Isolation of genes involved in nodulation competitiveness from *Rhizobium leguminosarum* bv. *trifolii* T24

(trifolitoxin/rhizobial antagonism/microbial ecology)

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ABSTRACT *Rhizobium leguminosarum* bv. *trifolii* T24 produces a potent anti-rhizobial compound, trifolitoxin, and exclusively nodulates clover roots when in mixed inoculum with trifolitoxin-sensitive strains of *R. leguminosarum* bv. *trifolii* [Schwinghamer, E. A. & Belkengren R. P. (1968) *Arch. Mikrobiol.* 64, 130–145]. In the present study, the isolation of trifolitoxin production and resistance genes is described. A cosmid genomic library of T24 was prepared in pLAFR3. No trifolitoxin expression was observed in the resulting *Escherichia coli* cosmid clones. One cosmid clone was identified that restored trifolitoxin production and nodulation competitiveness in three nonproducing mutants of T24. The recombinant plasmid from this cosmid clone, pTFX1, also conferred trifolitoxin production and resistance when transferred to symbiotically effective strains of *R. leguminosarum* bvs. *trifolii*, *phaseoli*, and *viciae*. Cosmid pTFX1 also conferred expression of trifolitoxin production when present in strains of *Rhizobium meliloti* and *Agrobacterium tumefaciens*. No trifolitoxin expression was observed in strains of *Bradyrhizobium japonicum* or *Rhizobium* sp. (cowpea) with pTFX1. Southern blot analysis with a biotinylated pTFX1 probe did not suggest that these genes were plasmid-borne. Transfer of pTFX1 to T24 or its derivatives resulted in 6- to 10-fold higher level of trifolitoxin production than wild-type T24.

Root nodule bacteria are responsible for symbiotic nitrogen fixation in legume nodules (1). The presence of inferior strains of *Rhizobium* in soil depresses legume productivity (2–4). Despite this knowledge, attempts to increase legume productivity by inoculation with superior rhizobia have sometimes failed because of the ability of indigenous strains to limit nodulation by the inoculum strains (5–9). When inoculation has been successful, the indigenous rhizobial populations usually have been small (10–12).

Many investigators have studied the factors involved in determining nodule occupancy by strains of *Rhizobium* [summarized by Dowling and Broughton (13)]. Despite these efforts, no solutions to the *Rhizobium* competition problem have been developed to date.

There are few reports of the genetic basis of the expression of nodulation competitiveness by *Rhizobium* strains. McLoughlin *et al.* (14) have described the isolation of competition-defective Tn5 mutants of *Rhizobium fredii*. Triplett and Barta (15) have also used transposon mutagenesis to generate mutants with decreased competitiveness compared with the wild-type strain. Dowling *et al.* (16) have reported the cloning of blocking factor genes from *Rhizobium leguminosarum* bv. *viciae* PF₂ that prevent nodulation of *R. leguminosarum* bv. *viciae* TOM on the Afghanistan pea.

One objective in this laboratory is to study the basis of the nodulation competitiveness expressed by a very competitive

Table 1. Plasmids used in this study with relevant characteristics

Plasmid	Relevant characteristics	Source or ref(s).
pGS9	p15A replicon, Km ^r (Tn5)	18
pLAFR3	Cosmid vector derived from pLAFR1, Tc ^r	19, 20
pRK2013	Mobilization helper, Km ^r	21
pRK415	RK2-derived cloning vector, Tc ^r	N. T. Keen
pTFX1	pLAFR3 clone with trifolitoxin genes, Tc ^r	This study

Km^r, kanamycin resistance; Tc^r, tetracycline resistance. N. T. Keen is at the University of California, Riverside.

strain of *Rhizobium* and to isolate competitiveness genes. These competitiveness genes then can be transferred to strains of *Rhizobium* to provide increased plant productivity. The strain chosen for this study is *R. leguminosarum* bv. *trifolii* T24. Schwinghamer and Belkengren (17) showed that this strain induces ineffective nodules on clover, prevents nodulation by other strains in mixed inoculum, and produces a potent anti-rhizobial compound. Transposon mutants of T24 were produced in this laboratory and it was demonstrated that the production of an anti-rhizobial compound, trifolitoxin, is the basis for the nodulation competitiveness of T24 (15). Trifolitoxin inhibits strains of *R. leguminosarum* bvs. *phaseoli*, *trifolii*, and *viciae* as well as *R. fredii* (15).

The objectives of the work described here were to isolate the genes for trifolitoxin production and resistance by cosmid cloning and to transfer those genes to strains of *R. leguminosarum* bv. *viciae* that are known to have high symbiotic nitrogenase activities.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The bacterial plasmids used in these experiments, along with their relevant phenotypes, are listed in Table 1. The properties of T24 and its derivatives are listed in Table 2. Many strains were tested for sensitivity to trifolitoxin produced by wild-type T24 or recombinant strains possessing cloned trifolitoxin genes (Tables 3 and 4). The sources of these strains were described by Triplett and Barta (15). Strains in the *Rhizobiaceae* used in this study were maintained on Bergersen's minimal medium (22), referred to as BSM. Strains of *Escherichia coli* were maintained on Luria-Bertani (LB) medium. The appropriate antibiotics were added as needed. For conjugation, the bacteria were grown on YM/KB medium (15).

Plant Culture and Inoculation. Clover (*Trifolium repens* L. cv. Dutch White) and vetch (*Vicia sativa* L.) plants were inoculated and cultured as described (15). Acetylene reduction assays of nodulated plants were performed with four replicates per treatment as described by Triplett and Barta (15).

Competition Experiment. A competition experiment was designed similar to that described by Triplett and Barta (15).

Table 2. Derivatives of *R. leguminosarum* bv. *trifolii* T24 used in these experiments and their relevant phenotypes

Strain	Relevant phenotypes	Source or ref.
T24	Trifolitoxin-producing, Fix ⁻	17
T24::Tn5 ₁	Trifolitoxin-minus, Fix ⁻	15
T24::Tn5 ₁ (pTFX1)	Trifolitoxin-producing, Fix ⁻	This study
T24::Tn5 ₄	Trifolitoxin-producing, Nod ⁻	15
T24::Tn5 ₄ (pTFX1)	Trifolitoxin-producing, Nod ⁻	This study

An effective strain of *R. leguminosarum* bv. *trifolii*, TA1, was co-inoculated with either T24 or one of its derivatives, T24::Tn5₁ or T24::Tn5₁(pTFX1). Turbid suspensions of each strain were prepared in water and diluted to an OD of 0.1 at 600 nm. The number of colony-forming units present in each inoculum was determined. Plants were inoculated with 6.5, 8.0, 7.5, and 4.5 × 10⁷ colony-forming units for strains TA1, T24, T24::Tn5₁, and T24::Tn5₁(pTFX1), respectively.

Clover seedlings were grown on sterile Jensen's agar in test tubes as described by Triplett and Barta (15). One day after transfer to the test tubes, the plants were inoculated with 0.1 ml of the rhizobial cell suspensions described above that were necessary for each treatment. After 50 days, plants were harvested and fresh weights of the shoots were recorded.

Bioassay for Trifolitoxin. A suspension of a trifolitoxin-sensitive strain of *R. leguminosarum* bv. *trifolii* in water was diluted to an OD of 0.1 at 600 nm, and 0.1 ml was spread on 100-mm (maximal diameter) plates containing BSM agar. A sterile cork borer was used to cut a 8-mm diameter hole in the center of the agar. In this hole was placed 100 μl of filter-sterilized culture fluid supernatant or a partially purified sample of trifolitoxin.

When screening transconjugants from the genomic library for trifolitoxin production, a suspension of each transconjugant was prepared in water; 5 μl of each suspension was placed on a BSM plate containing tetracycline at 12.5 μg/ml. After 24 hr at 28°C, each plate was sprayed with a suspension of a tetracycline-resistant derivative of *R. leguminosarum* bv. *trifolii* 2046, which was prepared by conjugating pRK415, a plasmid that confers tetracycline resistance, into 2046. The conjugation was performed as described above with pRK2013 as the helper plasmid. After 36 hr at 28°C, zones of inhibition were observed in strains carrying plasmids that conferred trifolitoxin production.

When screening recombinant or wild-type strains for trifolitoxin production, suspensions of test strains were prepared and diluted to an OD of 0.1 at 600 nm. These were spread (0.1 ml) on BSM plates and allowed to dry for 1 hr. Suspensions of each trifolitoxin-producing strain were prepared in water and diluted to an OD of 0.5 at 600 nm. Five

Table 3. Restoration of nodulation competitiveness by insertion of pTFX1 into T24::Tn5₁

Treatment(s)	Shoots, mg (fresh weight)	Nitrogenase activity of intact plants, nmol of C ₂ H ₄ per hr per plant
TA1/T24::Tn5 ₁	89.8 ± 9.6 ^a	3.2 ± 0.4 ^a
TA1	81.7 ± 12.8 ^a	3.8 ± 0.8 ^a
TA1/T24	15.2 ± 5.0 ^b	0.0 ± 0.0 ^b
Uninoculated	9.3 ± 0.7 ^b	0.0 ± 0.0 ^b
T24	8.6 ± 1.2 ^b	0.0 ± 0.0 ^b
TA1/T24::Tn5 ₁ (pTFX1)	8.7 ± 1.4 ^b	0.0 ± 0.0 ^b
T24::Tn5 ₁ (pTFX1)	8.5 ± 0.8 ^b	0.0 ± 0.0 ^b

Values represent the mean ± SEM of five replicates. Treatments followed by the same letter were not statistically different at the 1% level as determined by the Duncan's multiple range test.

Table 4. Sensitivity of various *Rhizobiaceae* strains to trifolitoxin produced by T24 and five recombinant *Rhizobiaceae* strains with pTFX1, including *R. leguminosarum* bv. *trifolii* T24::Tn5₁(pTFX1), *R. leguminosarum* bv. *trifolii* TA1, *R. leguminosarum* bv. *viciae* B518, *R. leguminosarum* bv. *phaseoli* 127K90, *R. meliloti* 102F3, and *A. tumefaciens* C58

Test strain	Trifolitoxin sensitivity, area of the zone of inhibition (cm ²)					
	T24	T24::Tn5 ₁	TA1	B518	102F3	C58
<i>R. leguminosarum</i>						
bv. <i>trifolii</i>						
T24	0.0	0.0	0.0	0.0	0.0	0.0
2046	5.5	14.3	7.5	5.9	8.8	7.9
TA1	5.8	8.3	3.5	1.8	3.6	2.9
bv. <i>viciae</i>						
128C84	7.4	17.8	9.2	8.8	12.4	7.8
128C41	6.4	12.3	9.4	7.9	10.0	7.3
128A1	4.0	16.4	9.0	9.4	11.2	8.4
128C78	2.9	10.0	6.1	4.8	4.8	7.3
128A12	10.0	16.4	11.8	10.0	11.1	9.6
128C15	5.5	6.1	5.5	7.4	3.5	12.4
128C1	10.6	23.0	13.4	11.0	15.5	7.9
3960	0.0	0.0	0.0	0.0	0.0	0.0
B518	3.6	6.0	2.9	1.5	4.3	3.2
bv. <i>phaseoli</i>						
127K115	2.6	9.4	5.2	2.9	5.5	6.9
127K60	9.4	19.5	11.6	9.2	13.9	7.9
127K42	5.1	15.5	8.2	5.5	10.3	4.8
127K30	4.7	8.4	6.3	4.0	5.1	2.6
127K8	2.3	8.3	3.8	2.6	3.5	3.3
127K12b	8.4	11.1	8.4	5.0	7.9	5.3
127K16	6.4	23.3	5.5	6.0	7.5	5.1
127K90	0.4	15.7	11.3	6.4	8.3	7.2
127K117	3.6	12.4	7.4	7.9	8.9	4.7
<i>R. fredii</i> 205	9.4	17.9	9.7	9.6	15.3	7.3
<i>R. meliloti</i>						
102F51a	0.0	0.0	0.0	0.0	0.0	0.0
102F34a	0.0	0.0	0.0	0.0	0.0	0.0
102F97	3.6	2.9	0.0	0.0	0.0	0.0
102F92	0.0	0.0	0.0	0.0	0.0	0.0
102D6	0.0	0.0	0.0	0.0	0.0	0.0
102F3	0.0	0.7	0.0	0.0	0.0	0.0
<i>B. japonicum</i>						
USDA 110	0.0	0.0	0.0	0.0	0.0	0.0
USDA 83	0.0	0.0	0.0	0.0	0.0	0.0
SM31	0.0	5.3	0.0	0.0	0.5	0.0
<i>A. tumefaciens</i>						
C58	0.0	0.0	0.0	0.0	0.0	0.0
B6	0.0	0.0	0.0	0.0	0.0	0.0
Ag63	0.0	0.0	0.0	0.0	0.0	0.0
<i>A. rhizogenes</i> A2	0.0	0.0	1.0	0.0	0.0	0.0
<i>A. radiobacter</i> K84	0.0	0.0	0.0	0.0	0.0	0.0

Strains possessing pTFX1 are T24::Tn5₁, TA1, B518, 102F3, and C58.

microliters of each suspension was placed in the center of a dried BSM plate. After 48 hr at 28°C, zones of inhibition were measured.

Preparation of Cosmid Library of T24. Total genomic DNA was prepared from a Nod⁻ Tn5 mutant of T24 as described by Marmur (23). A partial *Sau*3A digest of T24::Tn5₄ DNA was prepared and ligated to the pLAFR3 vector as described by Bender and Cooksey (19). T24::Tn5₄, the Nod⁻ mutant of T24, was used to prepare the genomic library, because this strain has a Tn5 marker outside the region of the trifolitoxin genes. With a library of this strain, the frequency of genes represented in the library was easily determined by replica plating onto kanamycin-containing medium. The resulting

recombinant pLAFR3 cosmid clones were mobilized into *Rhizobium* strains with *E. coli* HB101 carrying the helper plasmid pRK2013. Suspensions of the donor, recipient, and helper strains in water were prepared with an OD of 0.1 at 600 nm and mixed in a 1:1:1 (vol/vol) ratio, and 10- μ l portions were spotted on a YM/KB plate containing 3% (wt/vol) agar. After 48 hr at 28°C, the cells were suspended in 1 ml of water and spread in 0.1-ml aliquots on BSM agar plates containing tetracycline at 12.5 μ g/ml and streptomycin at 50 μ g/ml. The recombinant plasmid that possessed genes for trifolitoxin production and resistance is referred to as pTFX1.

Transfer of pTFX1 to Strains of *Rhizobiaceae*. Conjugations were done as described above to transfer pTFX1 to several recipient strains of *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium*. Transconjugants were selected by spreading cells on BSM plates with tetracycline at 50 μ g/ml.

DNA Hybridizations and Plasmid Visualization. Biotinylated probes of pTFX1 and pGS9 were prepared by nick-translation and were hybridized to DNA on agarose gels with kits purchased from and used as directed by Bethesda Research Laboratories. Low-stringency conditions were used for Southern blot analysis. Plasmid visualization was done by the in-well lysis method of Plazinski *et al.* (24).

Time Course of Trifolitoxin Production. Flasks containing 250 ml of BSM broth were inoculated with 25 μ l of a suspension of a trifolitoxin-producing strain with an OD of 1.0 at 600 nm. Tetracycline was added to a final concentration of 12.5 μ g/ml to each flask inoculated with a recombinant strain possessing pTFX1. At 24-hr intervals following inoculation, a portion of each culture ($\approx$ 4 ml) was removed from each flask. One milliliter of the sample was used to determine the OD at 600 nm. Serial dilutions of 0.1 ml of each sample were prepared and cultured on BSM plates to determine the number of colony-forming units. Tetracycline (12.5 μ g/ml) was added to each plate for the recombinant strains. Another portion of the sample ($\approx$ 3 ml) was filter-sterilized and assayed for trifolitoxin production as described above. The tetracycline-resistant derivative of 2046 was used as the trifolitoxin-sensitive strain in all of the trifolitoxin bioassays in this experiment.

Partial Purification of Trifolitoxin. Three flasks, each containing 500 ml of BSM broth, were inoculated with 1 ml of a suspension of T24, T24::Tn5₁(pTFX1), and *Rhizobium meliloti* 102F3(pTFX1). After 3 days of growth on a shaker at 28°C, the cells were harvested by centrifugation and discarded. The supernatant of each culture was placed on a chromatographic column containing 100 g of reverse-phase C₁₈ resin. A step gradient of 0 and 80% (vol/vol) methanol in water was applied to the column. Each fraction was assayed for trifolitoxin activity. Each fraction was evaporated to dryness to remove the methanol and resuspended in 10 ml of water and was assayed for trifolitoxin activity. The most active fraction was placed on a DEAE-Sephadex column equilibrated with water at pH7, and a step gradient of 0.0 and 1.0 M ammonium acetate was applied. Each fraction was lyophilized to remove the ammonium acetate, resuspended in 5 ml of water, and assayed for trifolitoxin activity. The most active fraction then was filter-sterilized and assayed against several strains in the *Rhizobiaceae*.

Insert Size of DNA in pTFX1. A complete digest of pTFX1 was prepared by incubating 1 μ g of pTFX1 DNA with 3 units of *Pst* I for 1 hr at 37°C. Following electrophoresis of the digest on a 0.9% agarose gel, the size of the *Pst* I fragments was determined by using *Hind*III fragments of λ phage DNA as molecular size markers.

RESULTS

Complementation of Trifolitoxin Production in Three Trifolitoxin-Minus Mutants by a Cosmid Clone from a T24

Library. Among the 1213 cosmid clones in the genomic library of T24::Tn5₄, 8 cosmid clones were found to be kanamycin-resistant—i.e., a gene frequency in the library of 0.7%. None of the *E. coli* cosmid clones in the library were capable of trifolitoxin production. Three hundred cosmid clones from this library were mated into a transposon mutant of T24 lacking trifolitoxin production. Among these, 1 cosmid clone restored trifolitoxin production in the trifolitoxin-minus mutant. Subsequent matings showed that this cosmid clone, referred to as pTFX1, also was capable of restoring trifolitoxin production in two other trifolitoxin-minus mutants of T24.

Overproduction of Trifolitoxin by the Complemented Mutants. The trifolitoxin-minus mutants of T24 that were complemented with pTFX1 produced larger zones of inhibition than wild-type T24 when tested in the trifolitoxin bioassay against the trifolitoxin-sensitive strain *R. leguminosarum* bv. *trifolii* 2046. The amount of trifolitoxin overproduction was measured by determining the area of the zone of inhibition during the growth of cultures of T24, T24::Tn5₁(pTFX1), T24::Tn5₄, and T24::Tn5₄(pTFX1). The two T24 mutants containing pTFX1 produced more trifolitoxin than did wild-type T24 or T24::Tn5₄ (Fig. 1).

Complementation of the Nodulation Competitiveness Phenotype. The recombinant plasmid pTFX1 restored nodulation competitiveness to a trifolitoxin-minus mutant of T24. Clover plants co-inoculated with the effective strain TA1 and either of the trifolitoxin-producing strains, T24 or T24::Tn5₁(pTFX1), were small and chlorotic with low symbiotic nitrogenase activities (Table 3). In contrast, plants co-inoculated with TA1 and the trifolitoxin-minus mutant of T24 T24::Tn5₁ were large and green with high symbiotic nitrogenase activities (Table 3). Preliminary experiments have shown that with this assay clover nodules are occupied exclusively by the ineffective strain when the plants are small and chlorotic (15). Thus, each trifolitoxin-producing strain was capable of preventing nodulation by the trifolitoxin-sensitive effective strain TA1. Without trifolitoxin production, the ineffective strain was incapable of preventing nodulation by the effective strain.

Table 5. Degree of sensitivity of various *Rhizobiaceae* strains to the partially purified trifolitoxin produced by T24, Tn5₁(pTFX1), and 102F3(pTFX1)

Test strain	Trifolitoxin sensitivity, area of the zone of inhibition (cm ²)		
	T24	T24::Tn5 ₁ (pTFX1)	102F3(pTFX1)
<i>R. leguminosarum</i>			
bv. <i>trifolii</i>			
TA1	19.9	14.7	12.7
2046	4.0	5.7	2.7
bv. <i>viciae</i>			
B518	2.3	8.1	5.7
3960	1.3	3.3	4.0
bv. <i>phaseoli</i> 127K117	6.1	6.6	6.6
<i>R. fredii</i> 205	10.9	17.6	16.9
<i>R. meliloti</i>			
102F3	0.6	11.5	10.3
102F97	1.0	6.6	6.1
<i>B. japonicum</i> USDA 110	0	0	0
<i>A. tumefaciens</i> C58	0	0	0

Transfer of Trifolitoxin Production to Effective Strains of *Rhizobium*. To determine whether pTFX1 contained all of the genes for trifolitoxin production, pTFX1 was transferred by conjugation into six strains of *Rhizobium* and one strain of *Agrobacterium*. Following conjugal transfer of pTFX1 each of the following strains expressed trifolitoxin production: *R. leguminosarum* bv. *viciae* strains 3960 and B518, *R. leguminosarum* bv. *trifolii* strain TA1, *R. leguminosarum* bv. *phaseoli* strain 127K90, *R. meliloti* 102F3, *R. meliloti* 102F97, and *Agrobacterium tumefaciens* C58. None of these strains exhibited anti-rhizobial activity prior to transfer of pTFX1. The transconjugants of these matings were all capable of trifolitoxin production. No expression of trifolitoxin production was observed following transfer of pTFX1 into *R. sp.* (cowpea) P132 and *Bradyrhizobium japonicum* USDA 110.

Transfer of Trifolitoxin-Resistance Function to *Rhizobium* Strains. All of the recombinant strains of *Rhizobium* containing pTFX1 produced trifolitoxin regardless of the sensitivity of these strains prior to the transfer of pTFX1. In addition, the growth of T24 was not inhibited in the presence of any strain containing pTFX1 (Table 4).

Effectiveness of a Strain of *R. leguminosarum* bv. *viciae* with pTFX1. The ability of *R. leguminosarum* bv. *viciae* B518 to express nitrogenase activity in vetch nodules following introduction of pTFX1 was determined. Nodulated roots of vetch plants inoculated with B518 or B518(pTFX1) had nitrogenase activities of 5.2 and 5.5 nmol of C₂H₄ per hr per plant, respectively. These values were not statistically different at the 1% level of confidence. The rhizobia isolated from nodules of plants inoculated with B518(pTFX1) were resistant to tetracycline indicating that the pTFX1 plasmid was maintained in the strain during the infection process. To date, no other strains of *Rhizobium* with pTFX1 have been tested for symbiotic nitrogenase activity.

Comparison of the Anti-Rhizobial Activity of T24 with That of pTFX1-Containing Strains. The capacity of five pTFX1-containing strains to inhibit a broad range of strains within the *Rhizobiaceae* was examined and compared to the range of strains inhibited by T24. None of these strains possessed anti-rhizobial activity in the absence of pTFX1. Each pTFX1-containing strain and wild-type T24 inhibited most strains of *R. leguminosarum* bvs. *trifolii*, *viciae*, and *phaseoli* as well as *R. fredii* (Table 4). Little or no inhibition of strains of *R. meliloti*, *B. japonicum*, or *A. tumefaciens* was observed with any pTFX1-containing strain or with T24 (Table 4).

Partial Purification of Trifolitoxin Activity from Cell Culture Supernatants of T24, T24::Tn5₁(pTFX1), and *R. meliloti*

FIG. 2. Ethidium bromide stain (lane A) and Southern blot hybridization with a biotinylated pTFX1 probe (lane B) of a plasmid profile of T24. The size in kDa of each indigenous T24 plasmid is shown.

102F3. The anti-rhizobial activity was partially purified from cell culture supernatants of wild-type T24 and two strains containing pTFX1. The anti-rhizobial activity from each culture absorbed to the reverse-phase column and was eluted with 20% (vol/vol) methanol. The active fractions after reverse-phase chromatography were then placed on an anion-exchange column. The anti-rhizobial activity from each culture did not bind to the anion-exchange resin.

Following these chromatographic steps, the active fractions then were assayed for trifolitoxin activity. Each partially purified preparation inhibited strains of *Rhizobium* but not *Agrobacterium* or *Bradyrhizobium* (Table 5). None of the preparations inhibited *E. coli*.

Localization of Trifolitoxin Genes. A biotinylated probe of pTFX1 was prepared and used to determine whether the trifolitoxin genes are plasmid-borne. Southern blot analysis of a plasmid profile of T24 did not show hybridization with any of the four indigenous plasmids of T24, even under low-stringency conditions (Fig. 2). Chromosomal DNA of T24 did hybridize with the pTFX1 probe, however. The pTFX1 probe also did not hybridize to either chromosomal or plasmid DNA of either *R. leguminosarum* bv. *trifolii* TA1 or *R. leguminosarum* bv. *viciae* 3960. A biotinylated probe of pGS9 was prepared for hybridization of Tn5. This probe hybridized to the chromosomal DNA of the trifolitoxin-minus, Tn5 mutants of T24, but no plasmid hybridization was detected (data not shown). The pGS9 probe did hybridize to the Sym plasmid of *R. leguminosarum* bv. *viciae* 3960 (data not shown), which possesses a Tn5 insertion in that large plasmid (25).

Size of the T24 Insert DNA in pTFX1. A complete digest of pTFX1 with *Pst* I showed that the insert size of pTFX1 was 24.2 kilobases.

DISCUSSION

The capacity of pTFX1 to restore trifolitoxin production and nodulation competitiveness in trifolitoxin-minus mutants of T24 is further evidence that trifolitoxin production plays an important role in the expression of nodulation competitiveness by T24. Other evidence in support of this view includes the observations that trifolitoxin-minus transposon mutants of T24 lack the competitiveness phenotype (15) and that the degree of sensitivity of *Rhizobium* strains to trifolitoxin is correlated with the extent to which T24 can regulate their nodule occupancy (17).

The pTFX1 cosmid appears to code for the production of the same anti-rhizobial compound that is produced by T24. The anti-rhizobial compounds produced by T24, T24::Tn5₁(pTFX1), and *R. meliloti* 102F3(pTFX1) seem to be similar chemically, as they co-chromatographed on both reverse-phase and anion-exchange columns. The anti-rhizobial compounds from each source also had similar biological activities; that is, each inhibited the growth of

Rhizobium strains with no effect on *Agrobacterium*, *Bradyrhizobium*, or *E. coli* strains.

The experiments described here further support the hypothesis that trifolitoxin production by T24 is unrelated to that strain's inability to fix nitrogen. The transfer of pTFX1 to an effective strain of *R. leguminosarum* bv. *viciae* did not decrease that strain's ability to reduce acetylene in vetch nodules, although the strain was capable of producing trifolitoxin. Also, a mutation in the trifolitoxin production genes does not alter the ineffectiveness of T24 on clover (15).

Both trifolitoxin-sensitive and -resistant strains of *Rhizobium* produced trifolitoxin and became resistant to inhibition by T24 following transfer of pTFX1. This suggests that pTFX1 contains all of the genes necessary for both trifolitoxin production and resistance. Hence, in *R. leguminosarum* bv. *trifolii* T24, the trifolitoxin production and resistance genes are linked in a region no larger than 24.2 kilobases. The size of the insert DNA in pTFX1 is large enough to code for many genes. Linkage of antibiotic production and resistance genes is common in bacteria. One example is in *Agrobacterium radiobacter* K84 in which all of the genes necessary for agrocin 84 production and resistance are linked on a 47.7-kilobase plasmid (25).

There is no evidence to suggest that the trifolitoxin genes are plasmid-borne. A biotinylated probe of pTFX1 does not hybridize to any of the indigenous plasmids of T24 even under low-stringency conditions (Fig. 2). Chromosomal localization of the trifolitoxin genes is consistent with the observation that all attempts to conjugate the trifolitoxin phenotype from wild-type T24 into other *Rhizobium* strains have failed (unpublished data).

Following transfer of pTFX1, expression of trifolitoxin production was observed only in species closely related to *R. leguminosarum* bv. *trifolii* T24 such as *R. leguminosarum*, *R. meliloti*, and *A. tumefaciens*. The pTFX1 cosmid did not confer expression of trifolitoxin in *Bradyrhizobium*, *R. sp.* (cowpea), or *E. coli*. Elucidation of the cause of this narrow range of expression may require a thorough analysis of the trifolitoxin genes as well as knowledge of the structure and mode of action of trifolitoxin. Similarly, it is not known how the transfer of pTFX1 to a T24 derivative can cause overexpression of the anti-rhizobial compound. A simple explanation is that two copies of trifolitoxin genes in a *Rhizobium* strain can induce overproduction.

The trifolitoxin genes were transferred to two strains of *R. leguminosarum* bv. *viciae*, 3960 and B518, that possess the symbiotic plasmid pIJ1008. This symbiotic plasmid has been shown to increase nitrogen fixation when transferred to various genetic backgrounds (26). However, the competitiveness of these pTFX1-containing strains has not been tested, because cosmid clones in *Rhizobium* and *Bradyrhizobium* are known to be unstable in the absence of selection pressure (27–29). Perhaps the transfer of trifolitoxin genes to the chromosomal DNA of 3960 and B518 will improve the nodulation competitiveness of these two superior strains.

The cloning of competitiveness genes described here differs significantly from that described by Dowling *et al.* (16). (i) The basis of the competitiveness expressed by T24 is known to be the result of the production of an anti-rhizobial compound (15, 17), whereas the mechanism of the nodulation blocking expressed by PF₂ is not known. (ii) No strain was developed by Dowling *et al.* (16) that possesses both the nodulation blocking genes and the ability to nodulate Afghanistan peas. Conversely, transfer of trifolitoxin genes to strains of *Rhizobium* has no effect on the ability of the recipient strains to nodulate or express nitrogenase activity.

(iii) The blocking factor genes are only useful in strains of *R. leguminosarum* bv. *viciae* that can infect the Afghanistan variety of pea. Conversely, the trifolitoxin genes can be transferred to and expressed in several species of *Rhizobium* and thus may be useful as a solution to the *Rhizobium* competition problem for a wide variety of leguminous crops.

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