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 tfxAABCDEFG 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 Tn5 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 tfxAABCDEFG 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 epidermis 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 tfxAABCDEFG into several genera of α-proteobacteria confers 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 Tn5-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 tfxAABCDEFG. 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 37°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 disrupt 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) for E. coli; 15 μg/ml, nal 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 Tn5GUS cassette in these strains. Sm alone was frequently used to maintain the transposon in T24-Tn5 derivatives.

The substrates for β-glucuronidase, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 5-bromo-4-chloro-3-indolyl-β-D-glucuronoside, and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide were used to detect β-glucuronidase activity.

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### TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>Rhizobium leguminosarum</em> (bv. trifolii)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANU794</td>
<td>TFX, Sm&lt;sup&gt;+&lt;/sup&gt; derivative of TA1</td>
<td>5</td>
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<tr>
<td>T24</td>
<td>TFX&lt;sup&gt;+&lt;/sup&gt;, wild type</td>
<td>29</td>
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<tr>
<td>Tn5-1</td>
<td>TFX&lt;sup&gt;+&lt;/sup&gt;, T24(tfxB::Tn5)</td>
<td>34, this work</td>
</tr>
<tr>
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<td>34, this work</td>
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<td>Tn5-3</td>
<td>TFX&lt;sup&gt;+&lt;/sup&gt;, T24(&lt;&lt;m&gt;3::Tn5&lt;/m&gt;)</td>
<td>34</td>
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<tr>
<td>Tn5-4</td>
<td>TFX&lt;sup&gt;+&lt;/sup&gt;, T24(&lt;&lt;m&gt;3::Tn5&lt;/m&gt;)</td>
<td>34</td>
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<td><strong>E. coli</strong></td>
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<td></td>
</tr>
<tr>
<td>C2110mal</td>
<td>nal&lt;sup&gt;+&lt;/sup&gt; derivative of C2110, polA</td>
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<tr>
<td>DH5&lt;sup&gt;ex&lt;/sup&gt;</td>
<td>nal&lt;sup&gt;+&lt;/sup&gt;, recA</td>
<td>Bethesda Research Lab,</td>
</tr>
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<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
<td>pBluescriptII KS+</td>
<td>cloning and sequencing vector, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBEETn526</td>
<td>pBluescriptII KS+::7.1-kb EcoRI Tn5 fragment</td>
<td>This work</td>
</tr>
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<td>pBEC631</td>
<td>pBluescriptII KS+::5.7-kb EcoRI fragment of pC6</td>
<td>This work</td>
</tr>
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<td>pBEC639</td>
<td>pBluescriptII KS+::5.7-kb EcoRI fragment of pC6</td>
<td>This work</td>
</tr>
<tr>
<td>pBEHTn526</td>
<td>pBluescriptII KS+::2.7-kb HpaI fragment of pBEETn526</td>
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<td>pBSC62</td>
<td>pBluescriptII KS+::6-kb SfiI fragment of pC6</td>
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</tr>
<tr>
<td>pDSK519</td>
<td>broad-host-range vector, Km&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>pDTXC-12</td>
<td>pDSK519::fxaC DCEF9 (confers TFX resistance)</td>
<td>35</td>
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<tr>
<td>pLAFR3</td>
<td>broad-host-range vector, Te&lt;sup&gt;+&lt;/sup&gt;</td>
<td>31</td>
</tr>
<tr>
<td>pC6</td>
<td>pLAFR3::36-kb Tn5-5 fragment that complements Tn5-5</td>
<td>This work</td>
</tr>
<tr>
<td>pRBC695</td>
<td>pRK415::6-kb EcoRI fragment of pC6</td>
<td>This work</td>
</tr>
<tr>
<td>pRBC696</td>
<td>pRK415::6-kb EcoRI fragment of pC6</td>
<td>This work</td>
</tr>
<tr>
<td>pRK2013</td>
<td>ColE1 mobilization helper (Tra&lt;sup&gt;+&lt;/sup&gt;, Mob&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>19</td>
</tr>
<tr>
<td>pRK415</td>
<td>broad-host-range vector, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>pRT42</td>
<td>pRK415::fxaABC DCEF9 (confers TFX production and resistance), tfx genes in the opposite orientation from P&lt;sub&gt;rec&lt;/sub&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pRTfuA1</td>
<td>pRK415::2.7-kb SacII-HpaI fragment of pBEC639 (fxaA), fxaA is in the same orientation as P&lt;sub&gt;rec&lt;/sub&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pRTfuA2</td>
<td>pRK415::2.7-kb SacII-HpaI fragment of pBEC639 (fxaA), fxaA is in the opposite orientation from P&lt;sub&gt;rec&lt;/sub&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pTFX&lt;sup&gt;+&lt;/sup&gt;</td>
<td>pBluescriptII KS+::7.1-kb MluI fxaABC DCEF9 fragment</td>
<td>4</td>
</tr>
</tbody>
</table>

* TFX<sup>+</sup>, TFX nonproducing; TFX<sup>+</sup>, TFX producing; TFX<sup>+</sup>, TFX sensitive; Tra, transfer; Mob, mobilization.
were assayed for TFX production. Where zones of inhibition were found, the zones were conjugated individually and the transconjugants were reasayed for TFX activity. Only one clone (pC6) gave transconjugants that produced large zones of inhibition, and this was also the clone that hybridized with the 0.6-kb Tn5 insertion site of Tn5-2. Plasmid pC6 contains an insertion of about 36 kb. A 5.7-kb EcoRI fragment of pC6 hybridized to the Tn5-2 DNA as well as to the 2.7-kb ScaI-HpaI fragment of pBEC639. The 5.7-kb EcoRI fragment of pBEC639 was subsequently ligated into both orientations into the EcoRI site of pBluescriptII KS+ to yield pBEC631 and pBEC639. The 5.7-kb EcoRI fragment of pBEC639 was subsequently ligated into both orientations into the EcoRI site of pBluescript II KS+ to yield pBEC652. Both clones complement the mutation in Tn5-2. To subclone mtxA, the 2.7-kb SacI-HpaI fragment of pBEC639 was blunt-end ligated in both orientations into pRK415 digested with PstI and EcoRI. The resulting plasmids are pRfuA1 and pRfuA2.

Region upstream of tfxA, a 6-kb SfiI fragment of pC6 containing part of tfxA and about 4.5 kb upstream of it was cloned into the SfiI site of pBluescript II KS+ to yield pBSC652. Plasmid pRT42 was constructed by blunt-end ligation. Vector pRK415 was digested with PstI and EcoRIII and ligated to the 7.2-kb BspHII fragment of pRTX24 (24).

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 BSA 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 BSA agar medium. Filter-sterilized filtrates placed in the cores were allowed to dry for 1 day before being filled with cell suspensions. 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 untranslated β-glucuronidase (GUS) activity was determined by addition of X-gluc 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 10⁵ CFU/ml of 0.5% 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 shaken at 275 rpm at 28°C. After 4152 BREIL ET AL. J. BACTERIOL.

RESULTS

Mapping the Tn5 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 Tn5 (34). Approximately 5,600 Tn5 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 Tn5 derivative of T24 which is unaffected in TFX production (34). When plasmids harboring the tfxAABCDEFG fragment were conjugated into each of the T24:Tn5 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 Tn5 insertion in each of Tn5-1, Tn5-2, Tn5-3, and Tn5-4. The Tn5 insertion sites of Tn5-1 and Tn5-3 were within the 7.1-kb MdiI 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. Tn5 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 Tn5 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. 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 cm²; T24 with Tn5-1, 36.5 ± 1.41 cm²; and T24 with Tn5-2, 35.5 ± 0.07 cm². 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 EcoRI 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...
is confirmed by the GUS mutants A9 and B7 since both of tfuA
TFXproduction (Table 2). Only one ORF overlaps this region,
that the region between positions 3007 and 4379 is involved in
predicted on the basis of sequence analysis, transcriptional
(Table 2). Descriptions of the open reading frames (ORFs)
screened for TFX production and
fall within the cloned fragment were conjugated into Tn5-2 and
TnGUS. GUS insertions sites of 31 Tn
were mutagenized with the transcriptional GUS fusion cassette
directly before position 4080. For more information about TnGUS
accession number U39409).

sequence AGGAGGT (30) are in boldface. The number of bases between the putative ribosome binding site and the
putative start methionine codon (GenBank
position 2467 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
Tn5 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 ThuA protein is described in Table 3. The
DNA sequence predicts a peroxidase motif at the carboxy end
of ThuA. Searching Swissprot with ThuA revealed no proteins
with significant similarity. Based on a Kyte-Doolittle plot (22),
ThuA does not appear to contain any transmembrane domains.

Rescue of the TFX phenotype by tfuA and tfxABCDEF.
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

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

<table>
<thead>
<tr>
<th>ORF</th>
<th>RBS*</th>
<th>No. of bases</th>
<th>ATG*</th>
<th>Stop†</th>
<th>No. of amino acids</th>
<th>Mass (kDa)</th>
<th>pl</th>
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</thead>
<tbody>
<tr>
<td>tfuA</td>
<td>CGGGGGT</td>
<td>10</td>
<td>2674</td>
<td>1764</td>
<td>4626</td>
<td>1</td>
<td>450.0</td>
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<tr>
<td>AGGAAGC</td>
<td>7</td>
<td>4980</td>
<td>6152</td>
<td>1</td>
<td>450.4</td>
<td>5.65</td>
<td></td>
</tr>
<tr>
<td>AGGAGCC</td>
<td>5</td>
<td>6149</td>
<td>6892</td>
<td>247</td>
<td>372.2</td>
<td>6.11</td>
<td></td>
</tr>
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</table>

* Putative ribosome binding site. Bases that match the Shine-Dalgarno sequence AGGAGGT (30) are in boldface.
† The number of bases between the putative ribosome binding site and the putative start methionine codon.
‡ Site of the first base of the putative start methionine codon (GenBank accession number U39409).
§ Number of amino acid residues in the putative translation product.

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

<table>
<thead>
<tr>
<th>Tn3GUS derivative</th>
<th>Site</th>
<th>TFX production</th>
<th>X-glc</th>
<th>ORF interrupted</th>
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<tbody>
<tr>
<td>G6 1764</td>
<td>+</td>
<td>W→</td>
<td>ORF2</td>
<td></td>
</tr>
<tr>
<td>G5 2313</td>
<td>+</td>
<td>W→</td>
<td>ORF2</td>
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<tr>
<td>B5 2546</td>
<td>+</td>
<td>W→</td>
<td>ORF2</td>
<td></td>
</tr>
<tr>
<td>A9 3007</td>
<td>+</td>
<td>B→ tfuA</td>
<td>ORF2</td>
<td></td>
</tr>
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<td>F7 3667</td>
<td>−</td>
<td>W→ tfuA</td>
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<tr>
<td>A8 6893</td>
<td>+</td>
<td>B→ ORF4</td>
<td>ORF2</td>
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</table>

* Nucleotide position within the map of Fig. 1 before which the Tn3GUS mutagenesis cassette is inserted.
†, complements the mutation in Tn5-2; −, does not complement the mutation in Tn5-2.
‡ 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.
§ ORF in which the insert is located (see Fig. 1).
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 TxF 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.

\( \text{CodA} \). The fragment of DNA initially sequenced yielded only part of the ORF2 sequence. The \( \text{E. coli} \) codA gene encodes a cytosine deaminase (6) that shows high similarity to this partial ORF. The upstream region 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. 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. The best matches were to the Bacillus subtilis activator of glutamate biosynthetic genes, Glic (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 were used in the BestFit, the identity increases to 51%.

\( \text{DISCUSSION} \)

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 \( \text{E. coli} \) DNA gyrase inhibitor, microcin B17 (38). The structural gene, \( mcbA \), is plasmid encoded (11). Downstream of \( mcbA \) are \( mcbBCDEFG \), whose products are responsible for post-translational 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 \( \text{E. coli} \), this gene could be involved in other cellular processes that exist in the heterologous hosts that produce TFX when harboring the \( tfxABCDEF \) 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.

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 \( \text{E. coli} \) 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. pRfuA2 is sufficient to complement the mutant phenotype of Tn5-2.
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-ethylbenzthiazoline sulfonyl 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 tfuA 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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