Toxin-Deficient Mutants From a Toxin-Sensitive Transformant of *Cochliobolus heterostrophus*

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ABSTRACT

Tox1 is the only genetic element identified which controls production of T-toxin, a linear polyketide involved in the virulence of *Cochliobolus heterostrophus* to its host plant, corn. Previous attempts to induce toxin-deficient (*Tox*$^-$) mutants, using conventional mutagenesis and screening procedures, have been unsuccessful. As a strategy to enrich for *Tox*$^-$ mutants, we constructed a *Tox1*$^+$ strain that carried the corn *T-urf13* gene (which confers T-toxin sensitivity) fused to a fungal mitochondrial signal sequence; the fusion was under control of the inducible *Aspergillus nidulans pelA* promoter which, in both *A. nidulans* and *C. heterostrophus*, is repressed by glucose and induced by polygalacturonic acid (PGA). We expected that a transformant carrying this construction would be sensitive to its own toxin when the *T-urf13* gene was expressed. Indeed, the strain grew normally on medium containing glucose but was inhibited on medium containing PGA. Conidia of this strain were treated with ethylmethanesulfonate and plated on PGA medium. Among 362 survivors, 9 were defective in T-toxin production. Authenticity of each mutant was established by the presence of the transformation vector, proper mating type, and a restiction fragment length polymorphism tightly linked to the *Tox1*$^+$ locus. Progeny of each mutant crossed to a *Tox1*$^+$ tester segregated 1:1 (for wild type toxin production *vs.* no or reduced toxin production), indicating a single gene mutation in each case. Progeny of each mutant crossed to a *Tox1*$^-$ tester segregated 1 : 1 (for no toxin production *vs.* no or reduced toxin production) indicating that each mutation mapped at the *Tox1* locus. Availability of *Tox*$^-$ mutants will permit mapping in the *Tox1* region without interference from a known *Tox1* linked translocation breakpoint.

COCHLIOBOLUS heterostrophus, a haploid Ascomycete, exists in nature as either of two races. Race T produces T-toxin, a family of 10–15 linear polyketides which vary in chain length from C_{35} to C_{45} (KONO and DALY 1979; KONO *et al.* 1981a,b), and is specifically virulent on corn containing Texas male sterile (T) cytoplasm (YODER 1980). Race O produces none of the members of the T-toxin family and has low virulence on T cytoplasm corn as well as on most other types of corn. When field isolates of race T and race O are crossed, progenies usually segregate 1 : 1 (Tox$^+$: Tox$^-$), thus defining the single genetic element *Tox1*, which controls production of T-toxin (BRONSON *et al.* 1990; LEACH *et al.* 1982b). Off ratios observed in progeny of certain field isolates (TAGA *et al.* 1985; YODER 1976; YODER and GRACEN 1975) have been explained by linkage of *Tox1* to an ascospore abortion factor, possibly a spore killer gene (BRONSON *et al.* 1990). Plant tests of progeny of all crosses reveal that without exception *Tox1*$^+$ progeny are highly virulent on T-cytoplasm corn whereas all *Tox1*$^-$ progeny are weakly virulent; thus T-toxin is highly associated with enhanced virulence of the fungus to T-cytoplasm corn (BRONSON *et al.* 1990; YODER 1980).

Sensitivity of T-cytoplasm corn to T-toxin is caused by a 13-kD T-toxin-binding protein found in the inner mitochondrial membrane of the host (LEVINGS and SIEDOW 1992). The protein is encoded by a mosaic gene (*T-urf13*), which is unique to the mitochondrial chromosome of T-cytoplasm corn. When *T-urf13* is expressed in *Escherichia coli* under control of an *E. coli* promoter, the cells are as sensitive to T-toxin as mitochondria of T-cytoplasm corn (DEWEY *et al.* 1988). These T-toxin-sensitive cells have been developed as the basis of an efficient and reliable microbiological assay for T-toxin (CIUFFETTI *et al.* 1992). In addition to expression in *E. coli*, *T-urf13* has been shown to function in yeast (HUANG *et al.* 1990), tobacco (VONALLMEN *et al.* 1991) and insects (KORTH and LEVINGS 1993). For yeast, a fungal mitochondrial signal sequence fused to *T-urf13* targeted the URF13 protein to the mitochondria; without the signal neither the cells nor the mitochondria themselves were sensitive to T-toxin. In contrast, when *T-urf13* without a mitochondrial signal sequence was expressed in tobacco or insects, both were sensitive to T-toxin.

Successful heterologous expression of *T-urf13* suggested that the URF13 protein might also function to confer T-toxin-sensitivity in *C. heterostrophus*. We predicted that expression in a *Tox1*$^+$ strain would be detrimental to the growth of the fungus, since it would be sensitive to its own toxin. This paper describes the successful construction of such a strain and its use to enrich for *Tox*$^-$ mutants in a population of mutagenized conidia. Nine *Tox*$^-$ mutants were collected; all mapped

to the *Tox1* locus. These mutants will be useful for mapping the *Tox1* region of the chromosome.

MATERIALS AND METHODS

Strains, media, crosses and transformation: *C. heterostrophus* strains C4 (*Tox1*$^+$; *MAT-2*; ATCC 48331), C5 (*Tox1*$^-$; *MAT-1*; ATCC 48332), C9 (*Tox1*$^+$; *MAT-1*) are members of an isogenic line generated by backcrossing progeny of field isolates (Leach *et al.* 1982a). Media, growth conditions, and storage of *C. heterostrophus* have been described previously (Turgeon *et al.* 1985) as have mating (Leach *et al.* 1982a) and transformation (Turgeon *et al.* 1993) procedures. CMX is CM (complete medium) with xylose instead of glucose (Tzeng *et al.* 1992); CMNS is CM with salts omitted. Polygalacturonic acid medium (PGA) consisted of 0.1 M sodium phosphate, pH 7.0, 0.5% polygalacturonic acid, 0.5% sorbose, 1% yeast extract and 1% enzymatic casein hydrolysate. Culture filtrate containing T-toxin was harvested from a seven day old still culture of strain C4 grown in Fries medium (Pringle and Braun 1957). Methomyl (DuPont), which mimics the biological effects of T-toxin (Klein and Koeppe 1985), was included in CMNS at a final concentration of 5 mM.

The plasmid carrying the *C. heterostrophus* restriction fragment length polymorphism (RFLP) marker G264 in pGem2 was supplied by C. Bronson (Tzeng *et al.* 1992). Plasmids pNL9LORF13, pHPG, and pRD301 were provided by A. Myers (Huang *et al.* 1990), W. Schäfer (Mönke and Schäfer 1993) and R. Dean and W. Timberlake, respectively. The *pelA* gene carried by pRD301 has been described (Dean and Timberlake 1989).

Plasmid constructions: Three vectors were made.

pHNU3: A 660 bp *Pst*I- *Eco*RI fragment from the plasmid pNL9LORF13 containing the *T-urf13* gene fused to the *Neurospora crassa* ATP synthase subunit 9 targeting peptide (MSS) was transferred to the same sites in the polylinker of pBluescript SK- (Stratagene). A 0.7 kb *Bam*HI-*Hin*dIII fragment, carrying the MSS::*T-urf13* sequence, was ligated to a 5.8 kb *Bam*HI-*Hin*dIII fragment of pHPG, resulting in plasmid pHNU2 (ca. 6.5 kb). A 1.3 kb *Sac*I fragment of pHNU2 carrying *C. heterostrophus* promoter1 (Turgeon *et al.* 1987) fused to the MSS::*T-urf13* sequence was cloned into the 5.3 kb fragment of *Sac*I digested pHPG to make pHNU3 (6.6 kb) which has the MSS::*T-urf13* gene under the control of the constitutive *C. heterostrophus* promoter1. The plasmid also carries the selectable marker *hygB* fused to *C. heterostrophus* promoter1.

pHNU3pelA and pHPGpelA: A 0.7-kb *Pvu*II fragment of pRD301, containing the *Aspergillus nidulans pelA* promoter, was cloned into the *Sma*I site of pUC19. A promoter clone in desired orientation was cut with *Kpn*I and *Bam*HI and a 0.7 kb fragment carrying the promoter was ligated into *Kpn*I-*Bam*HI digested pHNU3 or pHPG, replacing promoter1 5′ of the MSS::*T-urf13* fusion in the former and promoter1 5′ of the *GUS* gene in the latter. Plasmid pHNU3pelA (6.7 kb) thus carries the fusion *A. nidulans pelA*::*N. crassa* MSS::*T-urf13* and also contains the *C. heterostrophus* promoter1::*hygB* fusion for expression of *hygB* (Figure 1a). pHPGpelA (8.0 kb) differs in that the *GUS* gene, rather than the MSS::*T-urf13* fusion is under control of the *pelA* promoter.

Mutagenesis: Conidia of C4 urf13-1 (*Tox1*$^+$; *hygB*; *MAT-2*; *T-urf13*) and transformant C4gus-1 (*Tox1*$^+$; *hygB*; *MAT-2*; *GUS*) were treated with ethylmethanesulfonate as described previously (Leach *et al.* 1982a). After mutagenesis to 99% kill, conidia were spread on PGA medium and incubated for 10 days (Figure 1b). Survivors were subjected to the microbial assay for T-toxin (Ciuffetti *et al.* 1992). Those of interest were analysed genetically and assayed for virulence on corn plants (Yoder 1988).

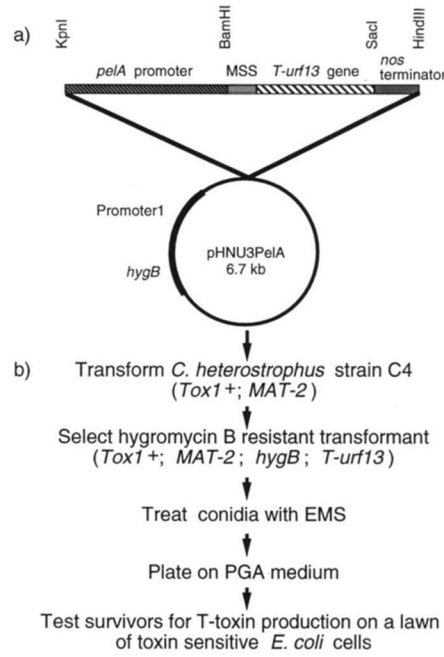

Figure 1.—Construction of a plasmid to confer T-toxin sensitivity on *C. heterostrophus*. (a) The *T-urf13* gene fused to the *N. crassa* ATP synthase subunit 9 mitochondrial targeting protein (MSS) and the *A. tumefaciens* nopaline synthase terminator (*nos*) was described by Huang *et al.* (1990). The *A. nidulans pelA* promoter, obtained from R. A. Dean and W. E. Timberlake (1989), was fused to the MSS; the entire cassette was inserted into a vector (pHPG; Mönke and Schäfer 1993) carrying the selectable marker *hygB* fused to *C. heterostrophus* promoter1 (Turgeon *et al.* 1987). (b) The resulting plasmid, pHNU3pelA, was transformed into *Tox1*$^+$ *C. heterostrophus* strain C4. Conidia of one transformant (C4 urf13-1) were mutagenized with EMS to 99% kill and plated on PGA medium (which induces *pelA*) to enrich for *Tox*$^-$ mutants. Survivors were screened for ability to produce T-toxin in a microbial bioassay (Ciuffetti *et al.* 1992).

RESULTS

Construction of T-toxin sensitive strains of *C. heterostrophus*: To determine if *T-urf13* could confer T-toxin sensitivity to *C. heterostrophus*, pHNU3 (promoter1::MSS::*T-urf13*) was transformed into *C. heterostrophus* strain C5 (*Tox1*$^-$). The recipient strain (C5) was necessarily *Tox*$^-$ since promoter1 is constitutive. Transformants grew normally on CMNS but were inhibited (Figure 2) on the same medium containing either 5 mM methomyl or culture filtrate from strain C4 (*Tox1*$^+$).

To determine if *T-urf13* could cause self-inhibition, pHNU3pelA (*pelA*::MSS::*T-urf13*) was transformed into *C. heterostrophus* strain C4 (*Tox1*$^+$). Control transformants carried pHPG*pelA* (*pelA*::*GUS*). Transformants expressing pHNU3pelA grew normally from mycelial inoculum on medium containing glucose (which represses the *pelA* promoter) but, on PGA medium

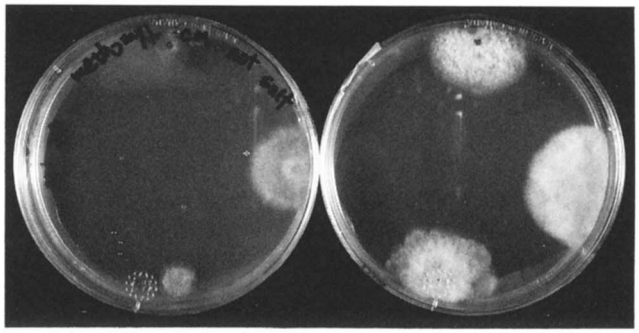

FIGURE 2.—Growth of *C. heterostrophus* on medium with or without methomyl. Right, CMNS; left, CMNS containing 5 mM methomyl. Colony on right: control strain C5 carrying pHPG (constitutive *C. heterostophus* promoter1 in front of GUS). Top and bottom colonies: transformants of C5 expressing *T-urf13* under control of constitutive *C. heterostophus* promoter1 (pHNU3). A similar result was observed when *C. heterostrophus* race T (strain C4) culture filtrate (1%) was substituted for methomyl. Photographed after 3 days of growth.

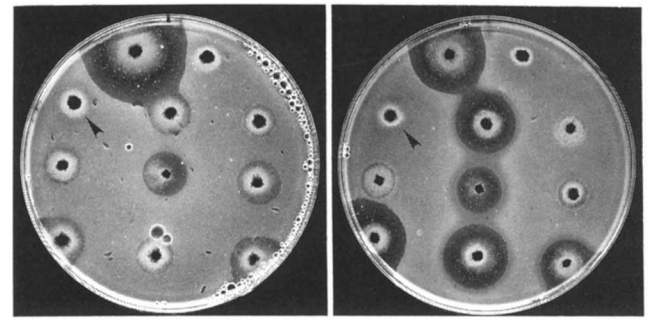

FIGURE 3.—Microbial assay of *C. heterostrophus* Tox$^-$ survivors of EMS mutagenesis. Plugs (4 mm) of agar medium bearing mycelium were placed, mycelium side down, on an overlay of T-toxin-sensitive *E. coli* cells on L broth plus ampicillin plates and incubated at 30° overnight. Top left: *Tox1*$^+$ control strain C4 (large halo). Top right: *Tox1*$^-$ control strain C5 (no halo). The remaining nine colonies are Tox$^-$ survivors, placed sequentially on the plate from left to right and top to bottom as follows: ctm45, ctm105, ctm106, ctm107, ctm108, ctm110, ctm111, ctm113, ctm118. Inocula were from colonies grown on CM (left plate), the standard culture medium, or Fries medium (right plate), which enhances T-toxin production. Note that ctm45 (arrowhead) caused no detectable halo when taken from either medium, whereas the remaining eight mutants caused small halos when taken from CM; some of them caused intermediate size halos (smaller than those produced by wild-type race T) when taken from Fries medium.

(which induces the *pelA* promoter), were only half the size after three days and 80% of the size after 1 week, of those grown on glucose as measured by colony diameter. Control transformants carrying pHPG*pelA* grew normally with either glucose or PGA. When conidial germination and germ tube elongation were monitored on PGA medium, those carrying *T-urf13* lagged behind those carrying *GUS*. For example, six hours after the start of germination 80% of the *GUS* conidia had germinated while only 10% of the *T-urf13* conidia had germinated. Within twelve hours however, germination was 95% for conidia of both strains. Similarly, germ tube elongation was initially slower in the *T-urf13* strain but by 12 hr germ tube lengths were the same for both strains. Thus, expression of *T-urf13* in a *Tox1*$^+$ strain was not lethal, but resulted in inhibition of fungal growth. We then determined whether this level of self inhibition was sufficient to allow enrichment for *Tox*$^-$ mutants.

Isolation of *Tox*$^-$ mutants: Conidia of transformant C4urf13-1 (*Tox1*$^+$; *MAT-2*; *hygB*; *T-urf13*) were treated with EMS and plated on PGA medium (Figure 1b). As a control, conidia of strain C4gus-1 (*Tox1*$^+$; *MAT-2*; *hygB*; *GUS*) were treated similarly. Survivors were tested for T-toxin production by microbial assay. In the first experiment with C4 urf13-1, 7 of 212 survivors were Tox$^-$ (defective in T-toxin production) and in a second experiment, two of 150 survivors were Tox$^-$. In the control test with C4gus-1, none of 306 survivors was Tox$^-$. Thus induced expression of *T-urf13* in *Tox1*$^+$ cells enriches for the Tox$^-$ phenotype when cells are plated on PGA medium.

To determine if pHNU3*pelA* integrated near the *Tox1* locus, one of the *Tox*$^-$ mutants, ctm45 (*hygR*; *Tox*$^-$, see Table 1) was crossed to tester strain C9 (*hygS*; *Tox1*$^+$) and progeny were scored for parental *vs* recombinant types. A ratio of 26 : 18 was obtained which is not different from 1 : 1 (at the 5% level of significance) and indicates that the two genes *hygB* and *Tox1* are not closely linked. To verify that the *pelA*::*MSS*::*T-urf13* sequence was intact and unrearranged in the genome, blots of DNA from strain C4urf13-1 (*Tox1*$^+$; *T-urf13*; *hygB*) were probed with *pelA*::MSS::*T-urf13*. Hybridization of the probe to a band identical in size to that on the transformation vector indicated that this sequence is intact in the genome of strain C4urf13-1.

Characterization of Tox$^-$ survivors: *T-toxin bioassay:* A microbial assay in which the nine Tox$^-$ survivors are compared to each other and to *Tox1*$^+$ and *Tox1*$^-$ wild type controls is shown in Figure 3. When inocula for the assay were taken from colonies growing on CM, one mutant (ctm45) did not produce a halo; the other eight produced halos of variable size, all clearly smaller than those produced by wild type *Tox*$^+$. When inocula were taken from colonies growing on Fries medium, which encourages high production of T-toxin, one strain (ctm45) caused no detectable halo while the other eight showed a range of halo sizes that generally were larger than those on CMX; none was as large as race T control halos. Thus, eight of the mutants appear to be leaky and one of the nine is tight.

Plant assays: Each mutant was inoculated on corn plants. Only one of them, ctm45, produced symptoms indistinguishable from those of a race O control strain in repeated assays (Figure 4). The remaining eight candidates showed weak race T type symptoms. These results are consistent with those of the toxin bioassay: the

FIGURE 4.—Assay of *C. heterostrophus* strains on T-cytoplasm corn. Blocks of agar medium bearing mycelium and conidia were placed in water in the whorls of two week old corn seedlings. Plants were held overnight in a mist chamber, then incubated five days in a growth chamber. For the photograph, three leaves were chosen from sets of plants inoculated with (from left to right): race T (*Tox1*$^+$) strain C4; race O (*Tox1*$^-$) strain C5; *Tox*$^-$ mutant ctm45; *Tox*$^-$ ascospore progeny (1151-3-1) of a cross of ctm45 × C9. Note that race T caused browning, complete tissue collapse and senescence of the leaves as well as yellow streaks (arrow), associated with T-toxin production in the noncollapsed tissue. The *Tox*$^-$ mutants caused symptoms indistinguishable from those of race O, *i.e.*, small necrotic lesions rather than tissue collapse, and no yellow streaks associated with T-toxin production.

tight mutant has a race O phenotype on plants whereas the leaky mutants have weak race T phenotypes.

Genetic analyses: To test the heritability of the apparent mutations, each of the nine Tox$^-$ survivors was crossed with *Tox1*$^+$ tester strain C9 and the progeny grown on CMX were tested for T-toxin production using the microbial assay. In all cases Tox$^+$:Tox$^-$ (toxin-defective) segregation was 4 : 4 in tetrads and 1 : 1 among random spores (Table 1). This indicates that each survivor carries a mutation which segregates as a single gene in meiosis.

The relationship between the mutations and the defined *Tox1* locus was determined by crossing each mutant to *Tox1*$^-$ tester strain C5. In the microbial assay for T-toxin, all tetrad and random spore progeny (grown on CMX) were Tox$^-$ (no halos or small halos). The absence of wild-type halos in any progeny indicates that each mutation maps at or near the *Tox1* locus (Table 2).

Analysis of tight and leaky mutants: Mutant ctm45 has a tight Tox$^-$ phenotype. Toxin production by ctm45

TABLE 1

Segregation of T-toxin-producing ability in progeny of crosses between race T (*Tox1*$^+$) and *Tox*$^-$ mutants

Strain[a]	No. of tetrads	Tox$^+$:Tox$^-$ [b]	No. of random spores	Tox$^+$:Tox$^-$ [b]
ctm45	6	4:4	44	22:22
ctm105	5	4:4	65	32:33
ctm106	5	4:4	50	27:23
ctm107	8	4:4	44	21:23
ctm108	4	4:4	58	29:29
ctm110	8	4:4	44	24:20
ctm111	4	4:4	54	24:30
ctm113	12	4:4	40	18:22
ctm118	6	4:4	44	21:23

Each *Tox*$^-$ mutant was crossed to tester strain C9 (*Tox1*$^+$; *MAT-1*). Progeny were grown on CMX and scored for T-toxin in the microbial assay. In control crosses, *Tox1*$^+$ × *Tox1*$^+$ produced 100% Tox$^+$ progeny, *Tox1*$^+$ × *Tox1*$^-$ produced 50% Tox$^+$ progeny.

[a] Each strain is a Tox$^-$ survivor of EMS mutagenesis (Figure 3).

[b] Tox$^-$ = toxin-defective; Tox$^-$ progeny of ctm45 produced no halo in the microbial assay for T-toxin; Tox$^-$ progeny of the remaining eight mutants produced halos smaller than those of wild-type race T.

TABLE 2

Segregation of T-toxin-producing ability in progeny of crosses between race O (*Tox1*$^-$) and *Tox*$^-$ mutants

Strain[a]	No. of tetrads	Tox$^+$:Tox$^-$ [b]	No. of random spores	Tox$^+$:Tox$^-$ [b]
ctm45	2	0:8	201	0:201
ctm105	2	0:8	114	0:114
ctm106	2	0:8	82	0:82
ctm107	1	0:8	102	0:102
ctm108	2	0:8	110	0:110
ctm110	2	0:8	40	0:40
ctm111	1	0:8	16	0:16
ctm113	5	0:8	183	0:183
ctm118	5	0:8	100	0:100

Each *Tox*$^-$ mutant was crossed to tester strain C5 (*Tox1*$^-$; *MAT-1*). Progeny were assayed as in Table 1. In control crosses, *Tox1*$^+$ × *Tox1*$^-$ produced 50% Tox$^+$ progeny. *Tox1*$^-$ × *Tox1*$^-$ produced 100% Tox$^-$ progeny.

[a] See Table 1, footnote *a*.

[b] See Table 1, footnote *b*.

was reassessed to determine if its tight phenotype was caused by a defect in toxin secretion. Filtrates and mycelial extracts were prepared from cultures grown in liquid Fries medium. These were tested, along with mycelial inoculum from colonies grown on CMX agar, in the microbial assay. No evidence for T-toxin production by ctm45 was detected in any case, suggesting that this tight *Tox*$^-$ mutant is not defective in toxin secretion.

Mutant ctm106 appears to be leaky (Figure 3). It causes a small halo in the microbial assay when inoculum is taken from colonies on CMX, and an intermediate size halo (smaller than wild type) when inoculum is taken from colonies on Fries medium. Ctm106 was chosen to determine genetic control of halo size. It was crossed to *Tox1*$^-$ (C5) and *Tox1*$^+$ (C9) testers and progeny assayed on *E. coli* plates. Progeny segregated 1 : 1 (no halo : small halo) when the cross was to the *Tox1*$^-$ tester and inoculum was taken from colonies on CMX and 1 : 1 (no halo : intermediate size halo) when inoculum was taken from colonies on Fries medium (Figure 5, a and b). Progeny of the cross to the *Tox1*$^+$ tester segregated 1 : 1 (large halo : small halo) when inoculum was taken from colonies on CMX and 1 : 1 (large halo : intermediate size halo) when inoculum was taken from colonies on Fries medium (Figure 5, c and d).

In a plant test, progeny from the cross of ctm106 to the *Tox1*$^-$ tester segregated 1 : 1 (wild-type race O symptoms : weak race T symptoms); progeny from the cross to the *Tox1*$^+$ tester segregated 1 : 1 (wild-type race T symptoms : weak race T symptoms). Thus, quantitative ability of ctm106 to produce T-toxin is heritable, as measured by both the microbial assay and the plant test. Halo size in the toxin assay depends on the culture medium used to produce inoculum.

Verification of mutant genotypes: To eliminate the possibility that the Tox$^-$ survivors were contaminants, all were assayed for three markers which identified the progenitor transformant C4 urf13-1: *hygB*, *MAT-2* and the *Tox1* linked polymorphism G264 (Tzeng *et al.* 1992). All nine were resistant to hygromycin B, were *MAT-2*, and carried the G264 polymorphism that is linked to *Tox1*$^+$; therefore none of the survivors is a contaminant.

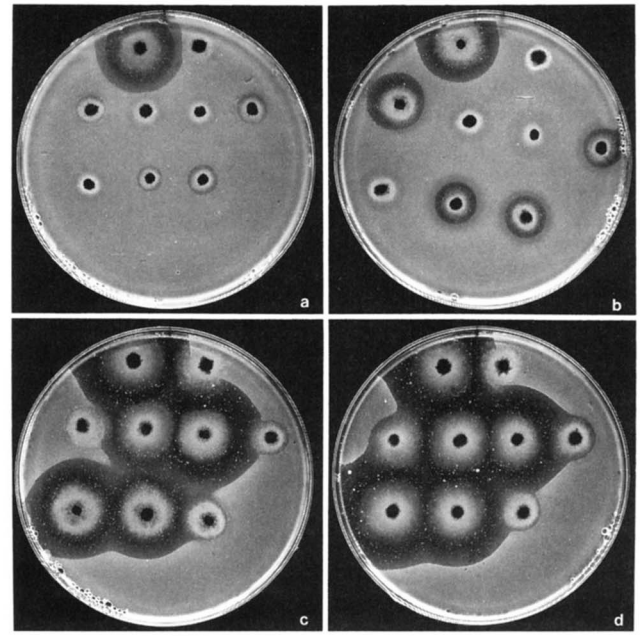

FIGURE 5.—Microbial assay of tetrad progeny of ctm106 (*Tox*$^-$; *MAT-2*) X strain C5 (*Tox1*$^-$; *MAT-1*), shown in plates a and b, or X strain C9 (*Tox1*$^+$; *MAT-1*), shown in plates c and d. Inocula were taken from colonies grown on CMX (plates a and c) or Fries medium (plates b and d). In each plate the top left colony is *Tox1*$^+$ control strain C4, the top right colony is *Tox1*$^-$ control strain C5, and the remaining colonies are seven of eight progeny from a single ascus. Note that progeny of the cross to race O (plates a and b) segregate 1 : 1 (no halo : small mutant type halo), whereas progeny of the cross to race T (plates c and d) segregate 1 : 1 (small mutant type halo : large wild-type race T halo). Thus, the Tox- phenotype segregates as a single gene in tetrads. Halos are larger if progeny are grown on Fries medium (plates b and d) than if grown on CMX (plates a and c).

DISCUSSION

We have demonstrated that a *Tox1*$^+$ strain of *C. heterostrophus* carrying the *T-urf13* gene from T-cytoplasm corn, which confers sensitivity to T-toxin, is sensitive to its own toxin and have taken advantage of this observation to isolate induced mutants of *C. heterostrophus* deficient in production of T-toxin. One of the *Tox*$^-$ mutants (ctm45) produces no detectable toxin in culture, either intra- or extracellularly, and causes typical race O symptoms on corn plants. Eight of the *Tox*$^-$ mutants produce detectable amounts of T-toxin in culture (but less than wild type race T strains) and cause weak race T symptoms in plant tests. *Tox*$^-$ progeny of either tight or leaky mutants have the same phenotypes on plants as their *Tox*$^-$ parents. These findings support the hypothesis that T-toxin is required by *C. heterostrophus* for high

virulence on T-cytoplasm corn. Previously, the most compelling evidence for this was the cosegregation of T-toxin production and high virulence in progenies of crosses between races T and O (YODER 1980). Now, an additional line of evidence, analysis of induced mutants, supports the conclusion that T-toxin is a virulence factor. None of the mutants, including ctm45 (which produces no detectable T-toxin), is non pathogenic; thus T-toxin does not appear to be required for pathogenicity. A similar conclusion was also drawn from analyses of segregating progeny from crosses of wild type race T and race O isolates (YODER 1980).

The mechanism by which *T-urf13* confers T-toxin sensitivity to cells of *C. heterostrophus* is not known. Although a mitochondrial targeting sequence was fused to the *T-urf13* gene used for transformation, we did not determine whether or not mitochondria of transgenic strains were sensitive to T-toxin. It is clear, however, that expression of *T-urf13* in a *Tox1*$^-$ strain of *C. heterostrophus* under the control of a constitutive promoter, or in a *Tox1*$^+$ strain under control of an inducible promoter, caused inhibition of fungal growth in the presence of T-toxin, supplied exogenously in the former case and produced endogenously in the latter case. Expression of *T-urf13* in the presence of T-toxin was not suicidal, but rather caused moderate inhibition of both conidial germination rate and hyphal growth. This modest inhibition was nevertheless sufficient to enrich for *Tox*$^-$ mutants in a population of mutagenized conidia. Nine Tox$^-$ mutants among 362 surviving conidia were collected when *T-urf13* was present compared with none among 306 survivors when the *GUS* gene was substituted for *T-urf13*. Presumably, if a transformant were constructed in which *T-urf13* expression was in fact suicidal, selection, rather than enrichment of *Tox*$^-$ mutants would result.

Until now the source of all Tox$^-$ strains has been the field collection of race O isolates, all of which differ from Tox$^+$ strains by heterozygosity at *Tox1* (BRONSON *et al.* 1990; TAGA *et al.* 1985; YODER 1976). The genetic nature of the naturally occurring difference between race O and race T is largely unknown and may be complex. A translocation breakpoint and several different repeated elements have been found, by conventional genetic and RFLP mapping, associated with the *Tox1* region (TZENG *et al.* 1992). Availability of *Tox*$^-$ mutants permits fine structure genetic mapping previously not possible. The existing map of the *Tox1* region (TZENG *et al.* 1992) places *Tox1* at the breakpoint of the reciprocal translocation and shows several RFLPs closely linked to *Tox1* (and therefore the breakpoint). The map was made, by necessity, with the progeny of a cross that was heterozygous both for *Tox1* and for the *Tox1*- linked translocation breakpoint. Since crossover frequencies can be distorted near translocation breakpoints (KAFER 1974), map distances between markers near the breakpoint may be misleading. The *Tox*$^-$ mutants make it possible to determine map distances using progeny of crosses in which the translocated chromosomes are homozygous, thereby reducing the likelihood of aberrant crossover frequencies. Furthermore, since markers on the two chromosomes involved in a reciprocal translocation appear linked to each other, the *Tox*$^-$ mutants also make it possible to determine whether or not these markers are linked. Toward this end we have analyzed the progeny of a cross between ctm45 (*Tox1*$^-$) and a *Tox1*$^+$ strain carrying *hygB* integrated at an RFLP marker (B88) which maps within one cM of *Tox1* (TZENG *et al.* 1992). Progeny were scored for resistance to hygromycin B and ability to produce T-toxin. Recombinants occurred as frequently as parental types, suggesting that when the breakpoint is homozygous, *Tox1* and B88 are not closely linked. A similar approach will be used to map additional markers near *Tox1*.

Crosses between naturally occurring race T and race O isolates usually segregate equal numbers of Tox$^+$ and Tox$^-$ progeny, consistent with the hypothesis that *Tox1* is a single gene. While each of the *Tox*$^-$ mutants collected in this study is a single site mutation and each maps at *Tox1*, the fact that most of them are leaky may be relevant to hypotheses for the pathway of T-toxin biosynthesis. Since T-toxin is a large polyketide (predominantly C_{41}) and very large polyketide synthase (PKS) genes, such as the 35-kb *eryA* gene of *Saccharopolyspora erythraea*, which produces the C_{13} polyketide erythromycin (DONADIO *et al.* 1991), and the 65-kb *avr* gene of *Streptomyces avermitilis*, which produces the C_{25} polyketide avermectin (MACNEIL *et al.* 1992), have been described, it is not unreasonable to speculate that the *Tox1* region encodes a giant PKS gene. Alternatively, the *Tox1* region may contain several genes, one of which may be a PKS. Our present ability to map the *Tox1* region without interference from the known translocation breakpoint data should lead to a reliable estimate of the size of *Tox1* and to its eventual cloning.

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