 (*Tox2*⁻). **A**, Time course of infection. **B**, Pathogenicity of 243-7 after disruption of its single copy of *HTS1* (isolate 560-2). The leaves in **B** came from three plants inoculated in parallel. Photographs were taken 7 days after inoculation.

intermediate to that caused by 367-2 (Tox2⁺) and 243-10 (Tox2⁻) (Fig. 2A). The lesions caused by 243-7 were fewer than those caused by 367-2 and more variable in size and shape. Isolate 243-10 caused only small flecks, typical of a resistance response in this disease interaction. At 7 days, the intermediate disease phenotype of 243-7 was still clear. Plants infected with the Tox2⁺ isolate 367-2 were completely dead as the fungus colonized the entire plant, including the stalks, whereas plants infected with the Tox2⁻ isolate 243-10 were healthy. The plants infected with 243-7, on the other hand, showed some lesions of highly variable size and irregular outline but the plants remained alive and continued to grow. Isolate 243-7 was never observed to invade the stalks of infected plants or to kill plants. We call this intermediate disease phenotype “reduced virulence” (RV).

When seedlings with only the flag leaf and one true leaf (2 to 3 cm tall) were inoculated with conidial concentrations of either 10⁴ per ml or 5 × 10⁴ per ml, all of the plants survived until 6 days post inoculation. The plants inoculated with 367-2 or 243-7 developed lesions only on the tips of the first true leaves. Plants inoculated with 243-7 had consistently fewer lesions than those sprayed with 367-2. At 6 or 7 days post inoculation, plants infected with 367-2 but not 243-7 began to lose turgor and became grayish in color, and they collapsed and died 2 to 3 days later. Plants inoculated with 243-10 or 243-7 continued to grow normally.

When plants were inoculated by soaking seeds in a conidial suspension, plants in all treatments emerged at the same time. Plants infected with 367-2 were brown in color from the first day of appearance, grew to a height of only 1 to 2 cm, and died 3 to 4 days after appearance. Plants from seeds inoculated with 243-7 or 243-10 resembled noninoculated plants and did not develop any visible symptoms.

Plants inoculated with conidia of isolate 367-2 at 5 × 10⁴ per ml or 10⁵ per ml through the soil looked healthy for the first 3 to 4 days after their appearance aboveground but then became grayish in color, wilted, and died by 10 days without the development of any visible leaf lesions. Plants inoculated with 367-2 at 10⁴ conidia per ml remained healthy. Plants infected by soil inoculation with isolates 243-10 or 243-7 showed no symptoms at any concentration at any time point.

In conclusion, the pathogenicity tests indicated that 243-7 has a virulence intermediate between those of wild-type Tox2⁺ and Tox2⁻ isolates. When inoculated directly onto leaves, isolate 243-7 could cause disease, but the lesions were fewer, more irregular in outline, and more variable than those caused by a wild-type Tox2⁺ isolate (Fig. 2A). Isolate 243-7 never caused wilting or death of seedlings, and could not cause any disease when inoculated onto seeds either directly or through the soil. Isolate 243-7 could not infect stems and therefore could not infect or spread systemically; it could cause disease only in leaf tissue that had come into direct contact with the primary inoculum.

To test whether the RV phenotype was due to defective appressorium formation or penetration, infection by 243-7 was examined microscopically. At 24 h, no differences were seen in spore germination, percentage of spores that formed appressorium, or percentage of germinating spores that penetrated the leaf either directly or through stomata (data not

shown). Therefore, the RV phenotype was not due to a defect in germination or penetration.

Co-segregation of the RV phenotype with the chromosomal aberration.

To test whether the RV phenotype was linked to the chromosomal deletion in strain 243-7, it was crossed to the Tox2⁺ isolate 243-1, which is a sibling of 243-7 from cross 243 (cross 625; Fig. 3). Of 53 progeny of this cross, 27 had three copies of *TOXC* and were fully virulent like the wild-type parent. Twenty-five progeny were missing copy 3 of *TOXC* and had an RV disease phenotype like the RV parent. The ratio of the two progeny classes (27:25) was close to 1:1 ($\chi^2 = 0.077$; $df = 1$; $0.70 < P < 0.90$), indicating that the two different-sized *TOX2* chromosomes behaved as homologs. The co-segregation of the RV phenotype with the chromosomal deletion indicated that the deletion is the cause of the phenotype.

One progeny of this cross (625-66), however, was missing only copy 3 of *TOXC* and copies 1 and 3 of *TOXD* (Fig. 4) and thus had a genetic composition different from that of either parent. It apparently arose de novo during the cross. Isolate 625-66 has the same haplotype as 512-48.

Growth and HC-toxin phenotype of 243-7.

Strain 243-7 sporulated normally in culture and its growth rate (0.84 ± 0.06 cm per day) was very close to that of the wild type (0.79 ± 0.04 cm per day). Growth of the different strains as surface still cultures was also indistinguishable. Therefore, the RV disease phenotype of 243-7 was not due to defective growth or development.

Isolates 243-7 and 367-2 (Tox2⁺) were grown in parallel and the culture filtrates extracted with chloroform and analyzed by high-pressure liquid chromatography (HPLC) followed by thin-layer chromatography (TLC). No HC-toxin could be detected in culture filtrates of 243-7 (data not shown). Based on the determination of the detection limit for HC-toxin by dilution of preparations from isolate 367-2, 243-7 made less than 1% of the wild-type level of HC-toxin.

Fig. 3. Crosses to generate deletion strains. Strains 243-1, 243-7, and 243-10 are the progeny of SB111 and SB114. Strain 367-2 was derived from 243-1 by backcrossing it three times to SB111.

Disruption of *HTSI* in isolate 243-7.

Two explanations for the RV phenotype seemed possible. One, supported by the in vitro HC-toxin analysis, was that strain 243-7 had completely lost the ability to produce HC-toxin and its residual virulence was due to an unrelated factor. An alternative hypothesis was that 243-7 still makes a low level of HC-toxin, perhaps only in planta, that is sufficient to allow some disease development.

To test the involvement of HC-toxin in the RV phenotype, the remaining copy of *HTSI* (copy 2, see Figure 1) in strain 243-7 was disrupted by transformation-mediated homologous integration of plasmid pCC119 (Panaccione et al. 1992) to create an *HTSI*-null strain. Five hygromycin-resistant transformants were analyzed by Southern blotting after digestion of total genomic DNA with *SalI*. The insert of pCC119 hybridized to a *SalI* fragment of 17.1 kb in the wild-type strain 243-7 (Panaccione et al. 1992). Integration of pCC119 was expected to result in the disappearance of the 17.1-kb band and appearance of two new bands of 12.5 and 10.6 kb, because pCC119 is 6.0 kb in size and contains one *SalI* site. The observed pattern of Southern hy-

bridization indicated that pCC119 had integrated at *HTSI* in transformant 560-2 as a single copy. pCC119 integrated into transformants 560-1, 560-3, 560-5, and 560-6 as multiple copies, as evidenced by the appearance of a hybridizing band at 6.0 kb in addition to the bands at 12.5 and 10.6 kb (data not shown). All of these transformants should be *HTSI* nulls.

Pathogenicity of the *HTSI*-null transformants was compared with that of 367-2 (Tox2⁺), 243-10 (Tox2⁻), and 243-7 (RV). All five were completely avirulent, that is, indistinguishable from the Tox2⁻ isolate 243-10 (Fig. 2B). Thus, the residual virulence of 243-7 is dependent on *HTSI*, which encodes the central enzyme in HC-toxin biosynthesis. The simplest explanation for this is that 243-7 still makes a low level of HC-toxin, at least in planta, and that this accounts for its residual virulence.

Isolation of additional strains with *TOX2* chromosomal aberrations.

In order to obtain an indication of the rarity of the chromosome break event that had occurred in 243-7, that is, of the instability of the *TOX2* chromosome, we examined progeny from additional crosses for deletions within the *TOX2* chromosome. The parents in cross 373 (Fig. 3) were Tox2⁺ isolates with homologous *TOX2* chromosomes of different sizes (namely, 3.5 and 2.2 Mb), and the parents in cross 512 (Fig. 3) were a Tox2⁺ isolate with a 3.5-Mb *TOX2* chromosome and a Tox2⁻ isolate lacking a chromosome of 3.5 Mb and all of the known *TOX2* genes (Ahn and Walton 1996; Canada and Dunkle 1997).

Thirty-four progeny were obtained and screened in cross 373 and 110 in cross 512 (Fig. 3). Progeny were initially screened by DNA blot analysis with *TOXC* as a probe (Fig. 4A), because copies 1 and 3 of *TOXC* form the boundaries of the mapped, approximately 540-kb region of the *TOX2* chromosome (Fig. 1). Strains were further analyzed with *TOXA* and *TOXD* as probes (Figs. 4B, C). *TOXD* is a gene found only in Tox2⁺ isolates, usually in three copies, and is regulated by *TOXE* (Ahn and Walton 1998), but disruption of it does not affect toxin production or pathogenicity. Among the progeny of both crosses, two isolates (373-39 and 512-24) lacked copy 3 of *TOXC*, copies 1 and 3 of *TOXD*, and copy 1 of *TOXA/HTSI*. Thus, these strains resembled 243-7 in their *TOX2* haplotype. All other strains that lacked copy 3 of *TOXC* (512-3, 512-8, 512-35, 512-48, and 512-98) still had copy 1 of *TOXA/HTSI*. Strain 512-35 grew slowly and was not analyzed further. Strain 512-48 lacked copies 1 and 3 of *TOXD* in addition to copy 3 of *TOXC* (Fig. 1C, Table 1). All strains that were tested still had at least one copy of the regulatory gene *TOXE*.

In cross 512, segregation with respect to the presence or absence of the *TOX2* chromosome was not statistically different from the expected Mendelian ratio of 1:1 (59 with the chromosome and 51 without; $\chi^2 = 0.582$; df = 1; $0.3 < P < 0.5$). In cross 373, none of the progeny lost the chromosome altogether, and none of seven progeny analyzed by pulsed-field gel electrophoresis (PFGE) had two *TOX2* chromosomes (data not shown). Therefore, despite the absence in Tox2⁻ isolates of a chromosome of the same size, the 3.5-Mb *TOX2* chromosome segregates normally.

From cross 373, all 34 progeny were screened for pathogenicity even if they were not missing any known *TOX2*

Fig. 4. Southern blot analysis of deletion strains. **A**, *TOXC*. Genomic DNA was cut with *XhoI* and the filter was probed with an internal fragment of *TOXC*. Fragment sizes of copies 1, 2, and 3 are 18, 14, and 12 kb, respectively (Ahn and Walton 1997). **B**, *TOXA*. Genomic DNA was cut with *BglI* and probed with an internal fragment of *TOXA*. Fragment sizes of copies 1 and 2 are 15 and 12 kb, respectively. **C**, *TOXD*. Genomic DNA was cut with *BamHI* and probed with an internal fragment of *TOXD*. Fragment sizes of copies 1, 2, and 3 are 15, 9, and 8 kb, respectively.

genes. All of the progeny had a fully virulent (Tox2⁺) phenotype, as expected, except 373-39, which had an RV phenotype. Therefore, the only progeny from this cross that had an RV phenotype harbored a chromosomal aberration.

The new deletion strains were analyzed further. Isolate 164R10, which, like 243-7, is a progeny of SB111 and SB114 (Fig. 3) was also included. Isolate 164R10 lacks copy 3 of *TOXC*, copies 1 and 3 of *TOXD*, and copy 1 of *TOXA/HTSI*, but has a wild-type Tox2⁺ virulence phenotype and produces HC-toxin in culture (Ahn and Walton 1997, 1998). The two RV isolates 243-7 and 373-39 had their single copies of *HTSI* on *PacI* fragments of 280 and 190 kb, respectively, whereas these *PacI* fragments were 290 kb or larger in all deletion strains that were still Tox2⁺ (Fig. 5). In some cases, the sizes of the *PacI* fragments were consistent with the maps of the deleted genes (strains 243-7, 373-39, 643-9, and 512-3), but in other strains the sizes were not consistent (strains 164R10, 512-48, and 625-66) (Fig. 1 and Table 1). That is, whereas some deletion strains could be explained by simple chromosome breakage, the chromosome in other strains had either undergone internal deletions or translocation of DNA from elsewhere in the genome.

In conclusion, the *TOX2* chromosome is unstable and can break in different locations (Table 1). However, in contrast to other fungal chromosomes containing pathogenicity genes, the *TOX2* chromosome of *C. carbonum* segregates normally in crosses (crosses 373, 512, and 625; Fig. 3; Canada and Dunkle 1997).

Phenotypes of the new deletion isolates.

The growth rates of 367-2, SB111, 141R, SB114, 243-7, 243-10, 373-39, 512-8, and 512-48 on either petri plates or in race tubes were statistically indistinguishable (data not shown). Isolate 512-24, on the other hand, grew slowly in culture. It was outcrossed to 243-6 (Tox2⁺, normal growth). Of 10 progeny analyzed, five grew at the same rate as the wild type, and, of these, two (643-8 and 643-9) had the same *TOX2* haplotype as 512-24 (Fig. 4). Therefore, the chromo-

somal aberration segregated away from the slow-growth phenotype.

Pathogenicity assays were done with strains with detectable deletions (Table 1). Strain 373-39 had an RV disease phenotype, and strains 512-3, 512-48, 643-9, 625-66, and 164R10 had a Tox2⁺ phenotype. Isolates 512-3, 512-48, and 164R10 produced wild-type levels of HC-toxin in culture but 373-39, like 243-7, produced no detectable toxin (data not shown).

Fig. 5. Physical mapping of representative wild-type and deletion strains. DNA was cut with *PacI*, fractionated by pulsed-field gel electrophoresis (PFGE), blotted, and probed with a fragment of *HTSI*. Locations of relevant *PacI* sites are shown in Figure 1. **A**, 367-2, 164R10, 512-3, 512-48, and 243-7. **B**, 373-39, 243-7, 367-2, 625-66, and 643-9.

Table 1. Summary of data on *Cochliobolus carbonum* strains

Strain	Genes missing ^a	Phenotype ^b	HTS activity ^c	HTS protein ^d	HC-toxin ^e	<i>PacI</i> fragment size (kb) ^f	<i>TOX2</i> chromosome size (Mb) ^g
SB111 367-2	All present	V	+	+	+	1000	3.5
141R	All present	V	+	+	+	ND ^h	2.2
243-10	None present	AV	–	–	–	–	–
243-7	H1, A1, C3, D1, D3	RV	+	+	–	280	2.1
560-2 ⁱ	H1, H2::hyg, A1, C3, D1, D3	AV	–	–	–	ND	ND
373-39	H1, A1, C3, D1, D3	RV	+	+	–	190	ND
643-9	H1, A1, C3, D1, D3	V	+	+	ND	290	ND
164R10	H1, A1, C3, D1, D3	V	+	+	+	900	3
512-48	C3, D1, D3	V	+	+	+	400	2.7
625-66	C3, D1, D3	V	+	+	+	490	ND
512-3	C3	V	+	+	+	450	2.3
512-8, 512-98	C3	V	+	+	ND	ND	ND

^a H = *HTSI*; A = *TOXA*; C = *TOXC*; D = *TOXD*. Numbers indicate the particular copy (see Figure 1).

^b V, virulent (Tox2⁺); RV, reduced virulence; AV, avirulent (Tox2[–]) (see Figure 2).

^c HC-toxin synthetase (HTS) activity measured by D-Ala-dependent ATP/PP_i exchange.

^d HTS protein detected by immunoblotting.

^e HC-toxin production in culture, analyzed by thin-layer chromatography.

^f Size of *PacI* fragment hybridizing to *HTSI* as measured by pulsed-field gel electrophoresis (see Figure 5).

^g Size of chromosome containing *HTSI*, *TOXA*, *TOXC*, and *TOXD*.

^h Not determined.

ⁱ 243-7 with remaining copy of *HTSI* mutated by targeted gene disruption.

The lack of detectable HC-toxin in the RV strains could be due to underexpression of one or more of the HC-toxin biosynthetic genes. To test this, all deletion strains were tested for the presence of *TOXA*, *TOXC*, and *TOXE* mRNAs. All were present in all deletion strains, including RV strains (data not shown). In addition, HTS protein (detected by immunoblotting) and HTS activity (defined as D-alanine-dependent ATP/PP_i exchange) were present in all deletion strains, including the RV strains. HTS activity was purified from the two RV isolates by anion exchange HPLC in parallel with Tox2⁺ and Tox2⁻ controls. The peak fractions of isolates SB111 (Tox2⁺), 243-7 (RV), 373-39 (RV), and 243-10 (Tox2⁻) had HTS activities of 9.0, 7.5, 7.0, and 0 pmol PP_i/min/μg protein, respectively. Therefore, the RV phenotype was not caused by down-regulation of any known *TOX2* gene.

DISCUSSION

C. carbonum requires HC-toxin for its high virulence on maize, and all of its known genes for HC-toxin biosynthesis are linked on the same chromosome. This chromosome is meiotically unstable, as evidenced by a reciprocal translocation polymorphism between different wild-type HC-toxin-producing isolates (Ahn and Walton 1996; Canada and Dunkle 1997), and, as shown here, by the fact that 8 of approximately 200 progeny (4%) from three crosses have deletions of one or more genes within the approximately 540-kb region of *TOX2*. Adding previously analyzed progeny from crosses A and 243 (Fig. 3), 10 (4.5%) of a total of approximately 220 progeny had deletions within the *TOX2* region. Because of a lack of markers outside the 540-kb *TOX2* region (Fig. 1), these results probably actually underestimate the instability of the *TOX2* chromosome. Strains such as 243-7 are missing between 240 kb and 1.4 Mb of the *TOX2* chromosome (Fig. 1). Because these strains had no growth phenotype and were fertile, the DNA that is missing apparently contains no essential genes.

Although the major translocation and most of the deletions cause no apparent change in virulence, certain deletions resulted in a unique RV disease phenotype. RV isolates still contained at least one functional copy of each of the known HC-toxin biosynthetic genes. Although RV strains are missing one copy of *HTS1*, this cannot account for the RV phenotype because engineered isolates lacking one of their two copies of *HTS1* are still fully pathogenic and produce HC-toxin in culture (Panaccione et al. 1992). The residual virulence of RV strains depended on the remaining functional copy of *HTS1*, but RV strains made no detectable HC-toxin in culture. One explanation for these results is that RV strains still produce a low level of HC-toxin, which is either undetectable in culture or is produced only in planta, and that this low level of HC-toxin is sufficient to support a reduced level of disease.

The simplest explanation to account for the putative low level of HC-toxin made by RV strains is that these strains are missing one or more as-yet-unknown genes that are present on the deleted DNA and that are required for full HC-toxin production. The missing gene or genes could have a structural (biosynthetic) or regulatory function. HC-toxin biosynthetic enzymes that are postulated to exist but have not yet been found include a fatty acid synthase alpha subunit to work to-

gether with the product of *TOXC*, and one or more enzymes to synthesize the 2-amino, 8-carbonyl, and 9,10-epoxide groups of Aeo. Deletion of an essential biosynthetic gene would be expected to result in the complete disappearance of HC-toxin production, whereas our results favor the hypothesis that RV strains still make a low level of HC-toxin. However, it is possible that the deleted gene or genes are partially substituted for by others that encode enzymes with overlapping functions. The plausibility of this hypothesis is supported by the fact that *C. carbonum* has other nonribosomal peptide synthetases (Nikolskaya et al. 1995). The function of one or more enzymes necessary for the biosynthesis of another nonribosomal peptide, for example, 4'-phosphopantetheinyl transferase (Lambalot et al. 1996), might partially overlap with those involved in HC-toxin biosynthesis.

The second possibility is that RV strains are missing a gene with a positive regulatory function, the loss of which results in reduced expression of one or more HC-toxin biosynthetic genes, decreased HC-toxin production, and therefore reduced virulence. *TOXE* is a known HC-toxin regulatory gene, deletion of which causes a complete loss of virulence (Ahn and Walton 1998), but RV strains still have one functional copy of *TOXE* (Fig. 1). The RV strains still express *HTS1*, *TOXA*, and *TOXC*, so any regulatory gene that is missing in RV isolates is not regulating these genes.

From a comparison of the virulence phenotypes of strains with chromosome breaks in different locations, a tentative location for the missing genes is between copy 1 of *TOXA* and copy 2 of *TOXC* (Fig. 1). A more precise location for the putative missing genes is not possible because it is not known if the *TOX2* chromosomes in these strains have undergone further alterations. Insofar as chromosome breaks must be stabilized by the addition of telomeres, some or all of the DNA between copy 1 of *HTS1* and copy 2 of *TOXC* in these strains could have come from elsewhere in the genome. The *TOX2* chromosome can undergo more than simple terminal deletions, based on comparison of the *PacI* maps of strains 164R10, 512-48, and 625-66 with their *TOX2* gene haplotypes (Fig. 1 and Table 1).

MATERIALS AND METHODS

Fungal culture growth and maintenance.

Conidia of *C. carbonum* SB111 (Tox2⁺; ATCC 90305), SB114 (Tox2⁻; Walton and Holden 1988), 367-2 (Görlach et al. 1998), 141R and 243-7 (Ahn and Walton 1996), 164R10 (Ahn and Walton 1997, 1998; Walton and Holden 1988), CC141 (Ahn and Walton 1996; Canada and Dunkle 1997), and others (this study) were stored at -80°C in 25% (vol/vol) glycerol and grown on V8 juice agar plates. For DNA or protein extraction, mycelial agar plugs (0.5 cm²) were inoculated into 1-liter Erlenmeyer flasks containing 125 ml of modified Fries's medium (Walton et al. 1982). Cultures were grown at room temperature (21 to 23°C) without shaking for 4 days.

Fungal crosses were done as described (Tzeng et al. 1992; Yoder 1988). Individual ascospores were picked and progeny were put through two rounds of single-spore reisolation to obtain nuclear homogeneity. For growth rate comparisons, cultures were grown, in duplicate, in horizontal race tubes containing potato dextrose agar, and linear growth was measured daily from 1 to 9 days.

Pathogenicity tests.

All pathogenicity tests were done at least twice and most were done three times. Plants were grown in 30-cm pots (three plants per pot) in soil composed of 4 parts Bactomix (Michigan Peat, Houston, TX) and 1 part sand in a greenhouse. Leaves of maize inbred Pr (genotype *hml/hml*) were sprayed until runoff with a suspension of conidia suspended in 0.1% Tween 20 with an atomizer. In every experiment, wild-type Tox2⁺ and Tox2⁻ isolates were included as controls. The plants were bagged in plastic overnight. Development of symptoms was observed each day for up to 21 days.

Seed inoculation was done with 9 seeds per treatment planted 3 per 15-cm-diameter pot. The seeds were soaked in a suspension of conidia (10⁴ per ml in 50 ml of 0.1% Tween 20) for 1.5 h. Soil inoculation was done in pots containing 3 seeds each by watering with 10 ml of a suspension of conidia at 10⁴ per ml, 5 × 10⁴ per ml, or 10⁵ per ml, after which the pots were covered overnight with plastic bags.

To assess spore germination, appressorial formation, and penetration, leaves (either individual excised leaves or leaves from inoculated plants) were collected 18, 24, and 48 h after inoculation with strains 367-2, 243-7, or 243-10 at a spore density of 5 × 10⁴ per ml in 0.1% Tween 20. Fungal hyphae were stained in cotton blue/lactophenol solution and examined at ×100 power.

Molecular genetics.

Transformation of protoplasts to hygromycin resistance and analysis of fungal DNA and RNA were as described (Pitkin et al. 1996; Scott-Craig et al. 1990). Transformants were purified by two rounds of single-spore isolation.

PFGE was performed as described, with a Bio-Rad (Richmond, CA) CHEF II system, with some modification (Ahn and Walton 1996). After proteinase-K treatment, the agarose plugs containing DNA were digested with 30 U of *Pac*I at 0°C for 15 h and then at 37°C for 2 h. For *Pac*I-cut DNA, gels of 1% chromosome-grade agarose (Bio-Rad) were run at 170 V with a switching time ramped from 2 to 25 s for 22 h and then ramped from 60 to 120 s for an additional 24 h. For separation of whole chromosomes, 0.8% agarose gels were run at 50 V for a total of 216 h.

Protein manipulations and HTS activity assays.

HTS enzyme extraction, partial purification by ammonium sulfate precipitation and anion exchange HPLC, and assay by ATP/PP_i exchange were done as described (Walton and Holden 1988). Protein was measured by the method of Bradford (1976). For anion exchange HPLC, 9 mg of protein was loaded onto the column, 1-ml fractions were collected, and 10 µl of each fraction was assayed. Reaction mixtures contained 20 mM D-alanine and 300,000 dpm [³²PP_i] in 125 µl.

Analysis of HC-toxin.

HC-toxin was purified from 24-day culture filtrates by solvent extraction and reverse-phase HPLC on a Beckman Ultrasphere ODS column (Walton et al. 1982). Elution conditions were 5 min 100% solvent A (water) followed by a 30-min linear gradient from 100% A to 60% B (acetonitrile) in 30 min. Flow rate was 1 ml/min and the eluant was monitored at 230 nm. HPLC fractions were lyophilized, redissolved in methanol, and analyzed by silica gel TLC. TLC plates were

developed in dichloromethane:acetone (1:1, vol/vol) and the toxin detected with an epoxide-specific spray (Hammock et al. 1974).

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