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# Mapping and Subcloning of the Trifolitoxin Production and Resistance Genes from *Rhizobium leguminosarum* bv. *trifolii* T24

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**In mixed inoculum, *Rhizobium leguminosarum* bv. *trifolii* T24 limits nodulation of clover roots by other strains of *R. l.* bv. *trifolii*. The nodulation competitiveness expressed by T24 is caused by that strain's ability to produce the antirhizobial peptide trifolitoxin. A recombinant plasmid, pTFX1, that confers trifolitoxin production in trifolitoxin-sensitive strains of *Rhizobium* has been identified in a genomic library of T24. In this study, transposon mutagenesis and restriction enzyme analysis were used to map and subclone the trifolitoxin genes in pTFX1. A 4.4-kb region of DNA, referred to as *tfx*, was found to be necessary for the expression of trifolitoxin production and resistance in *Rhizobium*. This region was subcloned into pRK415. The resulting recombinant plasmid, pTFX2, conferred the ability to produce trifolitoxin when conjugated into trifolitoxin-sensitive strains of *Rhizobium*. This demonstrates that pTFX2 possesses all of the genetic determinants necessary for trifolitoxin production and resistance. Chromatographic and bioassay evidence was used to confirm trifolitoxin production in transconjugants containing pTFX2. The genes for trifolitoxin resistance and production were separated by cloning a 10-kb fragment of pTFX1, containing part of *tfx*, into pDSK519. The resulting plasmid, pTFX4, conferred trifolitoxin resistance, but not production, in a trifolitoxin-sensitive strain of *Rhizobium* following conjugation.**

*Additional keywords*: microbial ecology, *Rhizobium* competition, symbiotic nitrogen fixation.

Trifolitoxin is a small peptide produced by *Rhizobium leguminosarum* bv. *trifolii* T24, which is bacteriostatic toward strains of *R. leguminosarum* and *R. fredii* (Triplett and Barta 1987; Triplett *et al.* 1988). The production of trifolitoxin confers on T24 the ability to limit the nodulation of clover roots by other strains of *R. l.* bv. *trifolii* in mixed inoculum with T24 (Schwinghamer and Belkengren 1968; Triplett and Barta 1987). The genes responsible for trifolitoxin production and resistance were isolated by cosmid cloning from a genomic library of a T24 derivative (Triplett 1988).

Trifolitoxin production was expressed in strains of *R. l.* bv. *trifolii*, *R. l.* bv. *viceae*, *R. l.* bv. *phaseoli*, *R. fredii*, *R. meliloti*, and *Agrobacterium tumefaciens* following the conjugal transfer of the trifolitoxin genes present on the recombinant plasmid pTFX1. In this study, a restriction enzyme map of pTFX1 was prepared by an analysis of Tn5 insertions in pTFX1. This map was used to determine the size and location of the trifolitoxin genes as well as to develop a strategy to subclone the trifolitoxin genes into the broad host range vector pRK415. In this work, the trifolitoxin production and resistance genes are referred to as *tfx*.

The ability of pTFX1 to confer trifolitoxin production as well as resistance in trifolitoxin-sensitive strains of *Rhizobium* suggests that these two phenotypes are linked. This study describes evidence that these two phenotypes are within a 4.4-kilobase (kb) region of pTFX1.

## MATERIALS AND METHODS

**Bacteria and plasmids.** The bacterial strains and plasmids used in this work are listed in Table 1. The sources of bacterial strains not listed in Table 1 can be found in Triplett and Barta (1987).

**Bacterial growth conditions.** *Rhizobium* strains were cultured at 28° C on Bergersen's synthetic medium (BSM) as described by Bergersen (1961). Strains of *Escherichia coli* were cultured at 37° C on Luria-Bertani (LB) medium. Antibiotics were added as needed at the following final concentrations: kanamycin (Km), 50 μg/ml; tetracycline (Tc), 12.5 μg/ml; spectinomycin, 50 μg/ml; streptomycin (Str), 50 μg/ml; and nalidixic acid (Nal), 10 μg/ml.

**Bacterial conjugations.** The conjugation of the pTFX1::Tn5 mutants into *Rhizobium* was performed as described previously (Triplett and Barta 1987) with some modifications. The donor, recipient, and helper strains were mixed in a 1:1:1 ratio in water, each at a cell density of approximately $5 \times 10^7$ per milliliter. After vortexing, a 5-μl suspension of this mixture was placed on a YM/KB plate (Triplett and Barta 1987) with 3% agar. After incubation for 2 days at 28° C, each mating was resuspended in 0.1 ml of water and spread on a BSM plate prepared with noble agar and supplemented with Tc and Str. The use of noble agar in the interruption media eliminated the background of growth on the plates. After 5 days, transconjugants were observed.

Conjugations involving the transfer of plasmid DNA between strains of *E. coli* were done as described above except that 5 μl of the mixture of donor, recipient, and helper strains was placed on an LB plate and incubated at 37° C overnight. Interruptions were done as described

above with the appropriate selective media on solid LB medium.

In the transfer of plasmid DNA from *E. coli* to *Rhizobium, E. coli* DH5a(pRK2013) was used as the helper strain. In the transfer of plasmid DNA between two strains of *E. coli, E. coli* HB101(pRK2073) served as the helper strain.

**DNA isolation.** Large-scale plasmid preparations were purified by the boiling method described by Holmes and Quigley (1981). For restriction enzyme analysis of small amounts of plasmid DNA, plasmids were purified from cells grown on solid medium by the alkaline lysis miniprep method described by Ausubel *et al.* (1987).

**Transposon mutagenesis.** The recombinant plasmid, pTFX1, was mutagenized with Tn5 by the method of Ditta (1986) with slight modifications. The plasmid pTFX1 was transformed into HB101::Tn5 as described by Hanahan (1983) with LB medium supplemented with Km and Tc for the selection of transformants. The transformants were pooled and conjugated with HB101(pRK2073) and C2110nal. The triparental matings were incubated overnight at 37° C. Cells were resuspended in water, and a dilution series was plated on LB medium supplemented with Km, Tc, and Nal. Transconjugants were pooled and plasmid DNA isolated by an alkaline lysis miniprep procedure as described by Ausubel *et al.* (1987). Fourteen separate matings were performed in order to enhance the prospects of obtaining independent mutations. Plasmid DNA was transformed into *E. coli* DH5a, and the subsequent transformants were selected on LB medium with Km and Tc.

Restriction enzyme analysis of 336 pTFX1::Tn5 mutants was done to provide the information necessary to construct a restriction enzyme map of pTFX1. Each mutant was also conjugated into *R. l.* bv. *trifolii* strain TA1 as described above to determine the trifolitoxin phenotype. The *tfx* genes are not expressed in *E. coli* (Triplett 1988).

The decision was made early in these experiments to conjugate all of these mutants into TA1 rather than one or more of the trifolitoxin-minus Tn5 mutants of T24 available in this laboratory. Determining the size of the trifolitoxin production region was considered to be more important than determining the size of the region required merely to complement one of our T24::Tn5 mutants. A further incentive to pursue this approach was that the frequency of transconjugants was much higher with conjugations of pTFX1::Tn5 mutants into TA1 than with those into T24::Tn5. This was an important consideration, because a few hundred conjugations were involved.

**Restriction enzyme analysis of pTFX1::Tn5 mutants.** Plasmid DNA of each pTFX1::Tn5 mutant was cleaved with the following restriction enzymes: *Eco*RI, *Kpn*I, *Dra*I, and *Mlu*I. To accurately map Tn5 insertions within each restriction enzyme fragment, selected pTFX1::Tn5 plasmids were cleaved with *Hpa*I, an enzyme with two symmetrical restriction enzyme sites within the inverted repeat sequences (IR) of Tn5 (Bruijn and Lupski 1984). *Hpa*I has two restriction enzyme sites in pTFX1. This enzyme was used for this purpose rather than *Bgl*II since there are no *Bgl*II restriction enzyme sites in pTFX1. Plasmid DNA was electrophoresed in 0.6% agarose at 100 V. For the separation of fragment sizes greater than 15 kb, field inversion electrophoresis was used. At 0.3-s intervals, the electric field was inverted between 100 V toward the anode and 60 V toward the cathode. Field inversion gels were run for 16 hr at 4° C. All gels were 10 cm in length.

**Trifolitoxin bioassay.** The ability of a strain to produce trifolitoxin was determined by bioassay as described previously (Triplett 1988).

**Southern hybridizations.** Southern analysis was

**Table 1.** Bacteria and plasmids

| | Genotype or relevant characteristics[a] | Source or reference |
|---|---|---|
| Bacteria | | |
| *Rhizobium leguminosarum* bv. *trifolii* | | |
| T24 | $Tfx^+$, $Fix^+$, $Cmp^+$ | Schwinghamer and Belkengren 1968 |
| TA1 | $Tfx^s$, $Fix^+$ | A. Gibson, CSIRO |
| *R. l.* bv. *viceae* | | |
| B518 | $Tfx^s$, $Fix^+$, $Km^r$, $Str^r$, $Nm^r$ | D. Phillips, Davis, CA |
| *R. l.* bv. *phaseoli* | | |
| Kim5 | $Tfx^r$, $Fix^+$, $Cmp^+$, $Sp^r$ | J. Handelsman, Madison, WI |
| CE3 | $Tfx^s$, $Fix^+$, $Cmp^-$, $Str^r$ | J. Handelsman, Madison, WI |
| *R. meliloti* | | |
| 102F97 | $Tfx^s$, $Fix^+$ | S. Smith, Milwaukee, WI |
| *Escherichia coli* | | |
| HB101 | pro, leu, thi, lacY, $Str^r$, endoI$^-$, recA$^-$, hsrA$^-$hsm$_k^-$ | Boyer and Roulland-Dussoix 1969 |
| HB101::Tn5 | As HB101 but::TN5 in the chromosome | Ditta 1986 |
| C2110nal | $Nal^r$, his, rha, polA$^-$ | Ditta 1986 |
| DH5a | | Bethesda Research Labs |
| Plasmids | | |
| pRK415 | Broad host range vector pRK404 derivative, $Tc^r$ | Keen *et al.* 1988 |
| pDSK519 | Broad host range vector pDSK509 derivative, $Km^r$ | Keen *et al.* 1988 |
| pRK2013 | $Km^r$, $Tra^+$, $Mob^+$, colE1 replicon | Figurski and Helinski 1979 |
| pRK2073 | pRK2073 $Sp^r$::Tn7 | Leong *et al.* 1982 |
| pTFX1 | pLAFR3 derivative with *tfx* genes, $Tc^r$ | Triplett 1988 |
| pTFX2 | pRK415 derivative with *tfx* genes, $Km^r$::Tn5, $Tc^r$ | This work |
| pTFX4 | pDSK519 derivative with *tfx* resistance genes, $Tfx^r$, $Km^r$ | This work |

[a]Tfx = trifolitoxin production or sensitivity; Fix = symbiotic nitrogen fixation; Tra = plasmid transfer function; Mob = plasmid mobilization function; Cmp = nodulation competitiveness; Km = kanamycin; Str = streptomycin; Sp = spectinomycin; Nal = nalidixic acid; Tc = tetracycline; Nm = neomycin; $^r$ = resistant.

determined with biotinylated probes of either pLAFR3 or pTFX1 as described previously (Triplett 1988).

**Partial purification of trifolitoxin.** Trifolitoxin was partially purified from the cell culture supernatants of T24 and various *Rhizobium* transconjugants containing pTFX2 by reverse-phase chromatography according to the methods of Triplett (1988).

**Subcloning of *tfx*.** An *Mlu*I digest of plasmid DNA from the pTFX1::Tn5 mutant 10-15 was blunted with T4 DNA polymerase as described by Ausubel *et al.* (1987). The insertion in pTFX1 mutant 10-15 is located 0.5 kb outside of the *tfx* region (Fig. 1). These fragments were ligated to an *Xmn*I digest of pRK415 treated with alkaline phosphatase. The resulting ligated DNA was transformed into *E. coli* DH5a competent cells, and transformants were selected on LB solid medium supplemented with Km and Tc. The presence of the appropriate insert in pRK415 was confirmed by *Eco*RI digestion of a plasmid miniprep from a transformant. The recombinant plasmid obtained by this procedure, referred to as pTFX2, was conjugated into the trifolitoxin-sensitive *Rhizobium* strains TA1 and KIM5. The resulting transconjugants were assayed for trifolitoxin production.

**Subcloning of the trifolitoxin resistance region in *tfx*.** The 10-kb *Kpn*I fragment of pTFX1 was ligated to the dephosphorylated *Kpn*I site of pDSK519. The ligation mixture was transformed into *E. coli* DH5a, and the transformants were selected on LB medium supplemented with Km. The presence of the insert DNA in pDSK519 was confirmed by a *Kpn*I digest of a plasmid miniprep. This plasmid, referred to as pTFX4, was conjugated into *R. l.* bv. *trifolii* TA1, and the resulting transconjugants were assayed for trifolitoxin production and resistance.

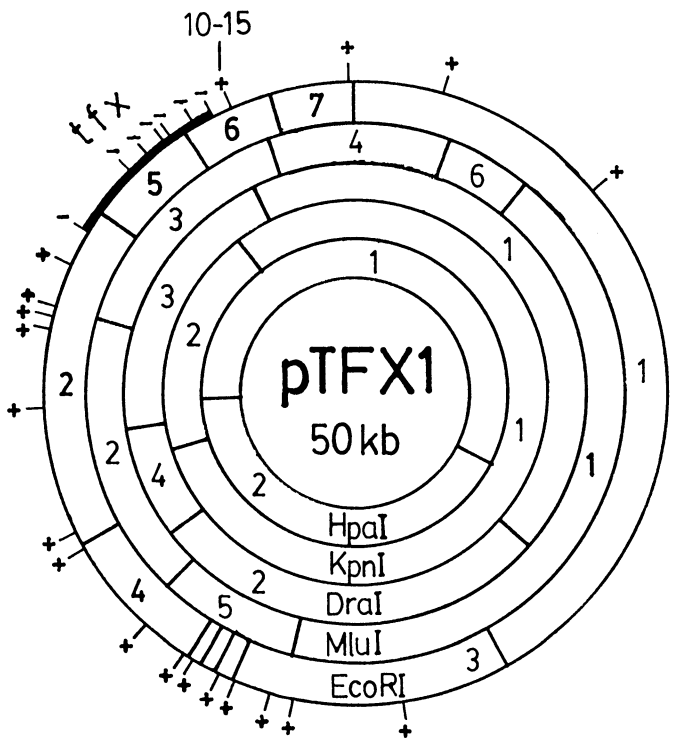


**Fig. 1.** Restriction map of pTFX1, a recombinant plasmid containing 29.5 kb of T24 DNA and 21.5 kb of vector (pLAFR3) DNA. Selected Tn5 insertions were accurately mapped by *Hpa*I digestion. Of 336 insertions, 37 failed to confer trifolitoxin production in *Rhizobium leguminosarum* bv. *trifolii* TA1. None of the insertions in *Eco*RI fragments 1, 3, 4, 7, 8, 9, and 10 affected trifolitoxin production. All of the insertions causing a trifolitoxin-minus phenotype in TA1 are mapped in the region depicted with the bold line. This region is 4.4 kb in size. The symbols + and − represent the ability and inability, respectively, of a plasmid containing a Tn5 insertion to confer trifolitoxin production in TA1. *Eco*RI fragment 1 contains the vector portion of the plasmid. The location of the insertion in the pTFX1::Tn5 mutant 10-15 is shown adjacent to the *tfx* region.

## RESULTS

**Insert size of pTFX1.** The previous estimate of the size of the insert in pTFX1, 24.2 kb, is inaccurate, owing to an underestimate of the size of the large restriction enzyme fragment of a *Pst*I digest of pTFX1 (Triplett 1988). Field inversion electrophoresis improves the resolution of restriction enzyme fragments between 15 and 50 kb in size. By the use of this technique, the insert size is now known to be 29.5 kb (data not shown).

**Mapping of the *tfx* genes.** A restriction enzyme map of pTFX1 was prepared by restriction enzyme analysis of 336 Tn5 mutations in pTFX1 (Fig. 1). Thirty-seven of these mutants failed to confer trifolitoxin production following conjugation in TA1. The restriction enzyme digestion patterns of each mutant were determined by means of the plasmid DNA with four restriction enzymes, *Eco*RI, *Mlu*I, *Dra*I, and *Kpn*I, which do not cleave the Tn5 sequence (Bruijn and Lupski 1984; Mazodier *et al.* 1985) and which cleave pTFX1 fewer than 10 times.

Selected mutations were accurately mapped by *Hpa*I digestion (Fig. 1). All of the 37 pTFX1::Tn5 plasmids that failed to confer trifolitoxin production in TA1 were accurately mapped, and all were found to be present in a 4.4-kb region (Fig. 1). Other insertions in *Eco*RI fragments 2 and 6 were also mapped accurately by *Hpa*I digestion in order to determine the maximum size of the *tfx* region. More than 200 insertions were observed in *Eco*RI fragments 1, 3, 4, 7, 8, 9, and 10. None of these insertions affected trifolitoxin production. Selected insertions in these fragments were accurately mapped, nevertheless, and are depicted in Figure 1. Four insertions were observed in *Eco*RI fragment 6; two of these prevented trifolitoxin production. Among 60 insertions observed in *Eco*RI fragment 2, only two prevented trifolitoxin production. All of the 33 insertions in fragment 5 caused a lack of trifolitoxin production when transferred to TA1.

Three lines of evidence suggest that the largest *Eco*RI fragment of pTFX1 is the pLAFR3 vector portion of pTFX1. First, only this fragment of the *Eco*RI digest hybridizes to a pLAFR3 probe. Second, the size of pLAFR3, 21.5 kb, is identical to the size of the largest *Eco*RI fragment of pTFX1. And, third, there is only one *Eco*RI restriction enzyme site in pLAFR3.

The maximum size of *tfx* appears to be no larger than 4.9 kb and no smaller than 4.4 kb. This is based on the mapping of the trifolitoxin-minus mutants of pTFX1 (Fig. 1). The frequency of insertion in the *tfx* region by Tn5 was 11%. On the assumption of random insertion of Tn5 in the 50-kb plasmid, the size of the *tfx* genes is estimated to be 5.5 kb. This agrees closely with the size range estimated by the mapped mutations. An overestimate of the size of *tfx* is expected by this calculation, since no mutations were selected in the 1.8-kb Tc resistance gene in the vector portion

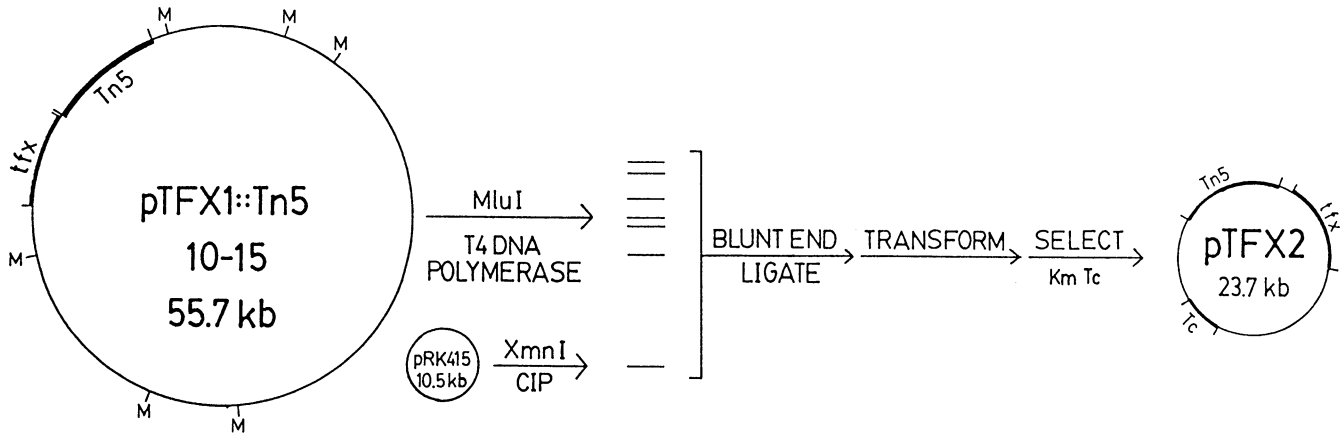


**Fig. 2.** Strategy for the subcloning of the *tfx* region. A derivative of pTFX1 was chosen that contained a Tn5 insertion downstream from *tfx* but within the same *Mlu*I fragment that contained *tfx*. This plasmid with *Mlu*I and the resulting fragments were blunted with T4 DNA polymerase. The blunted fragments were ligated to an *Xmn*I digest of pRK415 that was dephosphorylated with calf intestinal phosphatase. The plasmid of interest was selected on Luria-Bertani medium supplemented with kanamycin (Km) and tetracycline (Tc) following transformation into *E. coli* DH5a. The resulting plasmid, referred to as pTFX2, conferred trifolitoxin production and resistance following conjugation into a trifolitoxin-sensitive strain.

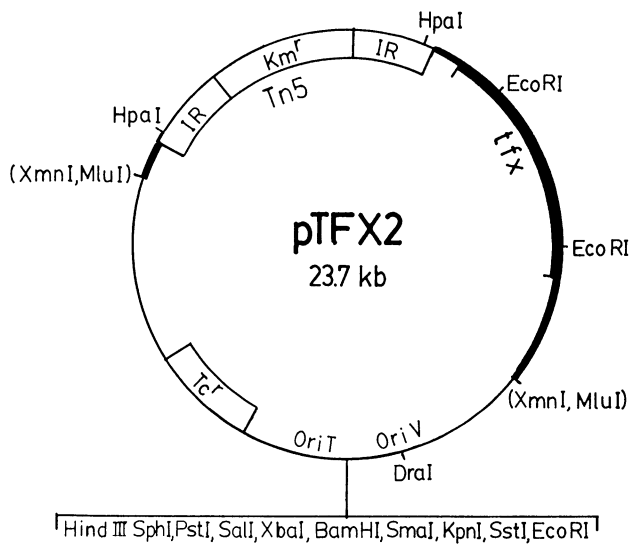


**Fig. 3.** Restriction map of pTFX2. The very bold line shows the *tfx* region. The bold line shows the 7.5 kb of insert DNA from pTFX1.

of pTFX1.

The region of DNA in pTFX1 required for trifolitoxin production was identical in each *Rhizobium* strain tested. That is, the pTFX1::Tn5 mutants that failed to confer trifolitoxin production in TA1 also lacked trifolitoxin production in CE3. Similarly, those that were trifolitoxin-producing in CE3 had the same phenotype when conjugated into TA1. The mutants also expressed the same trifolitoxin phenotype in KIM5 and B518.

**Subcloning of *tfx*.** The strategy for subcloning *tfx* is illustrated in Figure 2. One mutant of pTFX1 was chosen whose insertion was located within the 7.5-kb *Mlu*I fragment and 0.5 kb away from *tfx*. Mutant 10-15 possesses an insertion in the 7.5-kb *Mlu*I fragment 3 of pTFX1 that does not affect the expression of trifolitoxin production in *Rhizobium*. The ligation of the *Mlu*I fragments from 10-15, which contains both the intact trifolitoxin production genes and a Tn5 insertion on the same fragment, to the broad host range vector, pRK415, allowed for selection against the other possible ligation products. One transformant resistant to both Km and Tc was chosen for further study. Restriction analysis of the plasmid DNA from this transformant showed an insert size of 13.2 kb, as predicted on the basis of the size of *Mlu*I fragment 3 of pTFX1 with a Tn5 insertion. This plasmid is referred to as pTFX2.

**Restriction map of pTFX2.** A restriction enzyme map of pTFX2 was prepared on the basis of restriction enzyme sites known to be present in pRK415, the *Eco*RI restriction enzyme sites present in *Mlu*I fragment 3 in pTFX1, and double restriction enzyme digests of pTFX2 with *Sst*I, *Eco*RI, and *Hpa*I (Fig. 3). The *Xmn*I site in pRK415 and the *Mlu*I sites in the insert were eliminated by the blunt-end ligation of the insert into the vector.

**Trifolitoxin production by pTFX2 transconjugants.** To determine whether pTFX2 possessed functional *tfx*, this plasmid was conjugated into TA1 and KIM5. Trifolitoxin production by the resulting transconjugants was observed, as determined by bioassay. The pTFX2 transconjugants produced an antibiotic that inhibited strains of *R. fredii*, *R. l.* bv. *phaseoli*, *R. l.* bv. *viceae*, and *R. l.* bv. *trifolii* but had no effect on the growth of strains of *R. meliloti*, *Agrobacterium*, and *Bradyrhizobium* (Table 2). Similarly, partially purified preparations of cell culture supernatants of TA1(pTFX2) and KIM5(pTFX2) each possessed an antirhizobial activity that copurified with trifolitoxin from a T24 cell culture supernatant (Table 3) as described previously for *Rhizobium*(pTFX1) transconjugants (Triplett 1988). This narrow range of antibacterial activity is identical to that of trifolitoxin produced by T24, the strain from which *tfx* is derived.

The construction of pTFX2 confirms the location of *tfx* in pTFX1 illustrated in Figure 1. The subcloning of *tfx* also supports the observation that only 4.4 kb of DNA within pTFX1 is required for the expression of trifolitoxin production and resistance in *Rhizobium*.

**Expression of trifolitoxin resistance in pTFX2 transconjugants.** Strain TA1 is sensitive to trifolitoxin production by T24 (Triplett and Barta 1987). The growth of the transconjugant TA1(pTFX2) is not inhibited by T24 (Table 2). Furthermore, since this pTFX2 transconjugant produces trifolitoxin, it must possess the trifolitoxin

resistance phenotype to avoid self-inhibition. Identical results were observed following the transfer of pTFX1 to trifolitoxin-sensitive strains (Triplett 1988).

**Subcloning of the *tfx* resistance determinants.** None of the TA1(pTFX1::Tn5) transconjugants were sensitive to trifolitoxin, including those that failed to produce the peptide. Since none of the 336 mutations in pTFX1 affected trifolitoxin resistance, another subcloning exercise was initiated in order to determine the location of the trifolitoxin resistance phenotype expressed by pTFX1.

The trifolitoxin resistance phenotype must be within the 4.4-kb *tfx* region, for two reasons. First, none of the mutations outside of this region affected trifolitoxin production. If a transconjugant is capable of producing trifolitoxin, the resistance mechanism must be active as well. Second, the conjugation of pTFX2 into trifolitoxin-sensitive strains resulted in transconjugants capable of trifolitoxin production and having trifolitoxin resistance.

The *tfx* region includes a 1.9-kb fragment in which no Tn5 insertions were observed. All of the 37 Tn5 insertions in *tfx* were mapped, and none of those resulted in a trifolitoxin-sensitive phenotype following conjugation into *Rhizobium*. Thus the 1.9-kb region is a likely candidate for the location of the trifolitoxin resistance genes.

Evidence was obtained that supports the hypothesis that the 1.9-kb region in *tfx* is involved in trifolitoxin resistance. The pTFX4 transconjugant of TA1 was resistant to trifolitoxin. However, TA1(pTFX4) had no antirhizobial activity.

The ability of pTFX4 to confer trifolitoxin resistance in TA1 was expected on the basis of the map of pTFX1. This map shows that the 10-kb *Kpn*I fragment of pTFX1 contains 2.8 kb of the 4.4-kb *tfx* region. This 2.8-kb region includes all of the 1.9-kb portion of *tfx* presumed to be involved in trifolitoxin resistance.

**Table 2.** Sensitivity of various Rhizobiaceae strains to trifolitoxin produced by T24 and two recombinant *Rhizobium* strains with pTFX2, including *R. leguminosarum* bv. *trifolii* TA1 and *R. l.* bv. *phaseoli* KIM5

| | Area of zone of inhibition ($cm^2$) | | |
|---|---|---|---|
| **Test strain** | **T24** | **TA1(pTFX2)** | **KIM5(pTFX2)** |
| *R. leguminosarum* | | | |
| bv. *trifolii* | | | |
| T24 | 0.0 | 0.0 | 0.0 |
| TA1 | 11.5 | 11.6 | 11.8 |
| 2046 | 17.1 | 7.4 | 23.3 |
| bv. *viceae* | | | |
| 128C84 | 19.4 | 7.6 | 19.8 |
| 128C41 | 10.5 | 7.8 | 18.4 |
| 128A1 | 16.2 | 7.8 | 21.6 |
| 128C78 | 16.3 | 8.1 | 20.5 |
| 128A12 | 16.2 | 7.3 | 22.6 |
| 128C15 | 7.8 | 6.1 | 10.1 |
| 128C1 | 14.9 | 10.0 | 18.5 |
| 3960 | 0.8 | 0.0 | 0.0 |
| B518 | 21.8 | 12.3 | 28.6 |
| bv. *phaseoli* | | | |
| 127K115 | 10.5 | 4.5 | 16.8 |
| 127K60 | 16.3 | 8.7 | 18.2 |
| 127K42 | 9.9 | 3.7 | 15.5 |
| 127K30 | 12.7 | 6.8 | 16.1 |
| 127K8 | 9.9 | 5.0 | 14.4 |
| 127K12b | 13.6 | 7.9 | 15.8 |
| 127K16 | 15.6 | 7.8 | 16.1 |
| 127K90 | 12.9 | 7.9 | 16.7 |
| 127K117 | 15.6 | 9.4 | 23.0 |
| *R. fredii* 205 | 11.7 | 4.7 | 17.0 |
| *R. meliloti* | | | |
| 102F51a | 0.0 | 0.0 | 0.0 |
| 102F34a | 0.0 | 0.0 | 0.0 |
| 102F97 | 1.3 | 0.0 | 2.3 |
| 102F92 | 0.0 | 0.0 | 0.0 |
| 102D6 | 0.0 | 0.0 | 0.0 |
| 102F3 | 0.0 | 0.0 | 0.0 |
| *Bradyrhizobium japonicum* | | | |
| USDA 110 | 0.0 | 0.0 | 0.0 |
| USDA 83 | 0.0 | 0.0 | 0.0 |
| SM31 | 0.0 | 0.0 | 0.0 |
| *Agrobacterium tumefaciens* C58 | 0.0 | 0.0 | 0.0 |
| *A. radiobacter* K84 | 0.0 | 0.0 | 0.0 |

## DISCUSSION

The mapping and subcloning of *tfx* demonstrate that a 4.4-kb region in pTFX1 is responsible for trifolitoxin production and resistance. Within *tfx* is a 1.9-kb region in which no mutations have been mapped. This region contains the genes necessary for trifolitoxin resistance. The ability of pTFX4 to confer trifolitoxin resistance in a sensitive strain of *Rhizobium* supports this view. This plasmid contains all of the uncharacterized 1.9-kb region but lacks 1.6 kb required for trifolitoxin production. As expected, this plasmid fails to confer trifolitoxin production in TA1 but does confer trifolitoxin resistance. All of the available evidence suggests that these two phenotypes are located within the 4.4-kb *tfx* region.

It is surprising that no mutations in the 1.9-kb resistance region have been identified. All of the pTFX1::Tn5 plasmids were conjugated into the trifolitoxin-sensitive strain TA1. Mutations in the trifolitoxin resistance region of *tfx* may be lethal when conjugated into TA1. There were 44 pTFX1::Tn5 plasmids that failed to conjugate into TA1. All of these mutations were mapped outside of the *tfx* region. In each case, other insertions in pTFX1 were mapped very near those that failed to conjugate. The pTFX1::Tn5 plasmids that failed to conjugate are probably deficient in plasmid replication or in a regulatory function. All mutations found within *tfx* were conjugable into TA1.

This still leaves unresolved the observation that no mutations were observed in the 1.9-kb trifolitoxin resistance region. It is possible that a mutation in the trifolitoxin resistance region is lethal in *E. coli*. Although trifolitoxin production is not observed in *E. coli* with *tfx*, the lack of trifolitoxin production in this species may simply be an export problem. Trifolitoxin may be produced in *E. coli* but not exported and hence not observed by our

**Table 3.** Sensitivity of various Rhizobiaceae strains to the partially purified trifolitoxin produced by T24, TA1(pTFX2), and KIM5(pTFX2)

| | Area of zone of inhibition ($cm^2$) | | |
|---|---|---|---|
| **Test strain** | **T24** | **TA1(pTFX2)** | **KIM5(pTFX2)** |
| *Rhizobium leguminosarum* | | | |
| bv. *trifolii* | | | |
| TA1 | 5.8 | 9.4 | 18.8 |
| 2046 | 3.0 | 10.6 | 25.6 |
| bv. *viceae* 3960 | 0.0 | 0.0 | 0.0 |
| bv. *phaseoli* 127K117 | 4.5 | 9.4 | 21.3 |
| *R. fredii* 205 | 3.5 | 2.4 | 7.3 |
| *R. meliloti* 102F3 | 0.0 | 0.0 | 0.0 |
| *Bradyrhizobium japonicum* | 0.0 | 0.0 | 0.0 |
| USDA 110 | | | |
| *Agrobacterium tumefaciens* C58 | 0.0 | 0.0 | 0.0 |

bioassay.

In each case examined to date, the genes coding for the production of other antibiotics and resistance to them have been found to be linked and are often overlapping (Hutchinson 1987). Such genetic organization and coordination are essential for the self-protection of the antibiotic-producing strain. The trifolitoxin genes are no exception to this rule.

Trifolitoxin is a peptide of 10 amino acids with a UV-absorbing chromophore (Triplett *et al.* 1988). This peptide also includes a thiazoline ring similar to that found in bacitracin (Triplett *et al.* 1988). The synthesis of bacitracin, a peptide of 12 amino acids, requires a three-component complex, including proteins of 150, 210, and 380 kDa. A functional copy of the bacitracin synthesis genes has not yet been cloned, although progress has been made in recent years (Korsnes *et al.* 1986; Ishihara *et al.* 1989).

Peptide antibiotics having more than five amino acid residues are usually synthesized by a thiotemplate mechanism whereby a large multienzyme complex is responsible for synthesis (Kleinkauf and Dohren 1983). Each enzyme in the complex assembles no more than four residues (Kleinkauf and Dohren 1983). These complexes are usually very large. For example, gramicidin S is a peptide of 10 amino acid residues and is synthesized by two proteins, of 100 and 280 kDa. Edeine contains five amino acid residues, and its synthesis requires three components, of 210, 180, and 100 kDa.

Given these precedents, the observation that *tfx* is a region of only 4.4 kb was unexpected. This amount of DNA is sufficient to code for a 250-kDa protein. This is somewhat smaller than the enzyme complexes required for the synthesis of other antibacterial peptides. Furthermore, the 4.4-kb *tfx* region must code for trifolitoxin resistance.

Although conjugation of pTFX2 into *Rhizobium* does result in the expression of trifolitoxin production, these transconjugants have not been examined for improved nodulation competitiveness. The *tfx* genes on pTFX2 are likely to be very unstable in the absence of selection pressure (Lambert *et al.* 1987). Thus, *Rhizobium*(pTFX2) transconjugants are only likely to express trifolitoxin production and nodulation competitiveness in the presence of Tc or Km. The addition of these antibiotics in legume nodulation competition assays is not practical. This laboratory is studying methods for the stable transfer of *tfx* to the genomes of several strains of *Rhizobium*. Once stable expression of trifolitoxin production is observed in these strains in the absence of antibiotics, the competitiveness of effective, trifolitoxin-producing strains will be rigorously examined.

Other bacteriocinogenic strains of *Rhizobium* have been identified (Hirsch 1979; Schwinghamer 1971; Schwinghamer and Brockwell 1978; Schwinghamer and Reinhardt 1963). The bacteriocins produced by these strains have not been characterized. Thus, it is not known whether these bacteriocins are similar to trifolitoxin. To date, other than T24 and recombinant *Rhizobium* transconjugants containing pTFX1 or pTFX2, no strains of *Rhizobium* are known to produce trifolitoxin.

It is not known whether bacteriocin production by *Rhizobium* is a common means by which nodule occupancy is regulated. Schwinghamer (1971) conducted a survey of *R. l.* bv. *trifolii* strains in southeastern Australia and found that 35% of the strains produced dialyzable substances that were mildly antagonistic toward six indicator strains of *Rhizobium*. None of these strains were as inhibitory toward *Rhizobium* as was T24. One of the strains described by Schwinghamer (1971), CB782, was later shown by Hodgson *et al.* (1985) to be very competitive for nodulation of clover in mixed inoculum with bacteriocin-sensitive strains. However, the competitiveness of bacteriocin-minus mutants of CB782 was not determined.

In summary, the size and location of the *tfx* region in pTFX1 were determined by Tn5 mutagenesis and restriction enzyme analysis. This information was used to subclone *tfx* into the broad host range vector pRK415. The determinants of trifolitoxin resistance in *tfx* were subcloned, and their location was identified.

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