Genetic Control and Distorted Segregation of T-Toxin Production in Field Isolates of *Cochliobolus heterostrophus*

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ABSTRACT

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T-toxin is a family of linear polyketols responsible for the high specific virulence of race T of *Cochliobolus heterostrophus* on *cms-T* maize. To date, a single locus, *Tox1*, has been shown to be involved in T-toxin synthesis. In an attempt to find additional loci, 12 race T (Tox+) and 11 race O (Tox−) field isolates were examined. Crosses to genetically defined strains demonstrated that all the race T isolates had the *TOX1* allele and that all the race O isolates had the alternate allele, *tox1*. There was no evidence that these isolates differed at any other loci controlling T-toxin production. Five of the race O isolates carried a factor that caused distorted segregation at *Tox1*.

Additional keywords: ascomycete, ascospore abortion, *Bipolaris maydis*, *Drechslera maydis*, *Helminthosporium maydis*, fertility, segregation distortion, spore killer.

Southern leaf blight of maize is caused by the filamentous ascomycete *Cochliobolus heterostrophus* (Drechsler) Drechsler (anamorph: *Bipolaris maydis* (Nisik. & Miyake) Shoem. = *Helminthosporium maydis* Nisik. & Miyake = *Drechslera maydis* (Nisik. & Miyake) Subram. & Jain) (1). Two races of *C. heterostrophus* have been identified in field populations. Race O has low virulence toward most maize genotypes under temperate conditions. Race T is highly virulent on maize with Texas male-sterile cytoplasm (*cms-T*), producing large, elongated lesions. On maize with normal, male-fertile (N) cytoplasm, lesions of race T are slightly smaller than those of race O (9). The high virulence of race T to *cms-T* maize results from its production of a family of linear polyketols collectively known as T-toxin (3,18).

The identification of genes required for T-toxin production should help to elucidate the steps in its synthesis. Because T-toxin is a nonproteinaceous secondary metabolite, multiple genes may be involved. However, only a single genetic locus, *Tox1*, has been identified (11). The dominant or semidominant allele *TOX1* confers the ability to produce T-toxin; *tox1* specifies the inability to make this metabolite. This locus was defined by the analysis of C-strains, a set of closely related, genetically defined strains bred for use in research (10). In all laboratory strains and field isolates examined to date, alternate alleles at *Tox1* are tightly linked to the breakpoints of a chromosome rearrangement; the chromosome bearing *TOX1* in T-toxin-producing strains is reciprocally translocated with respect to the chromosome-bearing *tox1* in nonproducing strains (2,21, Chang and Bronson, *unpublished*).

Certain crosses involving field isolates have suggested that additional loci controlling T-toxin production might be found by an examination of wild populations. Because *Cochliobolus* is a haploid organism, a 1:1 ratio is expected for single-gene segregation. Segregation ratios for some crosses between race T and race O field isolates were significantly different from 1:1 (17,22,25). These crosses produced a deficiency of progeny with the ability to make T-toxin. Segregation ratios ranged from roughly 1:3 (Tox+:Tox−) to less than 1:7, suggesting that two, three, or possibly four loci had segregated. However, these crosses produced only incomplete tetrads, and no asci contained more than four viable ascospores. (*C. heterostrophus* undergoes a postmeiotic mitosis; the expected number of ascospores is eight.) Thus, the ratios that were not 1:1 could have been the result of preferential abortion of T-toxin-producing meiotic products. In those crosses showing 1:1 ratios, the identity of the locus that segregated was not determined. No firm conclusions could be drawn about the genetic control of T-toxin production in field isolates.

The objective of this research was to examine the naturally occurring genetic variation for T-toxin production in field isolates in order to identify additional loci involved in T-toxin synthesis. This objective was approached by crossing race T and race O field isolates to genetically defined C-strains. We report here that the genetic control of T-toxin production in the race T and race O field isolates examined differs by alleles at *Tox1* and that no other T-toxin loci were detected. Non-1:1 segregation was observed in crosses involving certain race O field isolates but is shown to be due to segregation distortion, probably brought about by nonrandom ascospore abortion.

MATERIALS AND METHODS

Strains and culture conditions. The strains of *C. heterostrophus* used in this study are listed in Table 1. C2, C3, C5, and C14 are near-isogenic C-strains constructed by Leach et al (10). The C-strains Q1, Q2, Q3, and Q4 are siblings from the second backcross of the *TOX1* allele from the C-strain 380.2.5 (*TOX1 Cyh1R MAT1-1*) into C3 and were produced during another study (9). All T-toxin-producing (Tox+) C-strains developed to date have the allele *TOX1*, and all C-strains not producing T-toxin

(Tox−) have the allele *tox1*. These strains are known to differ at no other T-toxin-production loci (9,11).

Strains were stored at 5 C on silica gel (15) or in 15–25% glycerol at −65 to −75 C (24). The fungus was cultured on complete medium (CM) and crossed on Sachs medium (10). Ascospores were isolated either as random spores or as tetrads (24). When ascospores were isolated as tetrads, the fertility of the crosses was monitored by counting the number of viable spores per ascus. In *C. heterostrophus*, asci mature asynchronously, and it is difficult to distinguish immature asci from those in which all ascospores have aborted. Therefore, only asci with at least one viable spore were counted.

T-toxin assays. Three T-toxin assays were employed.

T-toxin production by progeny of race T field isolates was analyzed by observing the killing of isolated maize protoplasts (5,17). Known *TOX1* and *tox1* C-strains were used as controls.

The race O field isolates were analyzed at a later date using a recently developed microbiological assay. T-toxin production was detected by observing the growth of cells of *Escherichia coli* transformed with *urf13-T*, the maize mitochondrial gene for T-toxin sensitivity. The procedure was adapted from the methods of Dewey et al (4) for use in a plate assay (26). Strain DH5α of *E. coli* containing pATH13-T was grown and induced as described by Dewey et al (4) and spread on the surface of Luria-Bertani (LB) agar containing 50 μg of ampicillin/ml (14). A piece (4 mm diameter) of mycelium of *C. heterostrophus*, grown on either solid or liquid CM, was placed on the lawn of bacterial cells and incubated overnight at 37 C. A clear halo of inhibited bacterial growth around the piece indicated the presence of T-toxin. Known *TOX1* and *tox1* C-strains were used routinely as controls.

The reliability of the *urf13-T* assay was determined by testing all progeny from crosses Hm540 × Q3 and Hm402 × Q3 (Table 2) in both the *urf13-T* assay and a maize-whorl assay (9). The whorl assay allows observation of both the chlorotic streaking produced by T-toxin-containing culture filtrates and lesion type due to infection. The assays gave identical phenotypic assignments. Some progeny from other crosses repeatedly gave ambiguous results (bacterial growth was suppressed but not completely inhibited) in the *urf13-T* assay. These progeny were retested by using the whorl assay and were found to have a Tox+ phenotype.

RESULTS

The genetic control of T-toxin production in field isolates was determined by crossing them with *TOX1* and *tox1* C-strains. Twelve race T (Tox+) field isolates obtained from diverse locations were examined (Table 3). The fertility in crosses of race T field isolates with C-strains was moderate; most crosses produced at least some asci containing complete tetrads (seven or eight ascospores). In crosses between the race T field isolates and the *tox1* C-strains, segregation among random spores was not significantly different from 1:1, and segregation in complete tetrads was always 4:4, indicating single-locus segregation in each cross. Each race T field isolate also was crossed with a *TOX1* C-strain. No Tox− recombinants were detected, indicating that the allele in the field isolates was *TOX1*. All isolates crossed with C14 also were crossed with the Tox+ field isolate Hm86. Again, no Tox− progeny were obtained. All race T field isolates examined, therefore, carry the *TOX1* allele and differ from the *tox1* C-strains at no other T-toxin-synthesis loci.

TABLE 1. Strains of *Cochliobolus heterostrophus* used in this study[a]

Strains of *C. heterostrophus*	T-toxin phenotype	Mating type	Origin	Genotype		
Field isolates						
Hm#9	Tox+	1	New York			
Hm79	Tox+	1	North Carolina			
HmF	Tox+	1	Kentucky			
Hm110	Tox+	1	Illinois			
Mon1	Tox+	1	Montana			
JC	Tox+	1	Iowa			
Hm2002	Tox+	1	Michigan			
Aus19679-2	Tox+	1	Australia			
PR1X412	Tox+	1	Mexico			
Italy F437	Tox+	1	Italy			
Italy F479	Tox+	1	Italy			
Hm86	Tox+	2	North Carolina			
Hm540	Tox−	1	North Carolina			
Hm402	Tox−	1	Florida			
Aus20239A3	Tox−	1	Australia			
Hm28	Tox−	2	North Carolina			
Hm653	Tox−	2	Florida			
Hm648	Tox−	2	Florida			
Dixon	Tox−	2	Illinois			
Hm9	Tox−	2	North Carolina			
NYSS1	Tox−	2	New York			
Aus20239A11	Tox−	2	Australia			
Hm813	Tox−	2	Japan			
C-strains						
C2				*TOX1*	*alb1*	*MAT1-1*
C3				*tox1*	*ALB1*	*MAT1-2*
C5				*tox1*	*ALB1*	*MAT1-1*
C14				*TOX1*	*alb1*	*MAT1-2*
Q1				*TOX1*	*ALB1*	*MAT1-1*
Q2				*tox1*	*ALB1*	*MAT1-2*
Q3				*TOX1*	*ALB1*	*MAT1-2*
Q4				*tox1*	*ALB1*	*MAT1-1*

[a] The nomenclature follows the recommendations of Yoder et al (27). *ALB1* specifies pigmented mycelia and conidia; strains with *alb1* are albino. *MAT1-1* and *MAT1-2* are complementary alleles for mating type. For the field isolates, the genotypes of which were not known at the initiation of this study, phenotypes are listed. Tox+ indicates T-toxin production; Tox− indicates failure to make T-toxin. Mating types 1 and 2 indicate ability to mate with *MAT1-2* and *MAT1-1* C-strains, respectively.

The genetic control of T-toxin production in the race O (Tox−) field isolates was more difficult to determine. Eleven race O field isolates were crossed with *TOX1* and *tox1* C-strains (Table 2). The fertility in all crosses of these field isolates with the *TOX1* C-strains was low. No asci contained more than four viable ascospores, and most contained only two. Six crosses gave segregation ratios not significantly different from 1:1, suggesting single-locus segregation. Five crosses, however, gave ratios significantly different from 1:1, with a deficiency of Tox+ progeny. This result could indicate that several T-toxin loci were segregating. However, the low fertility of these crosses also makes it possible that the non-1:1 ratios were due to segregation distortion brought about by preferential abortion of Tox+ ascospores. Fertility of these crosses tended to be, but was not always, lower than in crosses segregating 1:1. The average number of viable ascospores in asci having at least one viable spore ranged from 1.8 to 2.3 for crosses showing segregation distortion and from 2.2 to 2.6 for crosses segregating normally.

Testcrosses of the progeny were needed to determine the cause of the non-1:1 ratios. Testcrosses of the Tox− progeny to the *TOX1* C-strains were considered likely to be ambiguous because they could yield a deficiency of Tox+ progeny whether the cause of the non-1:1 segregation was multiple loci or preferential abortion of Tox+ ascospores. Testcrosses of the Tox− progeny to the *tox1* C-strains would be definitive only if Tox+ recombinants were obtained. Testcrosses of the Tox+ progeny to the *tox1* C-strains were considered less likely to be ambiguous. The *tox1* C-strains have all the alleles necessary for T-toxin production except one (*TOX1*). If the low frequencies of Tox+ progeny were due to multiple-locus segregation, then testcrosses of Tox+ progeny to *tox1* C-strains should yield 1:1 segregation. If, however, the non-1:1 ratios were due to segregation distortion, the distortion-inducing factor may have been inherited by the Tox+ progeny and would be capable of causing the abortion of Tox− ascospores. Such crosses should yield non-1:1 segregation with a deficiency of Tox− progeny.

Therefore, tests for segregation distortion by Tox+ progeny were performed. Tox+ progeny from all crosses showing non-1:1 segregation were crossed to *tox1* C-strains (Table 2). Segregation was significantly different from 1:1; this time, however,

TABLE 2. Segregation of T-toxin production in crosses of race O field isolates and their Tox+ progeny with *TOX1* and *tox1* C-strains of *Cochliobolus heterostrophus*[a]

Field isolate	Crosses of field isolates with *TOX1* C-strains		Crosses of Tox+ progeny with *tox1* C-strains[b]		Crosses of field isolates with *tox1* C-strains	
	C-strain	Tox+:Tox−[c,d]	C-strain	Tox+:Tox−[d,e]	C-strain	Tox+:Tox−[e,f]
Hm28	Q1	18:77*	Q2	71:38*	Q4	0:104
Hm653	Q1	6:116*	Q2	78:36*	Q4	0:114
Hm648	Q1	44:89*	Q2	89:17*	Q4	0:121
Dixon	Q1	13:120*	Q2	80:25*	Q4	0:125
Hm9	Q1	57:70			Q4	0:112
NYSS1	Q1	53:72	Q2	55:53	Q4	0:128
Aus20239A11	Q1	66:78	Q2	61:52	Q4	0:105
Hm813	Q1	70:67	Q2	55:56	Q4	0:118
Hm540	Q3	64:73	Q4	63:54	Q2	0:117
Hm402	Q3	13:122*	Q4	84:33*	Q2	0:109
Aus20239A3	Q3	56:71			Q2	0:105

[a] The *urf13-T* assay was used to test progeny for T-toxin production.
[b] Tox+ progeny were from the crosses of the field isolates with the *TOX1* C-strains.
[c] The phenotypes of all ascospores in each ascus harvested were determined. Chi-square values for tests of significance therefore were adjusted according to the methods of Rao and Scott (16) to reflect the number of ascospores per independent meiotic event. No asci had more than four viable spores. The number of viable spores per ascus containing at least one viable spore ranged from 1.8 to 2.6.
[d] Asterisks indicate ratios significantly different from 1:1 ($P \leq 0.05$).
[e] The phenotype of only one random ascospore per ascus was determined; therefore each ascospore represents an independent meiotic event.
[f] Isolates Hm540, Hm402, and Aus20239A3 also were crossed with C3 (*tox1*), and the maize protoplast assay was used to test progeny for T-toxin production. No Tox+ progeny were detected among the 138–160 random progeny tested per cross.

TABLE 3. Segregation of T-toxin production in crosses of race T field isolates with *tox1* and *TOX1* C-strains of *Cochliobolus heterostrophus*[a]

Field isolate	Crosses with *tox1* C-strains			Crosses with *TOX1* C-strains	
	C-strain	Tetrads segregating 4:4[b]	Random ascospores Tox+:Tox−[c]	C-strain[d]	Random ascospores Tox+:Tox−
Hm#9	C3	3/3	32:30	C14	108:0
Hm79	C3	5/5		C14	93:0
HmF	C3	7/7		C14	115:0
Hm110	C3	6/6		C14	101:0
Mon1	C3	6/6		C14	120:0
JC	C3		46:48	C14	113:0
Hm2002	C3	1/1	46:50	C14	102:0
Aus19679-2	C3	5/5		C14	105:0
Pr1X412	C3		28:26	C14	103:0
Italy F437	C3		35:40	C14	114:0
Italy F479	C3		43:35	C14	120:0
Hm86	C5	5/5		C2	90:0

[a] The maize protoplast assay (5,17) was used to test progeny for T-toxin production.
[b] Includes seven-spored asci segregating 4:3 or 3:4 (Tox+:Tox−). Numbers after the slashes are the numbers of tetrads tested. For traits under single-locus control, all asci should segregate 4:4. For traits controlled by two unlinked loci, the probability of all of five or more tetrads segregating 4:4 is less than 0.04.
[c] No ratios are significantly different from 1:1. All are significantly different from 1:3 or 3:1 at $P \leq 0.05$.
[d] All isolates crossed with C14 also were crossed with Hm86; 67–131 random progeny were assayed per cross, and no Tox− progeny were found.

there was a deficiency of Tox− progeny. These findings cannot be explained by multiple-locus segregation and suggest that the non-1:1 ratios are due to distorted segregation at *Tox1*. As a control, Tox+ progeny from some of the crosses that had shown 1:1 segregation also were crossed to *tox1* C-strains. These segregated 1:1 as expected, indicating a single-locus difference.

The non-1:1 segregation was examined in greater detail for isolate Hm28 (Table 4). In all three crosses of Hm28 or its progeny with C-strains, segregation was significantly different from 1:1 and there was a deficiency of ascospores having the C-strain parental phenotype. This deficiency of C-strain parental phenotypes was found no matter which *Tox1* allele the C-strain carried. Segregation ratios were 1:1 when Hm28 was crossed with its Tox+ progeny or when its Tox+ progeny were crossed with its Tox− progeny. These results cannot be explained by segregation of multiple loci. We conclude that there is no evidence that the race O field isolates differ from the *TOX1* C-strains at more than a single T-toxin locus. We also conclude that the non-1:1 segregation, when it occurred, was due to segregation distortion.

To determine the identity of the locus segregating for T-toxin production in the race O field isolates, the race O field isolates were crossed with *tox1* C-strains (Table 2). No Tox+ recombinants were found. Crosses also were performed among the race O field isolates and, again, no Tox+ recombinants were found (data not shown). We therefore conclude that all race O field isolates examined carry the allele *tox1* and that there is no evidence that they differ from the *TOX1* C-strains at other T-toxin loci.

DISCUSSION

Attempts to detect loci in addition to *Tox1* that control T-toxin production by the examination of naturally occurring variability in the population of *C. heterostrophus* were unsuccessful. Among 12 race T and 11 race O field isolates from several states and countries, the only difference detected in the genetic control of T-toxin production was for alleles at *Tox1*. All the race T isolates had the allele *TOX1*; all the race O field isolates had the allele *tox1*.

There are at least two possible causes for this seeming lack of variation for genes controlling T-toxin production. One race may have arisen from the other in a single mutational event or in several mutational events at the same locus. If so, mutations at other loci controlling T-toxin production are either infrequent or effectively lethal in natural populations. The latter could be true if the other loci also control important metabolic functions. For example, *Tox1* could control the modification of a normal class of nontoxic metabolic intermediates to toxic forms, or toxic intermediates to nontoxic forms (23). Alternatively, *TOX1* could be a cluster of tightly linked genes encoding all necessary biosynthetic functions, and there were no recombinants among the progeny analyzed. Clusters of functionally related loci are uncommon in fungi but have been reported (6). This possibility could be tested by looking for complementation in forced heterokaryons of naturally occurring variants and/or induced mutants.

Non-1:1 segregation for *Tox1* occurred in crosses involving approximately half of the race O field isolates. Some of these isolates (Hm28, Hm402, Hm648, and Dixon) and isolates not tested in this study had been reported previously to give non-1:1 segregation (17,22,25). In each of the previous instances, it was not clear whether the non-1:1 segregation was due to multiple loci or segregation distortion. We have shown in this study that, among the isolates we tested, non-1:1 segregation was due to segregation distortion. In the previous studies, both parental isolates in each cross were of field origin and not genetically characterized, making it impossible to determine which parent caused the distortion. In this study, non-1:1 segregation was associated with only the race O field isolates, indicating that the responsible factor is common in the naturally occurring race O population, but rare or absent in the race T population. The factor also must be absent in the C-strains.

For the five race O field isolates carrying the distortion-inducing factor, tests for the genetic control of T-toxin synthesis were confounded. Our study demonstrates that these isolates carry *tox1* but does not eliminate the possibility that they differ from the *TOX1* C-strains at additional T-toxin loci. However, given the lack of positive evidence for multiple loci and given the fact that none of the race O isolates lacking the distortion-inducing factor differed from the *TOX1* C-strains at other T-toxin loci, we believe that this possibility is unlikely. Further tests for additional T-toxin loci in these isolates could be performed by crossing their Tox− progeny to the *tox1* C-strains and looking for Tox+ recombinants.

The segregation distortion observed in crosses involving the race O field isolates could be accounted for if *Tox1* is linked to a locus causing abortion of meiotic products not containing it. Loci with such characteristics have been observed in *Neurospora* (19) and *Fusarium* (8) and are known as spore killers. In crosses heterozygous for the abortion-inducing locus, half of the meiotic products in an ascus should abort and all surviving progeny should carry the abortion-inducing allele. In coupling phase (*tox1* with the abortion-inducing allele), *TOX1* ascospores should abort, and in repulsion phase (*TOX1* with the abortion-inducing allele), *tox1* ascospores should abort. Low frequencies of progeny of the nonparental type should survive if there is crossing over between *Tox1* and the locus. The frequency of such progeny may be higher in backcross generations as homologues become more similar due to recombination and as pairing improves. In crosses between two strains carrying the abortion-inducing alleles, the locus should not cause either ascospore abortion or segregation distortion.

Results in this study are consistent with this model. All asci from crosses showing segregation distortion contained four or fewer viable spores. All testcrosses of progeny from non-1:1 crosses to C-strains showed segregation distortion (Tables 2 and 4), suggesting that all surviving progeny carried the putative abortion-inducing allele. Crosses among isolates that carry the putative allele did not show segregation distortion (Table 4). The degree of segregation distortion in the testcrosses of Tox+ progeny to *tox1* C-strains was generally less than in the original crosses (Table 2), suggesting an increased recombination frequency between the putative abortion-inducing locus and *Tox1*. The recombination frequency estimated by the fraction of surviving progeny with the nonparental phenotype averaged 15% in crosses of the field isolates to the *TOX1* C-strains and 27% in the crosses of their Tox+ progeny to the *tox1* C-strains. Because these Tox+ progeny were recombinant in the region between the locus and *Tox1*, pairing between homologues in the testcrosses may have been stronger, promoting more recombination.

Evidence for a spore killer as the cause of distorted segregation for alleles at *Tox1* in *C. heterostrophus* should be evaluated in light of the nature of the crosses performed. The crosses were designed and analyzed to determine the genetic control of T-toxin synthesis. The frequency of ascospore abortion in most of the crosses was not determined, and, for crosses heterozygous at *Tox1*, interpretation of abortion patterns would have been confounded by ascospore abortion due to heterozygosity for the reciprocal translocation linked to *Tox1* (2,21, Chang and Bronson, *unpublished*). Crosses heterozygous for *Tox1* abort half of their

TABLE 4. Segregation of T-toxin production in crosses involving the race O field isolate Hm28 and its progeny[a]

Cross	Tox+:Tox−[b]
Hm28 × Q1	18:77*
B81.P2.6.1 (Tox+) × Q2	71:38*
B81.P2.6.1 (Tox+) × Hm28	37:55
B81.P1.2.1 (Tox−) × Q1	19:87*
B81.P1.2.1 (Tox−) × B81.P2.6.1 (Tox+)	49:52

[a] The B81s are progeny from Hm28 × Q1. The *urf13-T* assay was used to test for T-toxin production.

[b] Except as noted for Hm28 × Q1 (Table 2, footnote c), the phenotype of only one random ascospore per ascus was determined; therefore, each ascospore represents an independent meiotic event. Asterisks indicate ratios significantly different from 1:1 ($P \leq 0.05$).

spores (*TOX1* and *tox1* spores equally) (2). This abortion may have contributed to the low fertility observed. In addition, the progeny used for the testcrosses were not chosen randomly but were selected for mating type because of initial plans to testcross the progeny to the field isolate parents as well as the C-strains. Many of the crosses back to the field isolate parent were infertile; therefore, with the exception of progeny from Hm28, this approach was abandoned.

The evidence for a spore killer as the cause of segregation distortion would be strengthened by the observation of ascospore abortion patterns in crosses homozygous for the translocation among otherwise highly fertile strains. Such a test may require the breeding of strains that are near-isogenic to the C-strains except for alleles for the putative spore killer. To date, alternate alleles at *Tox1* are associated absolutely with alternate forms of the translocation. Therefore, a marker other than *Tox1* will need to be scored to detect segregation distortion. The only other markers currently available in this region are restriction fragment length polymorphisms (20).

The likely effect of distorted segregation at *Tox1* on the frequency of T-toxin-producing strains in the population of *C. heterostrophus* is not clear. In sexually reproducing populations, segregation distortion can cause the frequency of alleles linked in repulsion to the distortion-inducing locus to decrease (7). The frequency of race T declined rapidly after the Southern leaf blight epidemic of 1970 and the switch away from T-cytoplasm maize. This decline has been attributed to a reduction in the fitness of strains carrying *TOX1* in the asexual cycle (9,13). The frequency of the sexual cycle of *C. heterostrophus* in nature is not known (12). If it is common, segregation distortion might have contributed to the decline of race T.

Leonard (12) hypothesized that race T is of recent origin. The seeming lack of variability for genes controlling T-toxin production in the population of *C. heterostrophus* and the observation that about 50% of race O isolates examined, but none of the race T isolates, carried the segregation distortion factor may reflect a recent origin of race T from a single, nondistorter, race O background.

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