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A Newly Discovered Gene, *tfuA*, Involved in the Production of the Ribosomally Synthesized Peptide Antibiotic Trifolitoxin

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Trifolitoxin (TFX) is a gene-encoded, posttranslationally modified peptide antibiotic. Previously, we have shown that *tfxABCDEFG* from *Rhizobium leguminosarum* bv. trifolii T24 is sufficient to confer TFX production and resistance to nonproducing strains within a distinct taxonomic group of the α-proteobacteria (E. W. Triplett, B. T. Breil, and G. A. Splitter, Appl. Environ. Microbiol. 60:4163–4166, 1994). Here we describe strain Tn5-2, a Tn*5* mutant of T24 defective in the production of TFX, whose insertion maps outside of the *tfx* cluster. It is not altered in growth compared with T24, nor does it inactivate TFX in its proximity. The wild-type analog of the mutated region of Tn5-2 was cloned. Sequencing, transcriptional fusion mutagenesis, and subcloning were used to identify *tfuA*, a gene involved in TFX production. On the basis of computer analysis, the putative TfuA protein has a mass of 72.9 kDa and includes a peroxidase motif but no transmembrane domains. TFX production studies show that extra copies of the *tfxABCDEFG* fragment increase TFX production in a T24 background while additional copies of *tfuA* do not. Lysate ribonuclease protection assays suggest that *tfuA* does not regulate transcription of *tfxA*. Upstream of *tfuA* are two open reading frames (ORFs). The putative product of ORF1 shows high similarity to the LysR family of transcriptional regulators. The putative product of ORF2 shows high similarity to the cytosine deaminase (CodA) of *Escherichia coli.*

Trifolitoxin (TFX) is a ribosomally synthesized, posttranslationally modified peptide antibiotic produced by *Rhizobium leguminosarum* bv. trifolii T24 (4, 29). A number of features make TFX an interesting antibiotic for study. Its spectrum of activity is quite narrow but includes bacteria that are plant symbionts and plant and animal pathogens (29, 34, 35). TFX has already been shown to limit nodules formed by TFX-sensitive strains (32–34) and so may provide a solution to the *Rhizobium* competition problem (9, 36). This problem arises when inoculant strains are not competitive for legume root nodulation against strains indigenous to soil. Its ribosomal synthesis makes it an easy substrate for drug modification (12, 16, 17, 27). Though the structure of TFX has not been completely elucidated, it is known to contain a thiazoline ring and another cyclic chromophore (23). The latter appears to be a novel, pyrimidine-like structure (unpublished results). It may be possible to use the enzymes that modify TFX to modify different substrates, thereby creating new molecules. EpiD from the epidermin system has been used to catalyze the oxidative decarboxylation of heptapeptides (21), thereby showing the very real possibility of using antibiotic posttranslational modification enzymes to catalyze reactions on molecules other than their natural preantibiotic target. Given the TFX system's agricultural, medical, and biochemical potential, elucidation of the genetics of TFX production is important.

A number of genes have been shown to be required for TFX production and resistance. TfxA is a prepeptide that contains the TFX backbone at the carboxy terminus preceded by a basic leader (4) that shares structural similarity to the leaders of other ribosomally synthesized peptide antibiotics (16, 17, 20, 27). Directly downstream of *tfxA* lies *tfxBCDEFG* (4). Evidence to date suggests that *tfxG* and possibly *tfxE* are involved in TFX resistance (4). The other genes are likely involved in the posttranslational modification and export of the peptide backbone and TFX export (4).

The gene organization of other ribosomally synthesized, posttranslationally modified peptides includes a gene cluster(s) located adjacent to the gene encoding the peptide backbone (16, 17, 20, 27). Previously, we have shown that the conjugation of plasmid-borne *tfxABCDEFG* into several genera of α-proteobacteria confers TFX production and resistance to the host bacteria (4, 35, 37). It is possible, however, that the heterologous hosts supply some of the necessary functions for TFX production and resistance and that other genes are involved in some aspect of the TFX system. In the systems of several other ribosomally synthesized, posttranslationally modified antibiotics, genes involved in the production of the antibiotic have been found that lie distal to the structural gene cluster (17, 20).

Strain Tn5-2 is a Tn*5*-generated mutant of T24 that fails to produce TFX. In preliminary studies, marker exchange was used to show that the mutation is causative, and Southern blotting revealed that the insertion is not within *tfxABCDEFG*. To continue the search for genes involved in TFX production, the region defined by the insertion in Tn5-2 was cloned and analyzed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and plasmids used in this study are described in Table 1. *Rhizobium* strains were maintained on Bergersen's synthetic minimal (BSM) medium (2) at 28°C, while *Escherichia coli* strains were cultured on Luria-Bertani medium (28) at 37°C. TFX assays were performed in BSM medium. Noble agar was used to solidify media used to interrupt conjugations and for TFX bioassays. Antibiotics were used in the following concentrations: 50 μg/ml, ampicillin (Ap); 34 μg/ml, chloramphenicol; 50 μg/ml, kanamycin (Km) for *E. coli*; 15 μg/ml, Km for *R. leguminosarum*; 20 μg/ml, nalidixic acid (nal); 15 μg/ml, tetracycline (Tc) for *E. coli*; 1.25 μg/ml, Tc for *R. leguminosarum*; 50 μg/ml, spectinomycin; and 50 μg/ml, streptomycin (Sm). The *bla* gene does not confer Ap resistance in the *Rhizobium* strains used here, so only Km was used to maintain the Tn*3*GUS cassette in these strains. Sm alone was frequently used to maintain the transposon in T24::Tn*5* derivatives. The substrates for β-glucuronidase, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Bacteria		
Rhizobium leguminosarum bv. trifolii		
ANU794	TFX^s, Sm^r derivative of TA1	5
T24	TFX$^+$, wild type	29
Tn5-1	TFX$^-$, T24(*tfxB*::Tn*5*)	34, this work
Tn5-2	TFX$^-$, T24(*tfuA*::Tn*5*)	34, this work
Tn5-3	TFX$^-$, T24(*tfxC*::Tn*5*)	34, this work
Tn5-4	TFX$^+$, T24(?::Tn*5*)	34
E. coli		
C2110nal	nal^r derivative of C2110, *polA*	8
DH5α	nal^r, *recA*	Bethesda Research Lab.
XL1-Blue	Tc^r host for single-stranded rescue	Stratagene
Plasmids		
pBluescriptII KS+	cloning and sequencing vector, Ap^r	Stratagene
pBEETn526	pBluescriptII KS+::Tn5-2 *Eco*RI Tn*5* fragment	This work
pBEC631	pBluescriptII KS+::5.7-kb *Eco*RI fragment of pC6	This work
pBEC639	pBluescriptII KS+::5.7-kb *Eco*RI fragment of pC6	This work
pBEHTn526	pBluescriptII KS+::.8-kb *Hpa*I fragment of pBEETn526	This work
pBSC62	pBluescriptII KS+::6-kb *Sst*I fragment of pC6	This work
pDSK519	broad-host-range vector, Km^r	19
pDTXC-12	pDSK519::*tfxB′CDEFG* (confers TFX resistance)	35
pLAFR3	broad-host-range vector, Tc^r	31
pC6	pLAFR3::36-kb Tn5-4 fragment that complements Tn5-2	This work
pRBC695	pRK415::6-kb *Eco*RI fragment of pC6	This work
pRBC696	pRK415::6-kb *Eco*RI fragment of pC6	This work
pRK2013	ColE1 mobilization helper (Tra$^+$, Mob$^+$), Km^r	10
pRK415	broad-host-range vector, Tc^r	19
pRT42	pRK415::*tfxABCDEFG* (confers TFX production and resistance), *tfx* genes in the opposite orientation from P_{Lac}	This work
pRtfuA1	pRK415::2.7-kb *Sac*II-*Hpa*I fragment of pBEC639 (*tfuA*), *tfuA* is in the same orientation as P_{lac}	This work
pRtfuA2	pRK415::2.7-kb *Sac*II-*Hpa*I fragment of pBEC639 (*tfuA*), *tfuA* is in the opposite orientation from P_{Lac}	This work
pTFX24	pBluescriptII KS+::7.1-kb *Mlu*I tfxABCDEFG fragment	4

[a] TFX$^-$, TFX nonproducing; TFX$^+$, TFX producing; TFX^s, TFX sensitive; Tra, transfer; Mob, mobilization.

(X-glc), and for cytosine deaminase, 5-fluorocytosine, were used at concentrations of 50 μg/ml and 20 μg/ml, respectively.

Growth curves and TFX production. T24 and Tn5-2 were grown on BSM plates, washed three times, and resuspended and adjusted to an optical density at 600 nm (OD_{600}) of 0.01 in BSM medium. Cultures were divided into five 50-ml cultures in 250-ml bottles and incubated at 28°C on a rotary shaker at 250 rpm. At time zero and at 12-h intervals, 500 μl was removed from each culture and mixed in 500 μl of 30% glycerol. Also, 1 ml from each culture was passed through a 0.2-μm-pore-size filter. Both filtrate and cells were stored at −80°C prior to analysis. Cell numbers were obtained by dilution plating. All TFX samples were analyzed simultaneously using the bioassay described below. Strain ANU794 (5) was used as the TFX-sensitive tester strain.

Coculture experiment. Cells were grown, washed twice, adjusted to a final OD_{600} of 0.5 in 15% glycerol, and stored at −80°C until used. Cell suspensions were mixed 1:1. Five microliters of each individual culture or 10 μl of each mixture was spotted on BSM plates. After incubating for 2 days at 28°C, plates were sprayed with a 0.1 OD_{600} suspension of ANU794. Zones of inhibition were recorded after 4 days. The mean zone area of two replicates was calculated after subtracting the area of the spot.

DNA biochemistry and transfer. All DNA isolations and manipulations were performed as described by Sambrook et al. (28) except where otherwise indicated. Kits and supplies were used according to the manufacturer's instructions. DNA fragments used for cloning were isolated from SeaPlaque (FMC BioProducts, Rockland, Maine) agarose gels by using β-agarase (New England Biolabs, Beverly, Mass.). Cohesive-end ligations were performed with T4 ligase. Blunt-end ligations were performed with the TaKaRa DNA Blunting Kit (TaKaRa Biochemicals Inc., Berkeley, Calif.), which uses T4 polymerase to blunt DNA overhangs. The helper phage VCS-M13 (Stratagene, La Jolla, Calif.) was used for the generation of single-stranded pBEC631 and pBEC639 DNA from the *E. coli* host XL1-Blue. PCR was performed in capillary tubes as described by Rumjanek et al. (26). Southern blots were performed with the ECL Direct Nucleic Acid Labeling and Detection System (Amersham Life Science, Little Chalfont, England). DNA sequencing was primarily performed by the University of Florida DNA Sequencing Core Laboratory. Reactions were performed by using the ABI sequencing kit (Perkin-Elmer, Foster City, Calif.). Some sequencing was performed with the USB Sequenase 2.0 kit (U.S. Biochemical Corp., Cleveland, Ohio). For the sequences cited in GenBank accession numbers U39409 and U31074, the sequence of both strands of DNA was determined.

Transformations were performed as described by Inoue et al. (15). Triparental conjugations were performed with pRK2013 as the helper plasmid (4, 10).

Mapping the Tn*5* insertions in Tn5-1 and Tn5-3. Southern analysis was used to map the Tn*5* insertion to a 3.6-kb *Eco*RI fragment within the 7.1-kb *Mlu*I fragment from T24 which contains *tfxABCDEFG*. PCRs were performed on genomic templates of Tn5-1 and Tn5-3 by using a primer that reads out of Tn*5* (AGGAGGTCACATGGAAG) paired with various primers designed to the 3.6-kb *Eco*RI TFX fragment. TFX-Tn*5* primer pairs were found that would yield fragments of 300 to 700 bases which were used as sequencing templates. An internal TFX primer was used to sequence into the Tn*5* transposon. The position of the insertion was determined by comparing this sequence with those of Tn*5* and the *tfx* region (4).

Cloning. All clones were transformed and maintained in DH5α. To clone the Tn*5* insert site of Tn5-2, total Tn5-2 DNA was digested with *Eco*RI, an enzyme that does not cleave Tn*5*, and ligated into the *Eco*RI site of pBluescriptII KS+. The ligation products were transformed into DH5α and the appropriate clone (pBEETn526) was found by selection with Ap and Km. A 0.8-kb *Hpa*I fragment of pBEETn526 was blunt-end ligated into the *Sma*I site of pBluescriptII KS+ to yield pBHTn526. This clone contains 0.2 kb of Tn*5* and 0.6 kb of T24 DNA from the insertion site of Tn5-2. This 0.6-kb fragment was sequenced. Primers were designed to its ends so it could be amplified and labeled for use as a probe to identify the wild-type region interrupted by Tn*5* in Tn5-2.

To isolate the clone that complements Tn5-2, a pLAFR3 (31) library of Tn5-4 DNA (34) was conjugated in groups of 12 into Tn5-2 and the transconjugants

were assayed for TFX production. Where zones of inhibition were found, clones were conjugated individually and the transconjugants were reassayed for TFX activity. Only one clone (pC6) gave transconjugants that produced large zones of inhibition, and this was also the only clone that hybridized with the 0.6-kb Tn*5* insertion site of Tn5-2. Plasmid pC6 contains an insertion of about 36 kb. A 5.7-kb *Eco*RI fragment of pC6 hybridized to the 0.6-kb insertion site and was ligated in both orientions into the *Eco*RI site of pBluescriptII KS+ to yield pBEC631 and pBEC639. The 5.7-kb *Eco*RI fragment of pBC6E39 was subsequently ligated in both orientations into the *Eco*RI site of pRK415 to yield pRBC695 and pRBC696. Both clones complement the mutation in Tn5-2. To subclone *tfuA*, the 2.7-kb *Sac*II-*Hpa*I fragment of pBEC639 was blunt-end ligated in both orientations into pRK415 digested with *Pst*I and *Eco*RI. The resulting plasmids are pRtfulA1 and pRtfuA2.

To subclone the region upstream of *tfuA*, a 6-kb *Sst*I fragment of pC6 containing part of *tfuA* and about 4.5 kb upstream of it was cloned into the *Sst*I site of pBluescriptII KS+ to yield pBSC62. Plasmid pRT42 was constructed by blunt-end ligation. Vector pRK415 was digested with *Pst*I and *Eco*RI and ligated to the 7.2-kb *Bss*HII *tfxABCDEFG* fragment of pTFX24 (4).

TFX and β-glucuronidase assays. In screens to determine TFX production, 5 μl of a suspension of the strain to be tested for TFX production was placed on the center of a BSM agar plate, permitted to grow 2 days at 28°C, and was then sprayed with a suspension of strain ANU794. After 3 days, plates were examined for the presence of zones of growth inhibition.

Filter-sterilized culture filtrates were similarly assayed. Cores of 6-mm diameter were bored in the agar of plates filled 2 days earlier with 18 ml of BSM agar medium. The filtrate was placed in the core and allowed to dry for roughly 6 h. A cell suspension of a tester strain was then sprayed on the plates. After 3 days of growth at 28°C, the diameters of the zones of inhibition were measured. The area of each zone was calculated, which included subtracting the area of the core.

Presence of *uidA*-encoded β-glucuronidase (GUS) activity was determined by addition of X-glc to agar plates (18).

TFX production in liquid culture. Cell numbers of cell stocks stored at −80°C in 15% glycerol were determined by dilution plating. Cell number was adjusted to 10^9 CFU in 0.8 ml of 15% glycerol, and this was added to 5.2 ml of BSM. At time zero, five 1-ml aliquots per culture were transferred to 15-ml test tubes and were shaken at 275 rpm at 28°C. One hundred microliters of the remaining 1 ml was added to 100 μl of 30% glycerol and stored at −80°C, and 500 μl was filter sterilized through a 13-mm (0.2-μm-pore-size) filter and frozen at −80°C. After 5.5 h, fractions of each culture were stored in glycerol or filter sterilized and stored as described above. Cell number at time zero was determined by plating serial dilutions of cell suspensions on BSM (Sm, Tc). TFX production was determined by testing 30 μl of each filter-sterilized culture as described above. Two and six plates were used for the 0 h and 5.5 h replicates, respectively. Half the plates were sprayed with the TFX-sensitive strain ANU794(pDSK519) and the other half were sprayed with the TFX-resistant strain ANU794(pDTXC-12) by using cell suspensions adjusted to an OD_{600} of 0.1. The lack of zones produced when spraying with the resistant strain is evidence that the zones of inhibition of the sensitive strain are due to TFX and not to some other phenomenon. To quantify the amount of TFX produced, serial dilutions of a known quantity of purified TFX were added to plate wells as above. TFX was purified and quantified as described by Lethbridge (23). The amount of TFX produced per cell per assay was determined by comparing the area of the zone of inhibition to a semilog plot of TFX (nanograms) versus the area of the zone of inhibition and then dividing by the cell number determined at time zero. Replicates were averaged and the standard deviation was calculated.

Tn*3*GUS mutagenesis and analysis. Tn*3*GUS and Tn*3*GUS mutagenesis are described by Breil et al. (4). Briefly, the Tn*3*GUS cassette includes Km and Ap resistance genes and a promoterless *uidA* gene that is preceded by translational stops in all three frames and a ribosome binding site. Tn*3*GUS derivatives lack the Tn*3* transposase, so insertions are stable.

To facilitate DNA isolations, plasmid DNA was isolated from the pool of C2110nal(pRBC695::Tn*3*GUS) derivatives and transformed into DH5α. Tn*3*GUS insertion sites were precisely mapped by double-stranded DNA sequencing (4). Several pRBC695::Tn*3*GUS plasmids whose insertions fell outside of the vector were conjugated individually into strain Tn5-2 and assayed for TFX production and β-glucuronidase activity. Those derivatives listed in Table 2 were also screened to ensure that they had not undergone gross rearrangements in Tn5-2 by isolating total DNA and hybridizing the *Eco*RV-digested DNA with pRK415.

DNA sequence analysis. All computer sequence entry and analysis was performed by using the programs of the Genetics Computer Group, Inc. (GCG; Madison, Wis.), maintaining their defaults (1). Figures were constructed by using the Clone/Enhance programs from Scientific and Educational Software, Inc. (State Line, Pa.).

Lysate ribonuclease protection. Levels of *tfxA* mRNA from T24 and Tn5-2 were compared by using the Direct Protect Lysate Ribonuclease Protection Assay from Ambion (Austin, Tex.). Cells from both strains were harvested during exponential growth, washed twice, and resuspended in lysis solution. Cells (2.7×10^5 CFU) were hybridized with two radiolabeled antisense riboprobes (*tfxA* and 16S rRNA) in the same reaction, digested with RNase, resolved on a 5% polyacrylamide gel (8 M urea), and exposed to X-ray film. The intensities of the protected *tfxA* mRNA and 16S rRNA bands were obtained by densitometric scanning. For each strain, the intensity of the *tfxA* band was compared with the corresponding 16S band, the results of four replicate assays were averaged, and the standard deviation was calculated.

The ^{32}P-radiolabeled antisense probes were made by using the MAXIscript transcription kit from Ambion. Templates for the antisense probes were made by PCR with T24 DNA as the template. The PCR primers for the *tfxA* template are TFXForward, TGATGAGCTCCGAAGTGAAGAAGGGCTCCA, and TFXReverse, GGATCCTAATACGACTCACTATAGGGAGGAGCGACGCAGCCCTGACGGC. Those for the 16S rRNA template are 16SForward, TGATGAGCTCGGTCCATTACTGACGCTGAG, and 16SReverse, GGATCCTAATACGACTCACTATAGGGAGGAAGGGCTGGTAAGGTTCTGC. A transcriptional promoter was integrated into the PCR-amplified templates by flanking the reverse primers with the T7 phage promoter sequence (underlined).

Nucleotide sequence accession numbers. The 7,405-base sequence whose restriction map is shown in Fig. 1 has been assigned the GenBank accession number U39409, and the 16S rRNA sequence of T24 has been assigned accession number U31074.

RESULTS

Mapping the Tn*5* insertions in Tn5-1 and Tn5-3. In initial studies of the genetics of TFX production, *R. leguminosarum* bv. trifolii strain T24 was mutagenized with the transposon Tn*5* (34). Approximately 5,600 Tn*5* mutants were screened. Three were found that could no longer produce TFX on minimal medium (BSM-N), i.e., Tn5-1, Tn5-2, and Tn5-3 (34). Tn5-4 is a Tn*5* derivative of T24 which is unaffected in TFX production (34). When plasmids harboring the *tfxABCDEFG* fragment were conjugated into each of the T24::Tn*5* derivatives, we noticed that the zone of inhibition produced by the Tn5-2 derivative was smaller than those produced by the Tn5-1 and Tn5-3 derivatives (data not shown). Southern analysis showed a single Tn*5* insertion in each of Tn5-1, Tn5-2, Tn5-3, and Tn5-4. The Tn*5* insertion sites of Tn5-1 and Tn5-3 were within the 7.1-kb *Mlu*I fragment containing the *tfx* region, while the insertion within Tn5-2 was elsewhere (data not shown). Insertion sites in Tn5-1 and Tn5-3 were precisely mapped as described above. Tn*5* in Tn5-1 lies before base 1620 within *tfxB* and that of Tn5-3 lies before base 2963 within *tfxC*. Base numbers refer to the *tfx* region described in GenBank accession number L06719.

Tn5-2 growth and TFX production. Given that the Tn*5* insertion was not within any known *tfx* gene, we became interested in determining if this insertion defined a new TFX locus. Marker exchange was used to show that the insertion in Tn5-2 was causative of the TFX$^-$ phenotype (data not shown). To examine the possibility that Tn5-2 was just defective in growth, the growth and TFX production of T24 and Tn5-2 were studied and compared. No significant growth differences between T24 and Tn5-2 were seen over 72 h. TFX activity was detected from the T24 culture supernatants beginning at the 12-h time point. No TFX activity was found in the Tn5-2 culture supernatants at any time point during the 72-h growth period.

Coculturing experiment. To test if Tn5-2 secretes TFX but then inactivates it, we used a coculture experiment. T24 alone, or T24 mixed with either Tn5-1 or Tn5-2, was assayed as described above. The mean areas (± standard deviations) of the zones produced were as follows: T24, 36.8 ± 0.21 cm^2; T24 with Tn5-1, 36.5 ± 1.41 cm^2; and T24 with Tn5-2, 35.5 ± 0.07 cm^2. Also, when T24 was placed 0.75 or 1.6 cm from either Tn5-1 or Tn5-2, the inhibition zones of growth inhibition were not different.

Transposon mutagenesis and sequence analysis. A 5.7-kb *Eco*RI fragment of T24 DNA that complements Tn5-2 was identified as described above and sequenced in both directions. The nucleotide sequence of this fragment comprises bases 1392 to 7405 in the sequence designated as GenBank accession number U39409. By subcloning, sequencing, and comparison

TABLE 2. Characterization of Tn5-2(pRBC695::Tn3GUS) transconjugants

Tn3GUS derivative	Site[a]	TFX production[b]	X-glc[c]	ORF interrupted[d]
G6	1764	+	W→	ORF2
G5	2313	+	W←	ORF2
B5	2546	+	W→	
A9	3007	−	B→	*tfuA*
F7	3667	−	W←	*tfuA*
H6	4198	−	W←	*tfuA*
C5	4319	−	W←	*tfuA*
B7	4379	−	B→	*tfuA*
C7	4695	+	B→	
B6	5102	+	W←	ORF3
E8	5779	+	W←	ORF3
H8	6452	+	W←	ORF4
F8	6888	+	W←	ORF4
A8	6893	+	B→	

[a] Nucleotide position within the map of Fig. 1 before which the Tn3GUS mutagenesis cassette is inserted.
[b] +, complements the mutation in Tn5-2; −, does not complement the mutation in Tn5-2.
[c] Color of colonies growing on X-glc (blue [B] or white [W]) and the direction of the *uidA* gene where *tfuA* is read left to right.
[d] ORF in which the insert is located (see Fig. 1).

to U39409, we found that the Tn*5* insertion in Tn5-2 lies directly before position 4080.

A plasmid that harbors the 5.7-kb fragment, pRBC695, was mutagenized with the transcriptional GUS fusion cassette Tn*3*GUS. GUS insertion sites of 31 Tn*3*GUS derivatives were mapped by sequencing. A number of those whose insertions fall within the cloned fragment were conjugated into Tn5-2 and screened for TFX production and β-glucuronidase activity (Table 2). Descriptions of the open reading frames (ORFs) predicted on the basis of sequence analysis, transcriptional fusions, similarity to known proteins, and complementation of Tn5-2 are summarized in Table 3.

On the basis of the transposon mutagenesis results, we find that the region between positions 3007 and 4379 is involved in TFX production (Table 2). Only one ORF overlaps this region, and it has been designated *tfuA*. Its orientation of transcription is confirmed by the GUS mutants A9 and B7 since both of these have β-glucuronidase activity and the *uidA* genes are inserted in the same orientation as *tfuA* (Table 2). The putative methionine start codon of this ORF lies 10 bases downstream of a site that differs from the Shine-Dalgarno sequence (30) by 2 bases (Table 3).

TABLE 3. Description of ORFs in Fig. 1 and their putative translation products

ORF	RBS[a]	No. of bases[b]	ATG[c]	Stop[d]	No. of amino acids[e]	Mass (kDa)	pI
1	**AGG**CAAC	9	61	963	300	33.3	10.28
2	TC**GAG**AC	5	996	2348	450	48.0	5.78
tfuA	C**GG**G**G**GT	10	2674	4626	650	72.9	5.54
3	**AGGA**ACA	7	4890	6152	420	45.4	5.65
4	**AGGAG**CC	5	6149	6892	247	27.2	6.11

[a] Putative ribosome binding site. Bases that match the Shine-Dalgarno sequence AGGAGGT (30) are in boldface.
[b] The number of bases between the putative ribosome binding site and the putative start methionine codon.
[c] Site of the first base of the putative start methionine codon (GenBank accession number U39409).
[d] Site of the last base of the stop codon (GenBank accession number U39409).
[e] Number of amino acid residues in the putative translation product.

Further analysis of the 5.7-kb *Eco*RI fragment revealed a number of other ORFs. Transconjugant A8 turns blue on X-glc and so this insertion predicts a gene at or near position 6893 that is transcribed from left to right (Fig. 1; Table 2). This insertion falls exactly after the stop codon of ORF4 (Fig. 1). The predicted start methionine codon of ORF4 overlaps the stop codon of ORF3 (Table 3). Both ORFs are preceded by reasonable Shine-Dalgarno sequences (Table 3). Genes in this orientation are consistent with the GUS fusion results (Table 2). We predict that these two ORFs are part of an operon. The predicted proteins of ORF3 and 4 showed no significant similarity to proteins within the Swissprot database.

Upstream of *tfuA* there are a number of potential ORFs. Transconjugants with GUS insertions within this region do not turn blue in the presence of X-glc. This suggests that there are no genes here, that the genes are expressed at extremely low levels, or that the ORF is truncated and the promoter has been lost. ORF2 fits into this last category. Recall that pRBC695 was used for the mutagenesis, so this ORF is truncated. Swissprot searches of the putative translation product of ORF2 revealed that it has high similarity with the *codA* gene product of *E. coli* (6).

tfuA. This ORF was subcloned as a *Sac*II-*Hpa*I 2.7-kb fragment in both orientations into pRK415 to give pRtfuA1 and pRtfuA2. When these clones were conjugated into Tn5-2, both conferred TFX production, suggesting that this fragment has promoter-operator sequences. Given the number of stops downstream of this ORF in all three frames and the distance of 264 bp from the 3′ end of *tfuA* to the 5′ end of the first likely ORF (ORF3), *tfuA* is probably expressed as a monocistronic unit. Derivative C7 turns blue on X-glc, suggesting the lack of a transcriptional stop signal between bases 4626 and 4695. Since Tn*5* insertions are usually polar, and since the *tfuA* gene is sufficient to complement the mutation in Tn5-2, if there are genes expressed as part of an operon with *tfuA*, they are not required for TFX production.

The predicted TfuA protein is described in Table 3. The DNA sequence predicts a peroxidase motif at the carboxy end of TfuA. Searching Swissprot with TfuA revealed no proteins with significant similarity. Based on a Kyte-Doolittle plot (22), TfuA does not appear to contain any transmembrane domains.

Rescue of the TFX$^-$ phenotype by *tfuA* and *tfxABCDEFG*. Plasmids pRtfuA2 and pRT42 and their parent plasmid, pRK415, were conjugated individually into Tn5-1, Tn5-2, and Tn5-4. Tn5-4 was selected as a control because it is unaffected in TFX production but has the same resistances as Tn5-1 and Tn5-2. The relative amount of TFX each transconjugant produced in liquid culture after 5.5 h was determined.

Plasmid pRtfuA2 fully restores TFX activity by Tn5-2 but does not significantly increase TFX activity by Tn5-4, nor does it rescue the mutation in Tn5-1. In contrast, extra copies of the *tfx* gene cluster boost TFX activity 26-fold in Tn5-4. Tn5-1(pRT42) produces 4.6-fold more TFX than Tn5-4(pRT42). This is consistent with cell suspension spot assays in which the zones of inhibition produced by Tn5-1(pRT42) are larger than those produced by Tn5-4(pRT42) (data not shown). Tn5-2(pRT42) does produce a zone of inhibition in a cell suspension spot assay, but this zone is smaller than that of Tn5-1(pRT42) (data not shown). In this assay we detected no TFX activity using Tn5-2(pRT42) and find that it produces at least 118 times less TFX activity than Tn5-1(pRT42).

Lysate ribonuclease protection. To test if *tfuA* is involved in regulating message levels of the TFX structural gene, *tfxA*, lysate ribonuclease protection was performed as described

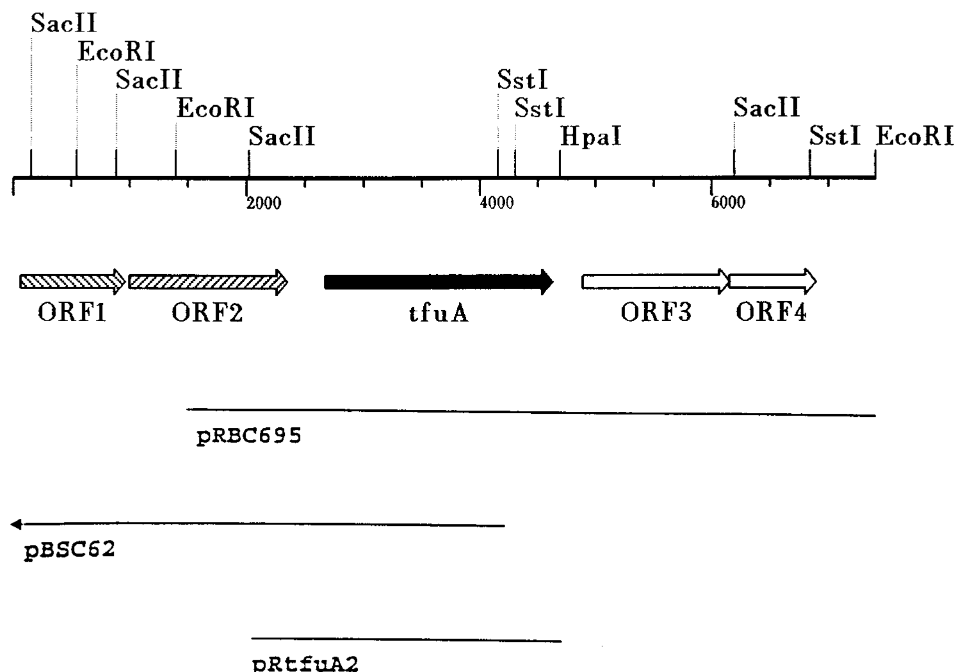

FIG. 1. Restriction map of the region described in GenBank accession number U39409. The scale of nucleotide numbers is shown below the restriction sites. ORFs are shown as boxes with the arrows indicating the direction of transcription. The putative ORF1 product has high similarity with the LysR family of transcriptional regulators, the putative ORF2 product has high similarity with the *E. coli codA* gene product, and *tfuA* encodes a protein required for TFX production. Inserts of important clones in this study are shown below the map. pRBC695 was used for transcriptional fusion mutagenesis. pBSC62 was used to obtain the sequence of ORF1 and the rest of ORF2. pRtfuA2 is sufficient to complement the mutant phenotype of Tn5-2.

above. The ratio of *tfxA* signal to that of the internal control 16S rRNA was not significantly different between T24 (77.8% ± 5.4%) and Tn5-2 (70.2% ± 10.3%). Given that TfxA is the substrate in TFX production, it is not surprising that the signal of *tfxA* is intense enough to compare with the signal of 16S rRNA.

CodA. The fragment of DNA initially sequenced yielded only part of the ORF2 sequence. The *E. coli codA* gene encodes a cytosine deaminase (6) that shows high similarity to this partial ORF. The region upstream of ORF2′ was cloned, sequenced, and reported as bases 0 to 1391 of GenBank nucleotide accession number U39409. The translation of bases 996 to 2348 would yield a protein that has 25.5% identity and 49.3% similarity to CodA over the full length of both proteins. The quality score was 24 standard deviations greater than the quality score based on 10 randomizations of ORF2 using the BestFit program from GCG.

To test for cytosine deaminase activity, T24 was streaked on BSM agar plates supplemented with 5-fluorocytosine. Cytosine deaminase catalyzes the conversion of this nontoxic substrate to the toxic compound, 5-fluorouracil. T24 did not grow.

LysR. ORF1 was found 32 bases upstream of ORF2. When the Blast program was used to compare the putative product of ORF1 with proteins in the Swissprot database, we found that it has high similarity to the LysR family of transcriptional activators (13). Its best BestFit match was to the *Bacillus subtilis* activator of glutamate biosynthetic genes, GltC (3). They share 30% identity and 51% similarity, and the quality score was 20 standard deviations greater than the quality score based on 10 randomizations. As is typical for members of the LysR family of regulators, identity is highest in the N-terminal region. When only the first 60 amino acids of each protein are used in the BestFit, the identity increases to 51%.

DISCUSSION

TFX belongs to a broad class of bacteriocins that are ribosomally synthesized, that have leader sequences that are cleaved, and whose peptide backbones undergo modification to yield unusual amino acids. Included within this group are the lantibiotics and the microcins (for reviews, see references 16, 17, 20, and 27). In these systems, the structural gene is found within a cluster of genes involved in antibiotic production. In some of these systems there are also genes distal to the cluster whose products are involved in some aspect of production of the antibiotic (17, 20). This is seen in the system of the *E. coli* DNA gyrase inhibitor, microcin B17 (38). The structural gene, *mcbA*, is plasmid encoded (11). Downstream of *mcbA* are *mcbBCDEFG*, whose products are responsible for posttranslational modification of McbA, microcin B17 export, and immunity and resistance to microcin B17 (20, 39, 40). MprA, a negative regulator of microcin B17 gene expression (7), PmbA, purported to be involved in leader processing (25), and OmpR, a positive regulator of microcin B17, *ompC*, and *ompF* gene expression (14, 24), are all chromosomally encoded. Similarly, we have found a gene, *tfuA*, that lies outside of the *tfx* gene cluster and is involved in TFX production. Like *ompR* in *E. coli*, this gene could be involved in other cellular processes that exist in the heterologous hosts that produce TFX when harboring the *tfxABCDEFG* genes. It is also possible that TfuA acts against a negative factor in T24 and so is not required in heterologous systems that lack this negative factor. Since the role of *tfuA* in TFX synthesis is not known, and since *tfuA* may be involved in other cellular processes, we chose not to use the *tfx* designation for this gene. *tfu* is derived from the terms trifolitoxin and unknown.

TFX activity has been shown to be pH, metabolite, and protease sensitive (29). It is easy to imagine mutations that could cause a strain to alter its environment in terms of pH, metabolites, or proteases. If Tn5-2 did produce TFX but in some way inactivated it after it had been secreted, Tn5-2 should have the ability to break down the TFX produced by T24. This was not the case in the coculture experiment. The coculture experiment also shows that Tn5-2 does not alter its extracellular environment in a way that inhibits TFX production.

The locus defined by the mutation in Tn5-2 has a role in TFX production. This role may be in regulation, synthesis, export, or intracellular stability. Cloning, complementation, sequencing, mutagenesis, and subcloning revealed *tfuA*, the wild-type gene interrupted in Tn5-2. The putative product of this gene shows no significant similarity to proteins in the Swissprot database. The TfuA peroxidase motif is interesting since some peroxidases carry out biosynthetic functions. Attempts to demonstrate increased peroxidase activity in crude cell extracts from cells with *tfuA* versus cells without *tfuA* using 2,2′-azino-bis(3-ethylbenzothiazoline sulfonic acid-6) as substrate failed. However, the conditions of the assay, including the substrate, may not be appropriate for detecting the peroxidase activity of TfuA.

A regulatory role for *tfuA* was originally suspected since Tn5-2 does produce some TFX activity when it harbors the *tfxABCDEFG* region on a multicopy plasmid. It was thought either that the increased copy number was sufficient to produce detectable TFX activity in the absence of an activator or that the extra copies titrated out an inhibitor. TfuA does not show significant similarity to known regulators or their motifs. When extra copies of *tfuA* are introduced into Tn5-4, TFX production does not increase, as one would expect if TfuA was a positive regulator unless TfuA was autoregulatory. Also, the interruption of *tfuA* had no detectable effect on *tfxA* message levels as determined by lysate ribonuclease protection assays.

The putative ORF2 product shows high similarity to the *E. coli codA* gene product. *codA* encodes cytosine deaminase, a protein responsible for the deamination of cytosine to yield uracil and ammonia (6). The inability of T24 to grow on media supplemented with 5-fluorocytosine suggests that T24 does have cytosine deaminase activity. In *E. coli*, *codA* is part of the *codBA* operon, in which CodB is a permease involved in cytosine transport and accumulation in cells (6). An ORF was found (ORF1), but this ORF predicts a protein resembling the LysR family of transcriptional regulators (13).

The structure of TFX is not fully known. Peptide sequencing predicted the TFX backbone to be DIGGSRXGCVA (23). The "X" is a UV-absorbing chromophore (23) that is likely derived from glutamine (4). Nuclear magnetic resonance-based experiments predict it to be a six-member ring that is pyrimidine-like (unpublished results). Given the similarity of ORF2 to CodA, the pyrimidine-like structure of the TFX chromophore, and the fact that genes involved in related functions often lie near each other, the idea that ORF2 plays a role in the synthesis of the chromophore is intriguing.

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