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Tagged mutations at the *Tox1* locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated integration

(mutagenesis/toxin/virulence/fungus)

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ABSTRACT **We have used the restriction enzyme-mediated integration insertional mutagenesis procedure to tag the *Tox1* locus in the filamentous Ascomycete *Cochliobolus heterostrophus*. Mutations at other, unselected, loci were also identified and a high proportion (30–50%) of them were tagged. This procedure may be of general utility for simultaneously mutating and tagging genes in fungi and in other eukaryotes. The *Tox1* locus of *C. heterostrophus* has been defined by Mendelian analysis as a single genetic element that controls production of T toxin, a linear polyketide involved in virulence of the fungus to its host plant, corn. To tag *Tox1*, protoplasts of a *Tox1*$^+$ (T-toxin producing) strain were transformed with a linearized, nonhomologous plasmid along with an excess of the restriction enzyme used to linearize the plasmid. Of 1310 transformants recovered, two produced no detectable T toxin in culture or on corn plants. In each of these transformants, the *Tox*$^-$ mutation mapped at *Tox1*, was tagged with the selectable marker (*hygB*) on the transforming plasmid, and was tightly linked to the other tagged *Tox*$^-$ mutation. The two mutations, however, represent two different points of plasmid insertion at the *Tox1* locus.**

Although transposons are known in filamentous fungi (1–3), transposon tagging has not been developed for routine production of marked mutations in these organisms. In a few cases, tagged mutations have been generated by random insertion, during transformation, of a plasmid that has no homology with the fungal genome (4–7). This procedure, however, is generally inefficient because the frequency of transformation with nonhomologous plasmids is often low. The restriction enzyme-mediated integration (REMI) procedure, first described for *Saccharomyces cerevisiae* (8) and refined for use with *Dictyostelium discoideum* (9, 10), offers the prospect of introducing random tagged mutations into the fungal genome at a relatively high rate and is the functional equivalent of transposon tagging in prokaryotes. We have used REMI for insertional mutagenesis of a particular genetic locus, *Tox1*, of the filamentous Ascomycete *Cochliobolus heterostrophus* (anamorph, *Bipolaris maydis*).

The *Tox1* locus controls production of a family of linear polyketides (T toxin), which render the fungus highly virulent toward Texas male sterile (T) cytoplasm corn (11). When *C. heterostrophus* race T, which produces T toxin, is crossed with a naturally occurring nontoxin-producing strain (race O), only parental progeny segregate, thus defining *Tox1* as a single locus that determines whether or not T toxin is produced (12, 13). Race T was unknown until 1969–1970, when it caused an epidemic that devastated the corn crop in the United States, largely due to vast planting of T-cytoplasm corn. Cloning and analysis of *Tox1* from race T and its counterpart from race O should lead to an understanding of the evolutionary origin of race T and the biosynthetic pathway for T-toxin production.

Recently, we developed a procedure to enrich for nontagged mutations at *Tox1* in a chemically mutagenized population of cells (14). For this, a conditionally T-toxin-sensitive strain was constructed by transforming *C. heterostrophus* race T with a plasmid carrying *T-urf13*, the gene that confers T-toxin sensitivity to T-cytoplasm corn (15). Nine mutants deficient in T-toxin biosynthesis were recovered after mutagenesis of this transformant. Each mutation mapped at the *Tox1* locus. These chemically induced mutants have established a correlation between loss of toxin production and reduction in fungal virulence, thereby contributing to the body of evidence that T toxin is a fungal virulence factor. They have also been valuable in fine structure mapping of *Tox1*. For cloning, however, the tagged mutations reported in this study are preferable to untagged, chemically induced mutations.

MATERIALS AND METHODS

Strains, Media, and Crosses. *C. heterostrophus* strains C4 (*Tox1*$^+$; *MAT-2*; ATCC 48331), C5 (*Tox1*$^-$; *MAT-1*; ATCC 48332), C9 (*Tox1*$^+$; *MAT-1*), CB15 (*Tox1*$^+$; *MAT-1*; isolation number B30.A3.R.89), and CB3 (*Tox1*$^-$; *MAT-1*; isolation number B30.A3.R.85) are members of a near-isogenic set of strains described previously (16, 17). Strain 1216-2-2 (*Tox*$^-$; *MAT-1*; *hygB*^{R}) is an ascospore progeny of C9 × REMI mutant R.C4.186 (*Tox*$^-$; *MAT-2*; *hygB*^{R}). Strain 1151-3-1 (*Tox*$^-$; *MAT-1*) is an ascospore progeny of C9 × Ctm45 (*Tox*$^-$; *MAT-2*; *hygB*^{R}), a chemically induced, nontagged, *Tox1*-linked T-toxin-deficient mutant described in a previous study (14). Media, growth conditions, and storage of *C. heterostrophus* have been described (14, 18), as have mating procedures (16). *C. heterostrophus* culture media included CMX [i.e., complete medium with xylose instead of glucose (19)], CMNS (i.e., CM with salts omitted), and Fries medium (20).

DNA Manipulations. pUCATPH (Fig. 1), which has no detectable homology to the *C. heterostrophus* genome, was constructed by ligating the 2.4-kb *Sal* I fragment from pDH25, containing *hygB* fused to the *Aspergillus nidulans trpC* promoter and terminator (21), into the *Sal* I site of pUC18. Plasmid DNA was purified by CsCl centrifugation (22) or on Qiagen-tip columns according to the supplier's directions (Qiagen). For REMI, plasmid DNA was digested with *Hin*dIII and used directly in the original digestion solution (100 μl) or after phenol extraction, ethanol precipitation, and resuspension in 100 μl of sterile TE (10 mM Tris·HCl/1 mM EDTA, pH 8.0). Isolation of genomic DNA

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Abbreviation: REMI, restriction enzyme-mediated integration.

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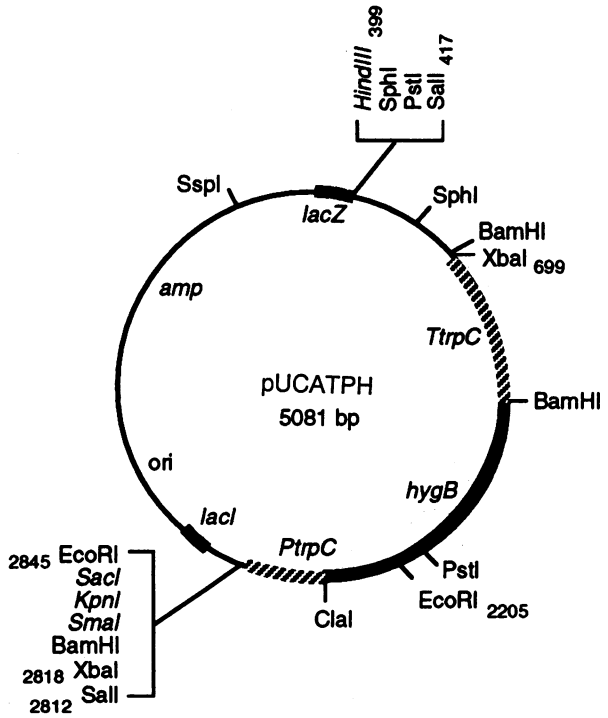

FIG. 1. pUCATPH, constructed as described in the text. Six-base-pair restriction enzyme sites are shown; numbers indicate map positions of sites referred to in the text (position 1 is the same as for pUC18). Italicized sites are unique. *amp*, Ampicillin resistance; *hygB*, hygromycin B resistance; *PtrpC*, *A. nidulans trpC* promoter; *TtrpC*, *A. nidulans trpC* terminator; *ori*, *Escherichia coli* origin of replication.

from *C. heterostrophus*, transfer of DNA from gels, and filter hybridizations were as described (23).

Transformation. Protoplasts were prepared from an overnight culture of *C. heterostrophus* strain C4 according to standard protocols (23). Approximately 1×10^6 protoplasts in 100 μl of STC (1.2 M sorbitol/10 mM Tris·HCl, pH 7.5/50 mM $CaCl_2$) were gently mixed with 30 μg of linearized plasmid in 100 μl of solution with or without restriction enzyme. The mixture was kept on ice for 5–10 min before addition of polyethylene glycol and completion of the usual transformation and plating protocols (23). Control protoplast mixtures included uncut plasmid and phenol extracted, ethanol precipitated, linearized plasmid resuspended in 100 μl of sterile TE. After 10 hr of incubation at 22°C, plates (each containing 20 ml of regeneration medium) were overlaid with 10 ml of 1% agar containing hygromycin B at 150 μg/ml and incubated at 30°C. Transformants appeared 5–15 days after plating.

Screening Transformants for T-Toxin Production. All hygromycin B-resistant transformants were transferred to CMX for single conidiation and then to fresh selective medium (CMNS containing hygromycin B at 100 μg/ml). Stable transformants were grown individually on CMX plates (diameter, 15 mm) for 7 days and then tested for T-toxin production with a microbial bioassay (24). Transformants that produced no halo after overnight incubation were transferred to Fries medium, which optimizes toxin production, and retested. Tox^- candidates were inoculated on plants of corn inbred W64A containing either T or N cytoplasm and incubated in a growth chamber as described (14).

RESULTS

Effect of REMI on Transformation Efficiency. Transformation of *C. heterostrophus* with plasmids having no homology to the *C. heterostrophus* genome occurs at very low frequency (ref. 18; Table 1). When circular pUCATPH was added to the protoplast suspension, no transformants appeared, whereas linear plasmid alone yielded a few transformants (up to 6 per 30 μg of DNA per 10^6 protoplasts). Addition of restriction enzyme increased the transformation rate. A total of 1310 REMI transformants were obtained from four experiments (Table 1) using linearized plasmid plus *Hin*dIII (as many as 107 transformants per 30 μg of DNA per 10^6 protoplasts).

The way in which the enzyme was added to the protoplast suspension influenced transformation efficiency (Table 1). Best results were obtained when pUCATPH was added directly to the suspension along with 100 units of *Hin*dIII used to digest the plasmid (107 transformants). This efficiency was ≈20-fold higher than when linear plasmid was used alone (5 transformants). More than 100 units of enzyme reduced the efficiency of transformation (Table 1, experiments 3 and 4 vs. experiments 1 and 2).

Detection of Tagged T-Toxin-Deficient Mutants. Two transformants (R.C4.186 and R.C4.350L) of the 1310 recovered failed to produce detectable T toxin on any medium (Fig. 2) and caused symptoms identical to those of race O when inoculated on T cytoplasm corn plants (Fig. 3). When either mutant was crossed to a $Tox1^+$ tester strain, progeny segregated 1:1 for both T-toxin production and hygromycin B resistance; all Tox^- progeny were resistant to hygromycin B, while all Tox^+ progeny were sensitive (Table 2), indicating that, in each mutant, a single mutation site was tagged with *hygB*. When either mutant was crossed to a $Tox1^-$ tester strain, all progeny were Tox^-, indicating that each mutation mapped at the genetically defined *Tox1* locus (Table 2). Segregation in complete tetrads (four sets of twins per ascus) isolated from crosses between either mutant and a $Tox1^+$; *MAT-1* tester was 4 Tox^+:4 Tox^-; $hygB^R$ (Fig. 4). In tetrads isolated from crosses between either mutant and a $Tox1^-$; *MAT-1* tester, segregation was 4 Tox^-; $hygB^R$:4 Tox^- (Fig. 4).

Linkage Among Tox^- Mutations. Progeny of a cross between REMI mutant R.C4.350L (*MAT-2*) and REMI mutant R.C4.186 (represented by strain 1216-2-2, a *MAT-1* ascospore progeny of a cross between R.C4.186 and C9) were 100% Tox^-; $hygB^R$, indicating that the two REMI mutations are tightly linked to each other (Table 2). Progeny of a cross between either REMI mutant and nontagged, *Tox1*-linked,

Table 1. Effect of REMI on *C. heterostrophus* transformation efficiency

	No. of stable transformants per 30 μg of DNA*				
			Linearized plasmid + *Hin*dIII‡		
Exp.	Circular plasmid	Linearized plasmid†	A	B	C
1	0	5	107	30	61
2	0	ND	79	ND	ND
3	0	6	57	25	32
4	0	3	10	6	11

Experiments 1, 3, and 4 consisted of five treatments (represented by the five columns), each in duplicate. Experiment 2 consisted of two treatments, each replicated eight times. ND, not done.

*Each value is the average of replicate transformation tubes. Total number of REMI transformants recovered, 1310, is the sum collected from replicate tubes (columns A, B, and C).

†pUCATPH was linearized with *Hin*dIII, phenol-extracted, ethanol precipitated, and suspended in 100 μl of TE.

‡One hundred units of *Hin*dIII was added to transformation tubes for experiments 1 and 2, 200 units was added for experiment 3, and 400 units was added for experiment 4. *Hin*dIII was added along with linearized plasmid in 100 μl of solution as follows: A, in original plasmid digestion solution; B, as fresh enzyme plus buffer, after removal of original enzyme by phenol extraction and ethanol precipitation; C, same as in B except buffer was omitted.

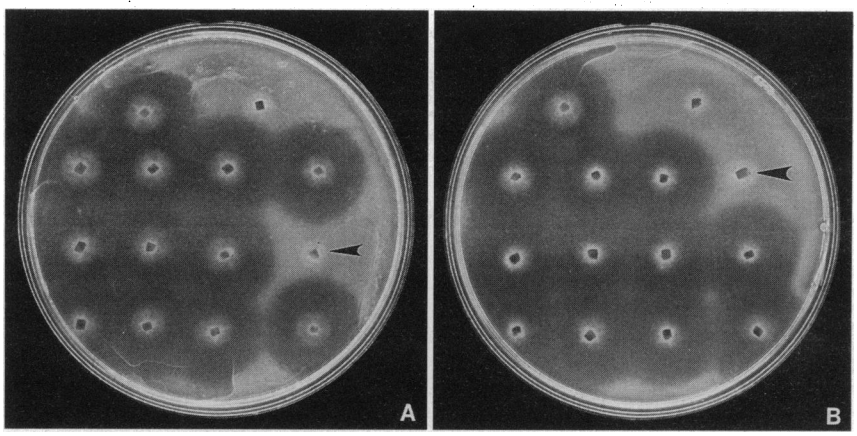

FIG. 2. Detection of T-toxin-deficient mutants among REMI transformants. In each plate, the two strains at the top are *Tox1*$^+$ (left) and *Tox1*$^-$ (right) controls; remainder of colonies are REMI transformants, one of which (arrowheads) failed to cause a halo: R.C4.186 (*A*) and R.C4.350L (*B*). Assay was performed as described (24). Results shown here were obtained whether mycelial inoculum was taken from CMX, CMNS, or Fries medium.

chemically induced *Tox*$^-$ mutant Ctm45 (represented by strain 1151-3-1, a *MAT-1* ascospore progeny of a cross between Ctm45 and C9) were 100% *Tox*$^-$ indicating that both REMI mutations map at the same position as the chemically induced *Tox1*-linked mutation.

The Linked REMI Mutations Are at Different *Hin*dIII Sites. DNA prepared from REMI mutants R.C4.186 and R.C4.350L was digested with *Eco*RI or *Xba* I (each of which has two sites in pUCATPH) or *Hin*dIII, electrophoresed, Southern blotted, and probed with pUCATPH. With *Hin*dIII, a single 5-kb band the size of pUCATPH (Fig. 1) was evident in DNA from both mutants (data not shown), indicating that the vector inserted at a *Hin*dIII site in both cases. Hybridization to DNA digested with the other two enzymes revealed fragments of sizes expected from the pUCATPH map (Fig. 1): for *Eco*RI, a vector-specific band of ≈640 bp and two border fragments greater than 1.8 and 2.2 kb; for *Xba* I, a vector-specific band of 2.1 kb and border fragments greater than 300 bp and 2.6 kb (Fig. 5). Sizes of the border fragments were different between the two REMI mutants, suggesting that, although both insertion points are *Hin*dIII sites that map at *Tox1*, they are at different positions on the chromosome.

DISCUSSION

The REMI procedure (9) has been used to mutagenize and simultaneously tag the *Tox1* locus of *C. heterostrophus*. Two *Tox*$^-$ mutants were identified in a screen of 1310 REMI transformants. Previously, we succeeded in collecting nine unmarked *Tox*$^-$ mutations after chemical mutagenesis and enrichment for mutants by using a strain of *C. heterostrophus* that was conditionally sensitive to its own toxin (14). Attempts to produce *Tox*$^-$ mutants by conventional mutagenesis and routine screening procedures have been uniformly unsuccessful. The relative ease with which we found tagged *Tox*$^-$ mutations with the simple REMI technique demonstrates that this is an effective approach to creating and marking mutations, possibly because REMI also increases

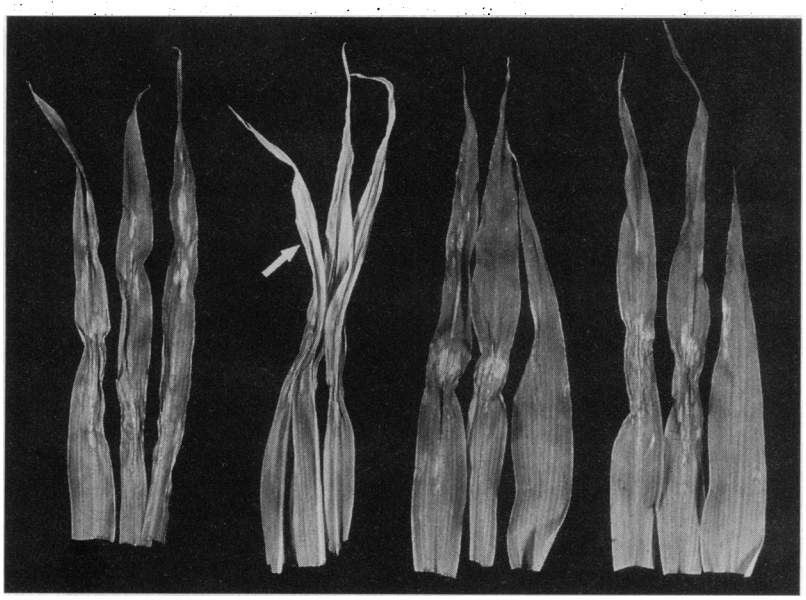

FIG. 3. Virulence of *C. heterostrophus* strains to T-cytoplasm corn. Assay was performed as described (14). Plants (each represented by three leaves) were inoculated with (left to right) race O strain C5 (*Tox1*$^-$ control), race T strain C4 (*Tox1*$^+$ control), REMI mutant R.C4.186 (*Tox*$^-$), and a *Tox*$^-$ ascospore progeny from the cross R.C4.186 × C9. Small localized lesions caused by R.C4.186 and its progeny are indistinguishable from those caused by the race O control strain. Yellow streaking (not visible in this photograph) and complete tissue collapse (arrow) typical of race T infection are lacking in plants inoculated with the mutant. Results with REMI mutant R.C4.350L were the same as those shown here.

Table 2. Progeny tests of crosses between *Tox*$^-$ REMI mutants and *Tox*$^+$ and *Tox*$^-$ testers

Parents		No. of progeny with genotype			
Mutant	Tester	*Tox*$^-$ *hygB*^{R}	*Tox*$^-$	*Tox*$^+$ *hygB*^{R}	*Tox*$^+$
R.C4.186	C9 (*Tox1*$^+$)*	53	0	0	67
R.C4.350L	CB15 (*Tox1*$^+$)*	57	0	0	61
R.C4.186	C5 (*Tox1*$^-$)*	30	30	0	0
R.C4.350L	CB3 (*Tox1*$^-$)*	27	21	0	0
R.C4.186	1151-3-1 (*Tox1*$^-$)†	46	54	0	0
R.C4.350L	1151-3-1 (*Tox1*$^-$)†	61	48	0	0
R.C4.350L	1216-2-2 (*Tox1*$^-$; *hygB*^{R})‡	59	0	0	0

Strains R.C4.186 and R.C4.350L are *hygB*^{R}; *Tox*$^-$; *MAT-2* REMI mutants of wild-type strain C4 (*Tox1*$^+$; *MAT-2*).

**MAT-1* testers, near isogenic with wild-type strain C4.

†Strain 1151-3-1 is a *Tox*$^-$; *MAT-1* progeny of a cross between C9 (*Tox1*$^+$; *MAT-1*) and Ctm45 [*Tox*$^-$; *MAT-2*; *hygB*^{R} (a chemically induced, *Tox1*-linked, nontagged *Tox*$^-$ mutant; ref. 14)].

‡Strain 1216-2-2 is a *Tox*$^-$; *MAT-1* progeny of a cross between C9 (*Tox1*$^+$; *MAT-1*) and R.C4.186 (*Tox*$^-$; *MAT-2*; *hygB*^{R}).

the transformation frequency. Routinely, no transformants result when *C. heterostrophus* is transformed with a circular, nonhomologous plasmid (Table 1). Linearization of the plasmid improves this slightly; however, linearization and addition of restriction enzyme increases the yield of transformants ≈20-fold, resulting in a workable pool of transformants with random insertions to screen for mutant phenotypes of interest.

Our specific goal, at the outset, was to make tagged T-toxin-deficient mutants. During the course of these experiments, however, we also screened REMI transformants for auxotrophy and for defects in mating ability, conidiation, color, colony morphology, and pathogenicity (S.L., L.L., C.B., O.C.Y., and B.G.T., unpublished data). We found no auxotrophs or nonpathogens but several mutants were recovered representing the remaining four classes. The efficiency of tagging these mutations varied between 30% and 50% per experiment. Thus, in *C. heterostrophus*, the REMI procedure yields mutants with a variety of phenotypes and the frequency of tagging is high. Although the cause of the nontagged mutations was not investigated, we suspect they result from improper repair of digested restriction enzyme sites. These results with *C. heterostrophus* suggest that REMI may be generally effective for tagging mutations in filamentous fungi.

Analysis of the vector insertion points in mutants R.C4.186 and R.C4.350L by Southern blotting and hybridization re-

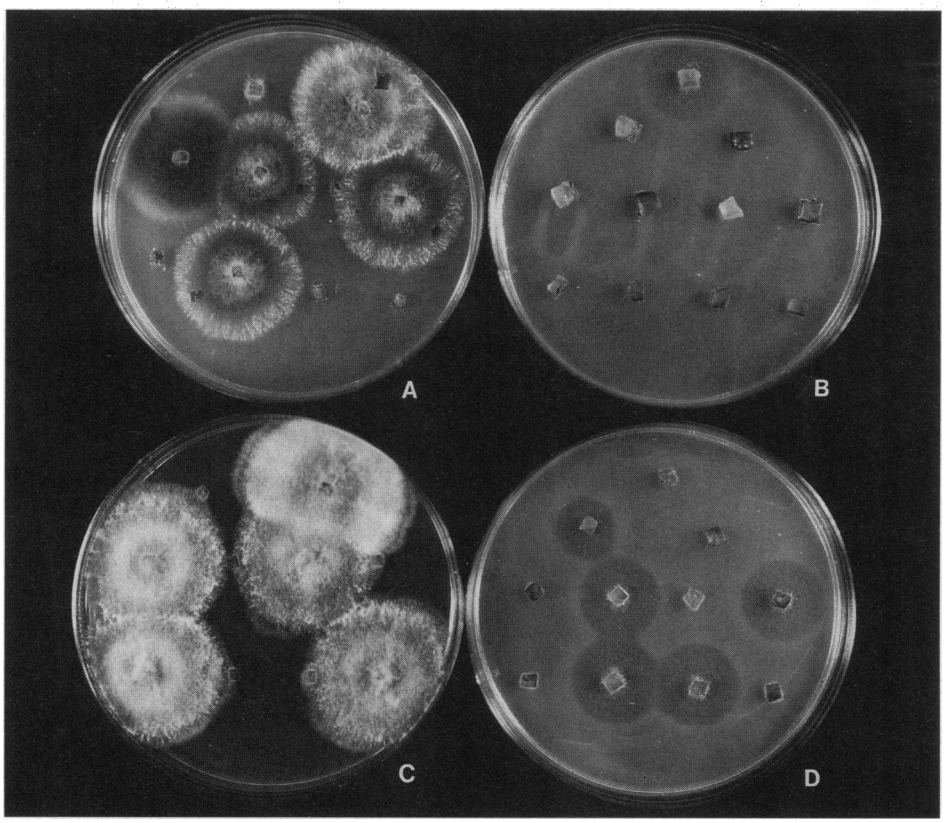

FIG. 4. Segregation of the *Tox*$^-$ mutation and *hygB* in complete tetrads isolated from crosses between REMI mutant R.C4.186 and *Tox1*$^-$ (*A* and *B*) and *Tox1*$^+$ (*C* and *D*) testers. (*A* and *C*) CMNS with 100 μg of hygromycin B per ml. (*B* and *D*) *E. coli* assay for T toxin (24). In all plates the three colonies at the top are controls: *Tox1*$^+$ tester (with halo in *B* and *D*); *Tox1*$^-$ tester and *Tox*$^-$ mutant (each with no halo in *B* and *D*). Note that the *Tox1*$^+$ tester in *A* and the *Tox1*$^-$ tester in *C* were overgrown by the *Tox*$^-$ mutant control and are not visible. In all four plates, the remaining eight strains represent a complete tetrad. In *C* and *D* each hygromycin B-resistant progeny is *Tox*$^-$ and each hygromycin B-sensitive progeny is *Tox*$^+$, indicating that the *Tox*$^-$ mutation is tagged.

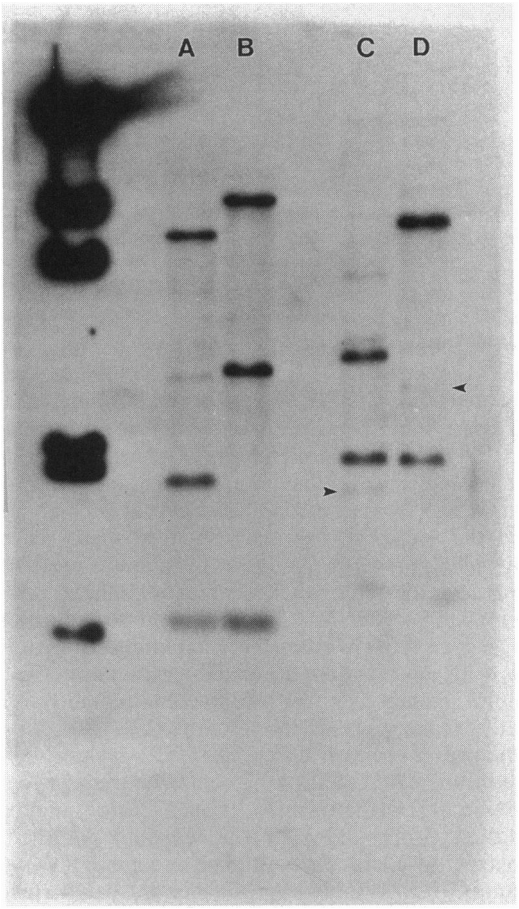

FIG. 5. Southern blot showing that plasmid insertion points in REMI mutants R.C4.186 (lanes A and C) and R.C4.350L (lanes B and D) are different. Genomic DNA of each mutant was digested with *Eco*RI (lanes A and B) or *Xba* I (lanes C and D), separated in an agarose gel, transferred to a nylon membrane, and probed with REMI vector pUCATPH. Vector-specific sequences (Fig. 1) are represented by bands of 0.6 kb (lanes A and B) and 2.1 kb (lanes C and D). Remaining bands represent vector/chromosome border fragments and are polymorphic between the two mutants, indicating different points of vector insertion. Arrowheads, border fragments that hybridized weakly because they contain a very short stretch of vector sequence. Leftmost lane, λ DNA digested with *Hin*dIII.

vealed that in both cases *Hin*dIII sites flank the transforming vector. Since *Hin*dIII was used to both linearize the vector and digest genomic DNA, this result confirms that the transforming DNA inserted into the genome at sites made available by digestion with the enzyme (8). Restriction mapping of the insertion point with additional enzymes revealed that the two insertion points were different. Crosses of the two mutants to *Tox1*$^-$ testers indicated that both were tightly linked to *Tox1* and crosses of the two mutants to each other suggested that the two mutations were tightly linked to each other. Southern analysis allowed us to further refine these linkage data and determine that, while the two mutations are closely linked, they are in fact different. Furthermore, when transforming DNA plus flanking DNA is recovered from the two mutant genomes, sequence data from two different positions within the *Tox1* locus will be in hand.

The recovery of two tagged mutations at *Tox1* among only 1310 REMI transformants suggests that the locus is large and/or rich in sites for *Hin*dIII, the enzyme used in the REMI procedure. Since *Tox1* controls production of T toxin, a family of large polyketides (predominantly C_{41}), we have hypothesized that one function of *Tox1* is to encode a very large polyketide synthase, for synthesis of a long-chain polyketide (11, 14). Polyketide synthase genes are known to be large. For example, the *eryA* gene of *Saccharopolyspora erythraea*, which produces the C_{13} polyketide erythromycin, is encoded by 35 kb of DNA (25) and the *avr* gene of *Streptomyces avermitilis*, which produces the C_{25} polyketide avermectin, has 65 kb of coding sequence (26). Since T toxin is larger than either erythromycin or avermectin, it would not be unreasonable to suggest that *Tox1* may encompass >100 kb of DNA. The ease with which we identified two Tox$^-$ mutations in only 1310 transformants and the discovery that the two mutations are at different *Hin*dIII sites may reflect the large size of this locus.

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