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Characterization of the *tet*(M) Determinant of Tn*916*: Evidence for Regulation by Transcription Attenuation

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The nucleotide sequence of the tetracycline resistance determinant *tet*(M), located on conjugative transposon Tn*916* of *Enterococcus faecalis*, was determined and found to encode a 72,486-dalton protein exhibiting a high degree of homology with other *tet*(M) determinants. A short open reading frame corresponding to a 28-amino-acid peptide and containing a number of inverted repeat sequences was noted immediately upstream of *tet*(M), suggesting that regulation might occur by a mechanism involving transcriptional attenuation. Transcription analyses found this to indeed be the case, showing that the expression of *tet*(M) resulted from an extension of a small transcript representing the upstream leader region into the resistance determinant. Exposure of cells to tetracycline resulted in a significant increase in the amount of *tet*(M) transcription; this increase could be explained on the basis of increased transcriptional read-through from the upstream transcript. A model suggesting how transcriptional attenuation might operate in this system is presented.

Tn*916* (16.4 kb) is the prototype of a large family of conjugative transposons commonly found in enterococci and streptococci (7, 9). These elements exhibit a broad host range, and essentially all of them carry a tetracycline resistance determinant of the *tet*(M) variety, as originally designated by Burdett et al. (6). The *tet*(M) gene product has been found to associate with ribosomes and mediate resistance at the level of protein synthesis (3, 5). Burdett recently purified the protein (5) and found it to consist of a single polypeptide with a molecular weight of approximately 68,000. Interestingly, it was found to have a ribosome-dependent GTPase activity. Tet(M) was also reported to be present in increased amounts when cells were grown in the presence of tetracycline (3).

In this report, we present the results of a nucleotide sequence analysis of *tet*(M) and flanking DNA and, in addition, provide evidence that the regulation of expression occurs at the level of transcription. After we had completed the sequence analysis, Burdett (4) reported the results of an independent investigation of the same sequence. Although our data are in good agreement (a few minor differences), we wish to point out here features of the sequence that we believe are related to gene expression. Aspects of the structure of the region immediately upstream of *tet*(M), along with our mRNA analyses, support the view that the regulation of Tet(M) biosynthesis involves transcriptional attenuation. A plausible model pointing out the mechanism by which this regulation may occur is presented.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 lists the bacterial strains and plasmids used in this study. Figure 1 illustrates the construction of pBΔfo, pAM1005, pAM1006, and pAM1008. Plasmid pAM620 is a derivative of pACYC184 into which an *Eco*RI fragment containing all of Tn*916* was cloned (41). Whereas pAM1008 conferred tetracycline resistance on its *Escherichia coli* host, pAM1005 and pAM1006, which contained DNA only on one side of the single *Hin*dIII site and on one side of the single *Kpn*I site in Tn*916*, respectively, did not. This result was expected, since earlier observations had suggested that a *Hin*dIII site was present within *tet*(M) (14).

Strain S5-10, which was used in the RNA (Northern) blot analyses, is a derivative of RH120 in which Tn*916* was substituted for Tn*916ΔE* by an allelic exchange technique. Tn*916ΔE* is a derivative of Tn*916* constructed by Rubens and Heggen (29) and has an *erm* determinant in place of *tet*(M). RH120 was kindly provided by C. Rubens and was shown by Southern blot hybridizations to contain a single copy of the transposon (8). [The use of this strain to replace *erm* with *tet*(M) was originally done for the purpose of comparing Tn*916* and Tn*916ΔE* when located in identical positions on the chromosome; results specifically related to such comparisons will be described elsewhere.] Suicidal in *Enterococcus faecalis*, pAM1008 (0.5 to 1 μg/ml) was used to replace the *erm* determinant by electroporation and allelic exchange. Six independent transformation experiments resulted in a total of 29 tetracycline-resistant derivatives. Seven of these were susceptible to erythromycin, implying that replacement had occurred. One of these was designated S5-10. Southern blot analysis showed that a single copy of the transposon was present. The probes used were the DNA oligomers 916RL-1 [hybridizes upstream of *tet*(M)] and 916LR-A (hybridizes downstream of the sequence shown in Fig. 2). When chromosomal DNA was cleaved with both *Hin*dIII [single site in the transposon and located in *tet*(M)] and *Eco*RI (no sites in the transposon), blotted, and probed, the upstream probe (916RL-1) hybridized to a single band that was the same size for DNAs from RH120 and S5-10. The downstream probe (916RL-A) revealed the other *Hin*dIII-*Eco*RI fragment, but the fragment from RH120 ran slightly slower than the corresponding fragment from S5-10, reflecting an expected 400-bp difference in size (29).

Media. *E. faecalis* strains were grown in Todd-Hewitt

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TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or relevant properties	Plasmid content	Reference or source
Strains			
Enterococcus faecalis			
OG1SS	*str spc*	None	13
RH120	Same as OG1SS; *erm* (Tn*916*Δ*E*)	None	C. Rubens
S5-10	Same as OG1SS; *tet*(M) (Tn*916*)	None	This study
Escherichia coli			
DH1	F^- *recA1 endA1 gyrA96 thi-1 hsdR17 supE44* λ^-	None	24
DH1-669	*erm*; same as DH1	pAM669	34, 41
AM620	Same as DH1; λ prophage; *erm tetM*	pAM620	41
XL1-Blue	*recA1 lac endA1 relA1 gyrA96 thi hsdR17 supE44* (F′ *proAB lacI*^{q} *lacZ*Δ*M15* Tn*10*)	None	Stratagene
XL1-pBL	*bla*; same as XL1-Blue	pBluescript II KS (−)	Stratagene
AL1-pBΔ	*bla*; same as XL1-Blue	pBΔfo	This study
Y68	Same as XL1-Blue; *bla*	pAM1005	This study
Y69	Same as DH1; *bla*	pAM1006	This study
Y72	Same as DH1; *bla tet*(M)	pAM1008	This study
Plasmids			
pBluescript KS M13(−)	*bla lacZ*		Stratagene
pBΔfo	*bla lacZ*		This study; Fig. 1
pAM620	*erm tet*(M) (Tn*916*)		41
pAM669	*erm tet*(M) (Tn*916*) *kan* (Tn*5*)		34
pAM1005	*bla*		This study; Fig. 1
pAM1006	*bla*		This study; Fig. 1
pAM1008	*bla tet*(M)		This study; Fig. 1

broth (Difco Laboratories), whereas the growth medium for *E. coli* strains was LB medium (1). Solid medium 2×YT (1) with 5 mM isopropyl-β-thiogalactopyranoside and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml (both from Sigma) was used to detect inserts into pBΔfo. For electroporation, *E. faecalis* RH120 was grown in 30% Todd-Hewitt broth with 5% glycine and 0.5 M sucrose (10). When present in selective plates, antibiotics were used at the following concentrations: tetracycline, 100 μg/ml for *E. coli* XL1-Blue, 4 μg/ml for *E. coli* DH1(pAM620) or DH1 (pAM1008), and 10 μg/ml for *E. faecalis* strains containing Tn*916*; ampicillin, 100 μg/ml; erythromycin, 150 μg/ml for *E. coli* AM620 and 25 μg/ml for *E. faecalis* strains carrying Tn*916*Δ*E*; and kanamycin, 25 μg/ml.

Enzymes and reagents. Restriction endonucleases, T4 DNA ligase, exonuclease III, mung bean nuclease, DNase I (RNase free), reverse transcriptase, deoxyribonucleoside triphosphates, and dideoxyribonucleoside triphosphates were obtained from Boehringer Mannheim Biochemicals. T4 polynucleotide kinase and an RNA ladder (0.24 to 9.5 kb) were obtained from Bethesda Research Laboratories. A universal sequencing primer (catalog no. 1211) and an M13 reverse primer (catalog no. 300304) were purchased from New England BioLabs and Stratagene Inc., respectively. Other primers were synthesized by the DNA facility of the University of Michigan. Antibiotics, lysozyme, and RNase A were obtained from Sigma. Radioactive compounds were obtained from Amersham Corp. Sequenase kits were obtained from United States Biochemical Corp.

General DNA techniques. Chromosomal and plasmid DNAs were isolated as described elsewhere (1, 14). General procedures for cloning and DNA manipulations were as described by Ausubel et al. (1). DNA was introduced into bacteria by electroporation by the method of Dower et al. (12) for *E. coli* and the method of Cruz-Rodz and Gilmore (10) for *E. faecalis*. Electroporation was done with a Gene Pulser (Bio-Rad Laboratories).

DNA sequencing analyses. The DNA sequence in Fig. 2 was determined from templates of pAM1005, pAM1006, and pAM1008 and their nested deletion derivatives by use of a modified T7 DNA polymerase (Sequenase) in accordance with the recommendations of the manufacturer (United States Biochemical Corp.). A previously described modification of the annealing process was used (38). The sequence was determined for both DNA strands. The location of Tn*5* in pAM669 was determined by use of synthetic primer 916LR-G (Fig. 2). Sequences were stored, matched, processed, and analyzed by use of a Macintosh SE/30 computer and by use of MacVector 3.5 software and its GenBank data base (18a).

DNA hybridization methods. DNA blot transfer and hybridization by use of nitrocellulose transfer membranes (BA-S NC; Schleicher and Schuell) were performed by the method of Southern (37) with modifications described by Wahl et al. (39). Oligonucleotides (916RL-1 and 916LR-A) were end labeled with [^{32}P]ATP by use of T4 polynucleotide kinase (26). 916RL-1 (5′-CCTTTTTTAGGAGGGC-3′) hybridizes to a sequence upstream of the *tet*(M) determinant (Fig. 2), whereas 916LR-A (5′-ATTGACTGTATGGGAG-3′) hybridizes to a site downstream of the sequence presented in Fig. 2. Washing was done at 5°C below the calculated melting temperature.

Transcription analysis. RNA was extracted from 50-ml cultures of *E. faecalis* OG1SS and S5-10 by a hot-phenol protocol (35) and treated with DNase I (RNase free) (1). Northern blot analyses were done as described by Ausubel et al. (1), with hybridization reactions being conducted at 42°C for 18 to 20 h. The probes used were oligonucleotides 916LR-O (5′-GGACGACGGGGCTGGCAAACAGGTTCA CCGG-3′) and 916LR-P (5′-AGGCATACAAAGCATACAG

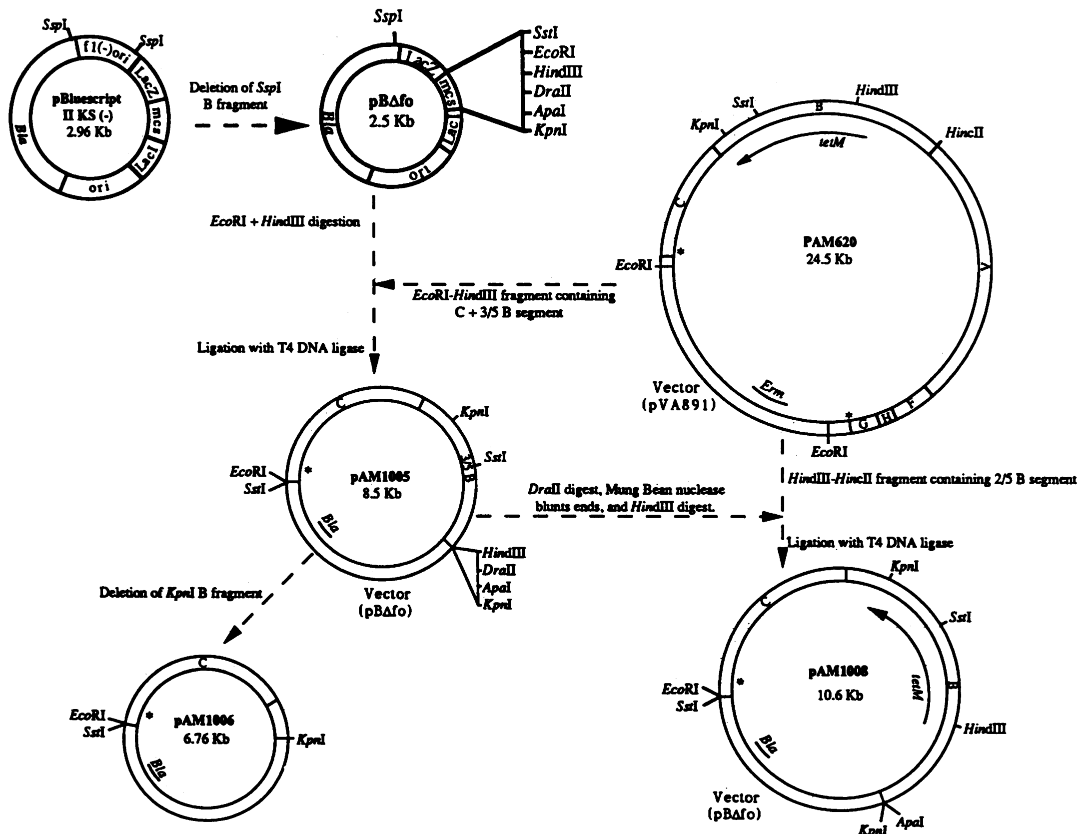

FIG. 1. Construction of plasmids. DNA segments of pBluescript II KS (−) and pBΔfo corresponding to their functions are separated with lines. mcs, multiple cloning sites. Asterisks indicate the ends of Tn*916*. A, B, C, F, G, and H represent *Hin*cII fragments of Tn*916* (41). Only relevant restriction enzyme sites are indicated.

ATATTCTCCGG-3') (Fig. 2) with the 5' ends labeled with [^{32}P]ATP and T4 polynucleotide kinase (26). A laser densitometer (LKB Ultroscan XL) was used to scan autoradiographs to estimate the extent of read-through from the leader region into *tet*(M). Primer extension and RNA sequencing were done by the method of Guild et al. (17).

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence presented here is M85225. This nucleotide sequence is related to that determined by Burdett (4), for which the EMBL accession number is X56353.

RESULTS

Sequence. The nucleotide sequence of *tet*(M) and its deduced amino acid sequence are shown in Fig. 2. The sequence from bp 298 to 2505 is essentially identical to that independently determined and recently published by Burdett (4), with a few nonidentical nucleotides indicated. The differences result in two amino acid changes (noted in Fig. 2). The deduced Tet(M) product represents a 639-amino-acid protein with a molecular weight of 72,486, a value in reasonably good agreement with the 68,000-dalton size estimated by Burdett (5) from electrophoretic measurements of purified Tet(M). A ribosome binding site (GGAGG) is located 9 bases upstream from the probable translational initiation site of the *tet*(M) determinant; 275 bp downstream of the stop codon are inverted repeats that would give rise to a stem-loop in mRNA with a free energy of −31.9 kcal/mol (ca. 133.5 kJ/mol) followed by UUU. This step-loop would appear to be a factor-independent transcriptional termination site, $T_{tet(M)}$. (This region was not included in the Burdett [4] analysis.)

A relatively short open reading frame was deduced in the region upstream of *tet*(M); its product would correspond to a 28-amino-acid protein with a molecular weight of 3,376. A potential ribosome binding site (GGAG) is suitably located upstream, and a putative promoter (−10 and −35 boxes) is indicated upstream (also see below). The stop codon (UGA) is very close to the ribosome binding site of *tet*(M). Interestingly, there are four methionines present within the first eight amino acid residues of the short open reading frame. As indicated in Fig. 2, the reading frame contains several pairs of inverted repeats; these are noted as 1 through 8, with potential pairings as 1:2, 3:4, 5:6, and 7:8. The various stem-loop structures that may be formed in mRNA, according to the energy model of Salser (31), are shown with their corresponding free energies in Fig. 3. The 5:6 pairing is shown in a short version (5S:6S) as well as a long version (5L:6L). The small open reading frame and the potential mRNA secondary structures are reminiscent of regions known to be involved in the regulation of downstream genes by virtue of transcriptional attenuation phenomena. Of particular note in this regard is the run of U's that are present immediately after the stem-loop indicated as 5S:6S, which is probably a factor-independent transcriptional terminator (T_{leader}) for a leader transcript (see below). A possible model

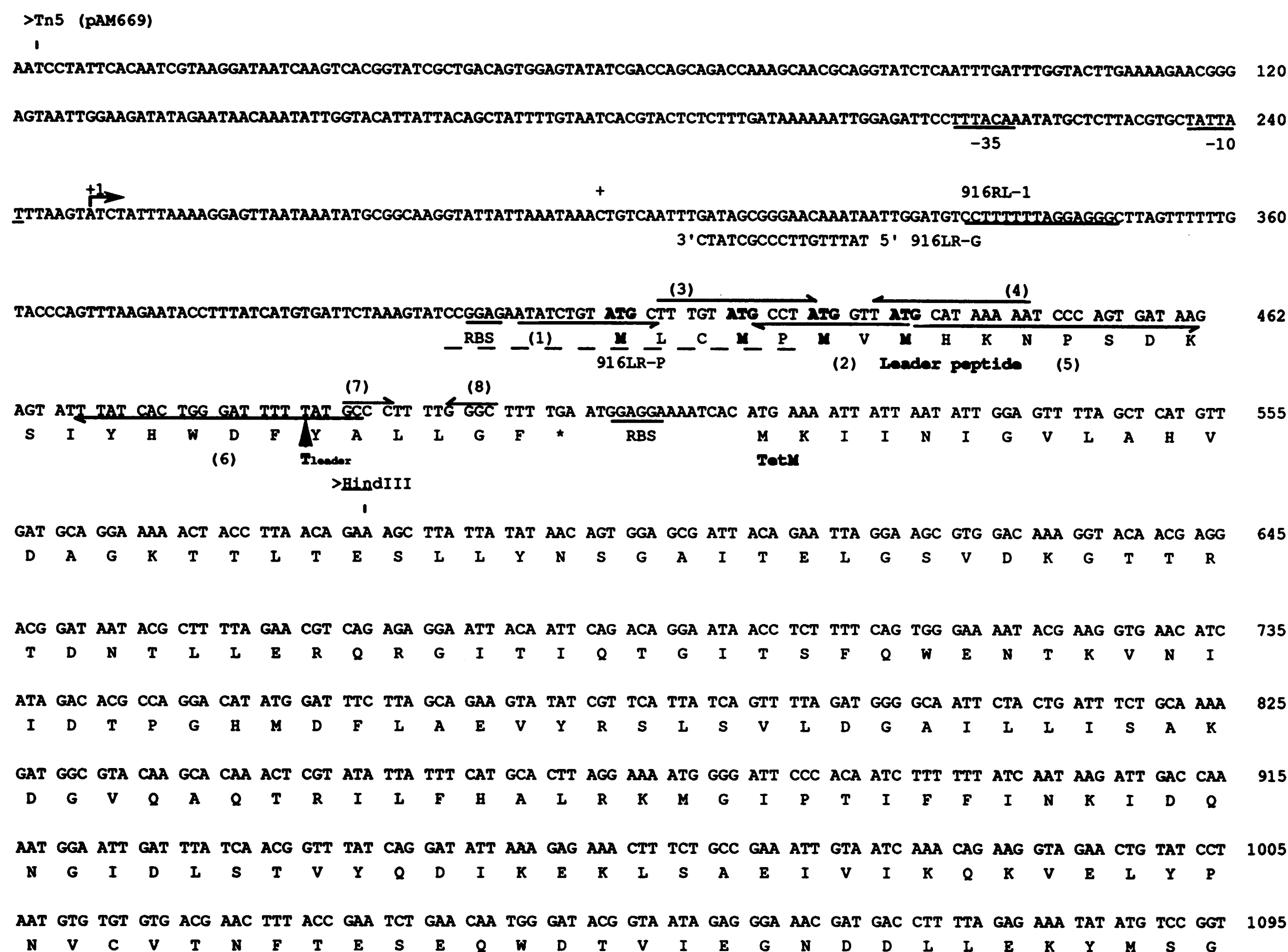

FIG. 2. Nucleotide sequence of the *tet*(M) gene and flanking regions. The deduced amino acid sequence of the Tet(M) protein and the leader peptide is given in single-letter code under the nucleotide sequence. >, the Tn*5* insertion or specified restriction site is to the right of the nucleotide indicated by a vertical line. The arrow marked +1 indicates the transcriptional initiation site of the leader and *tet*(M) mRNAs. The arrowheads labelled T_{leader} and $T_{tet(M)}$ indicate probable transcriptional termination sites of the leader and *tet*(M) transcripts, respectively. Promoter $P_{tet(M)}$ (indicated by −35 and −10), the sequence corresponding to primer 916RL-1, ribosome binding sites (RBS), and inverted repeats 1, 2, 5, and 6 and the pair downstream of the *tet*(M) determinant are underlined. Inverted repeats 3, 4, 7, and 8 are overlined. 916LR-G is a primer used to determine the Tn*5* insertion site in Tn*916* (in pAM669) and the site of transcriptional initiation. The broken lines underline the sequences complementary to probes 916LR-O and 916LR-P. The nucleotide sequence between the two plus signs (from 298 to 2,505 bp) is essentially identical to that published by Burdett (4), with the exception of a few nucleotides, which are indicated in parentheses above the nucleotide sequence. The letters in parentheses under the amino acid sequence represent residues deduced from the discrepant nucleotides. *, stop codon. Methionines in the leader peptide are in boldface type.

for how this region could regulate *tet*(M) expression is presented in the Discussion.

Evidence for regulation by transcriptional attenuation. Northern blot hybridization analyses were performed with two different probes. One (916LR-P) complements a segment of DNA upstream of the translational start site of *tet*(M) but downstream of the putative promoter; the other (916LR-O) complements a region within *tet*(M) near the 3′ end (Fig. 2). As shown in Fig. 4A, the probe specific for the region upstream of *tet*(M) revealed a small transcript of approximately 0.25 kb. In addition, transcripts of 1.8 and 2.5 kb were detected. The 1.8-kb band was present in transposon-free cells (lane 1) and thus would appear to represent a host transcript that cross-reacted with the probe. The 2.5-kb transcript increased significantly in intensity when the cells were exposed to tetracycline for 4 h. Although exposure to 0.05 μg of drug per ml (lane 3) resulted in little difference from the results for the unexposed control (lane 2), concentrations of 0.5 (lane 4), 5.0 (lane 5), and 20.0 (lane 6) μg/ml gave rise to sequentially higher band intensities. The amount of the 2.5-kb RNA detected when 20 μg of drug per ml was present during growth was approximately 15-fold higher (as determined by densitometer tracings and normalization to the relatively constant 1.8-kb host RNA) than that detected when the cells were not exposed to the antibiotic. Little increase was noted for the 0.25-kb transcript. When the probe corresponding to *tet*(M) was used (Fig. 4B), both the 1.8- and the 2.5-kb bands were revealed, but the smaller, 0.25-kb band was not detected. The 1.8-kb band was again present in the transposon-free host. The 2.5-kb band increased in intensity when the cells were exposed to increasing concentrations of tetracycline. The increases were sim-

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AAA TCA TTA GAA GCA TTG GAA CTC GAA CAA GAG GAA AGC ATA AGA TTT CAG AAT TGT TCT CTG TTC CCT CTT TAT CAT GGA AGT GCA AAA  1185
 K   S   L   E   A   L   E   L   E   Q   E   E   S   I   R   F   Q   N   C   S   L   F   P   L   Y   H   G   S   A   K

AGT AAT ATA GGG ATT GAT AAC CTT ATA GAA GTT ATT ACT AAT AAA TTT TAT TCA TCA ACA CAT CGA GGT CCG TCT GAA CTT TGC GGA AAT  1275
 S   N   I   G   I   D   N   L   I   E   V   I   T   N   K   F   Y   S   S   T   H   R   G   P   S   E   L   C   G   N

GTT TTC AAA ATT GAA TAT ACA AAA AAA AGA CAA CGT CTT GCA TAT ATA CGC CTT TAT AGT GGA GTA CTA CAT TTA CGA GAT TCG GTT AGA  1365
 V   F   K   I   E   Y   T   K   K   R   Q   R   L   A   Y   I   R   L   Y   S   G   V   L   H   L   R   D   S   V   R

GTA TCA GAA AAA GAA AAA ATA AAA GTT ACA GAA ATG TAT ACT TCA ATA AAT GGT GAA TTA TGT AAG ATT GAT AGA GCT TAT TCT GGA GAA  1455
 V   S   E   K   E   K   I   K   V   T   E   M   Y   T   S   I   N   G   E   L   C   K   I   D   R   A   Y   S   G   E

ATT GTT ATT TTG CAA AAT GAG TTT TTG AAG TTA AAT AGT GTT CTT GGA GAT ACA AAA CTA TTG CCA CAG AGA AAA AAG ATT GAA AAT CCG  1545
 I   V   I   L   Q   N   E   F   L   K   L   N   S   V   L   G   D   T   K   L   L   P   Q   R   K   K   I   E   N   P

CAC CCT CTA CTA CAA ACA ACT GTT GAA CCG AGT AAA CCT GAA CAG AGA GAA ATG TTG CTT GAT GCC CTT TTG GAA ATC TCA GAT AGT GAT  1635
 H   P   L   L   Q   T   T   V   E   P   S   K   P   E   Q   R   E   M   L   L   D   A   L   L   E   I   S   D   S   D

CCG CTT CTA CGA TAT TAC GTG GAT TCT ACG ACA CAT GAA ATT ATA CTT TCT TTC TTA GGG AAA GTA CAA ATG GAA GTG ATT AGT GCA CTG  1725
 P   L   L   R   Y   Y   V   D   S   T   T   H   E   I   I   L   S   F   L   G   K   V   Q   M   E   V   I   S   A   L

                                        (A) (C)
TTG CAA GAA AAG TAT CAT GTG GAG ATA GAA CTA AAA GAG CCT ACA GTC ATT TAT ATG GAG AGA CCG TTA AAA AAT GCA GAA TAT ACC ATT  1815
 L   Q   E   K   Y   H   V   E   I   E   L   K   E   P   T   V   I   Y   M   E   R   P   L   K   N   A   E   Y   T   I
                                        (I) (T)

CAC ATC GAA GTG CCG CCA AAT CCT TTC TGG GCT TCC ATT GGT TTA TCT GTA TCA CCG CTT CCG TTG GGA AGT GGA ATG CAG TAT GAG AGC  1905
 H   I   E   V   P   P   N   P   F   W   A   S   I   G   L   S   V   S   P   L   P   L   G   S   G   M   Q   Y   E   S

>SstI
|
TCG GTT TCT CTT GGA TAC TTA AAT CAA TCA TTT CAA AAT GCA GTT ATG GAA GGG ATA CGC TAT GGT TGT GAA CAA GGA TTG TAT GGT TGG  1995
 S   V   S   L   G   Y   L   N   Q   S   F   Q   N   A   V   M   E   G   I   R   Y   G   C   E   Q   G   L   Y   G   W

AAT GTG ACG GAC TGT AAA ATC TGT TTT AAG TAT GGC TTA TAC TAT AGC CCT GTT AGT ACC CCA GCA GAT TTT CGG ATG CTT GCT CCT ATT  2085
 N   V   T   D   C   K   I   C   F   K   Y   G   L   Y   Y   S   P   V   S   T   P   A   D   F   R   M   L   A   P   I

GTA TTG GAA CAA GTC TTA AAA AAA GCT GGA ACA GAA TTG TTA GAG CCA TAT CTT AGT TTT AAA ATT TAT GCG CCA CAG GAA TAT CTT TCA  2175
 V   L   E   Q   V   L   K   K   A   G   T   E   L   L   E   P   Y   L   S   F   K   I   Y   A   P   Q   E   Y   L   S

CGA GCA TAC AAC GAT GCT CCT AAA TAT TGT GCG AAC ATC GTA GAC ACT CAA TTG AAA AAT AAT GAG GTC ATT CTT AGT GGA GAA ATC CCT  2265
 R   A   Y   N   D   A   P   K   Y   C   A   N   I   V   D   T   Q   L   K   N   N   E   V   I   L   S   G   E   I   P

                                                                                                          >KpnI
                                                                                                           |
GCT CGG TGT ATT CAA GAA TAT CGT AGT GAT TTA ACT TTC TTT ACA AAT GGA CGT AGT GTT TGT TTA ACA GAG TTA AAA GGG TAC CAT GTT  2355
 A   R   C   I   Q   E   Y   R   S   D   L   T   F   F   T   N   G   R   S   V   C   L   T   E   L   K   G   Y   H   V

                                                                                                                 (A)
ACT ACC GGT GAA CCT GTT TGC CAG CCC CGT CGT CCA AAT AGT CGG ATA GAT AAA GTA CGA TAT ATG TTC AAT AAA ATA ACT TAG TGTATTT  2446
 T   T   G   E   P   V   C   Q   P   R   R   P   N   S   R   I   D   K   V   R   Y   M   F   N   K   I   T   *
    ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
                            916LR-O
 (A)                                                       +
TATGTTGTTATATAAATATGGTTTCTTGTTAAATAAGATGAAATATTTTTTAATAAAGATTTGAATTAAAGTGTAAAGGAGGAGATAGTTATTATAAACTACAAGTGGATATTGTGTCC  2565

TGTATGTGGAAATAAAACACGATTAAAGATAAGGGAAGATACTGAATTAAAAAAATTCCCCCTCTATTGTCCGAAATGCAGACAAGAAAATTTAATTGAAATAAAGCAGTTCAAAGTAA  2684

CTGTGATTACAGAGCCAGACGCAAAGACGCAGAGCCGATAAAATGAGATTAATACAATCTCATTTTATCGGCTCTTTCCGTTATGTATGGATTCTTTTAATTAGTC  2790
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FIG. 2—*Continued.*

ilar in magnitude to those observed with the other probe. In addition, there was an approximately fivefold increase in the total amount of 0.25- plus 2.5-kb RNAs. The data imply that the 2.5-kb transcript is an extension of the 0.25-kb transcript and that exposure to tetracycline stimulated its appearance. The fact that the 0.25-kb RNA remained relatively constant (or possibly increased slightly with exposure to tetracycline [Fig. 4A]) indicates that the initiation of its transcription must also be enhanced.

It is interesting that the read-through evident in cells exposed to 5 μg of drug per ml for 10 min (Fig. 4, lane 7) appeared to be significantly greater than that in cells exposed

FIG. 3. Secondary structures (in RNA) of the putative *tet*(M) leader transcript. Ribosome binding sites (RBS) are underlined. The translational start sites of the putative leader peptide and Tet(M) are indicated by boldface letters. The free energy (ΔG) for each stem-loop is indicated in parentheses (1 cal = 4.184 J). The arrowheads indicate the presumed termination sites (T_{leader}) of the leader transcript. Translational initiation of the leader peptide disrupts the formation of stem-loop structures 1:2 and 3:4, since ribosome binding covers nucleotides −20 to +13 relative to the first nucleotide of the initiation codon (15, 42). As soon as the valine residue is incorporated via translational elongation, the ribosome sterically hinders the formation of stem-loop 5S:6S, resulting in RNA polymerase continuing transcription of the *tet*(M) determinant. Without coupled translation, stem-loop structures 1:2, 3:4, 5L:6L, and 7:8 can induce the pausing of RNA polymerase, whereas stem-loop 5S:6S terminates transcription. (See the text for details.)

to this same concentration for 4 h (Fig. 4, lane 5). This result suggests that the response to tetracycline is relatively high during early exposure but that it later decreases to a steady-state level that is dependent on the concentration of the drug.

It is noteworthy that a low level of transcription appeared to extend even beyond the putative transcriptional terminator toward the left end of the transposon, as transcripts larger than the 2.5-kb RNA can be seen in Fig. 4. Although the sizes of these transcripts appeared variable, they were more prevalent in cells exposed to tetracycline; a band corresponding to about 3.2 kb could be resolved when the drug concentration was 20 μg/ml (Fig. 4, lane 6).

Sites of initiation of the leader and *tet*(M) transcripts. By a combination of primer extension and RNA sequencing (Fig. 5), RNA was found to initiate with an adenylate residue 170 nucleotides upstream of the start site of the leader open reading frame. The distance from the transcriptional initiation site to the assumed leader and *tet*(M) termination sites (T_{leader} and $T_{tet(M)}$ [Fig. 2]) encompasses 237 and 2,521 nucleotides, respectively. The values are in good agreement with the 0.25- and 2.5-kb RNAs estimated from the Northern blot data in Fig. 4 and fit well with the positions of the −10 and −35 boxes noted in Fig. 2. A Tn*5* insertion (Fig. 2) upstream of the promoter (i.e., in derivative pAM669) was previously shown to have no effect on the expression of resistance (41).

Homology with other determinants. The *tet*(M) determinant of Tn*916* exhibits 95, 97, and 99% amino acid sequence identity with the Tet(M) proteins of Tn*1545* (25), *Staphylococcus aureus* (27), and *Ureaplasma urealyticum* (33), respectively. There is also 76% identity with the *tet*(O) determinants of both *Streptococcus mutans* (21) and *Campylobacter jejuni* (23). In addition, there are sequence similarities with translational elongation factors, as pointed out originally by Sanchez-Pescador et al. (32) and more recently

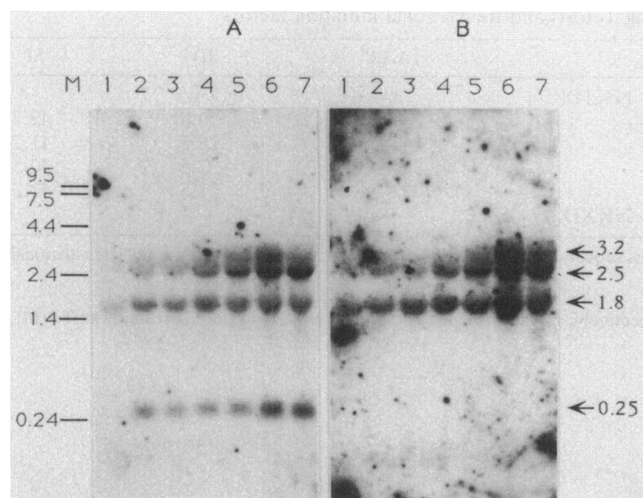

FIG. 4. Identification of transcripts by Northern blot hybridization. RNA was extracted from *E. faecalis* S5-10 cells exposed to different concentrations of tetracycline. Lanes: 1, RNA from a transposon-free control strain (OG1SS); 2 through 6, RNAs from S5-10 cells exposed for 4 h to 0.0, 0.05, 0.5, 5.0, and 20.0 μg of tetracycline per ml, respectively; 7, RNA from cells exposed to 5.0 μg/ml, but for only 10 min. The probe used for panel A was 916LR-P (a 30-mer; Fig. 2), which is homologous to the region upstream of *tet*(M). For panel B, the probe used was 916LR-O (a 31-mer; Fig. 2), which is homologous to a region within *tet*(M) near the 3′ end. Molecular weight markers (M) and the sizes of the designated transcripts are indicated in kilobases.

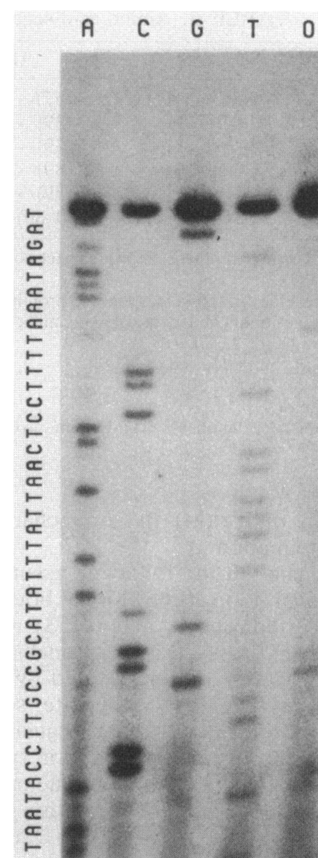

FIG. 5. Determination of the initiation site of the leader and *tet*(M) transcripts by primer extension and RNA sequencing. In lanes A, C, G, and T, the reaction was carried out with reverse transcriptase in the presence of deoxyribonucleoside triphosphates, appropriate dideoxyribonucleoside triphosphates, and total RNA extracted from *E. faecalis* S5-10 cells exposed to 20 μg of tetracycline per ml for 4 h. In lane 0, no dideoxyribonucleoside triphosphates were present. The primer used was oligonucleotide 916LR-G (5′-TATTTGTTCCCGCTATC-3′), which was 5′ end labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The top dark bands across all five lanes represent the base T complementary to the 5′ initiation base A of the transcripts. See also Fig. 2.

by Burdett (5). Interestingly, our computer analyses found that the amino-terminal 131 amino acids of Tet(M) share a significant degree of homology with about 120 amino acids in the middle of translational initiation factor 2 of both *E. coli* (19, 30) and *Bacillus* spp. (2, 36). The similarities are shown in Table 2. It was pointed out by Burdett (5) that elongation factor (EF)-G and EF-Tu homologies were related to GTP binding domains; this is also the case for translational initiation factor 2.

In the case of the leader region upstream of *tet*(M), there were only one to four differences in nucleotides at corresponding positions for the three different *tet*(M) genes noted above, whereas the *tet*(O)-related sequences showed similarity only from a point 3′ of proline (P) codon CCC (and including this codon). No similarity was observed with regions upstream of the initiation and elongation factor genes noted.

DISCUSSION

The *tet*(M) sequence reported here is in close agreement with that derived independently by Burdett (4). (Differences at only a few nucleotide positions were observed.) The Burdett sequence, however, did not include promoter $P_{tet(M)}$ and the apparent factor-independent transcriptional terminator, $T_{tet(M)}$, downstream of the gene. Our observation of several inverted repeat sequences within a short open reading frame (28 amino acid residues) ending immediately upstream of the start site of *tet*(M) suggested that regulation might involve transcriptional attenuation (20, 40). That such a phenomenon was indeed involved was supported by mRNA analyses which showed that a 2.5-kb transcript homologous to the *tet*(M) determinant was synthesized as an extension of a small (0.25-kb) transcript. The size of the small RNA agreed well with the distance between $P_{tet(M)}$ and T_{leader}, whereas the size of the larger transcript was consistent with an extension to $T_{tet(M)}$. Exposure of cells to tetracycline resulted in a significant increase in the amount of the 2.5-kb RNA relative to the smaller transcript; this result is strongly suggestive of enhanced read-through. However, since the amount of the 0.25-kb transcript did not decrease and the total amount of RNA (i.e., 0.25- + 2.5-kb transcripts) increased (e.g., about fivefold at 20 μg of drug per ml), the antibiotic would also appear to enhance transcriptional initiation. An alternative interpretation is that transcriptional initiation at the promoter, as well as read-through, remains constant but that the extended transcript is more stable in the presence of the drug. It is difficult, however, to envision how this would work. It is conceivable, though, that the longer transcript is more stable than the shorter, regardless of the mechanism(s) governing the induction phenomenon. On the basis of the structure of the region upstream of *tet*(M), subsequently referred to as the leader transcript, we suggest the following model for the regulation

TABLE 2. Amino acid sequence similarities among Tet(M) and translational initiation factors

Protein	Alignment[a]	AAR[b]	ID[c]	SI[c]
Tet(M)	AHVDAGK----(57)--- DTPG ---(50)----NKID	1–131		
BSUIF2[d]	G · · · H · · ----(39)-- · · · · · · · · ---(50)--- · · · · · · ·	217–329	29.0	42.7
BSTIF2G[e]	G · · · H · · ----(39)-- · · · · · · · · ---(50)--- · · · · · · ·	243–354	28.5	41.2
ECOIFA2A[f]	G · · · H · · ----(39)-- · · · · · · · · ---(50)--- · · · · · · ·	389–501	27.5	40.5
ECOIF2B[g]	G · · · H · · ----(39)-- · · · · · · · · ---(50)--- · · · · · · ·	232–344	27.5	41.2
Consensus[h]	GXXXXGK-- (40–80)--DXXG--(40–80)--NKXD			

[a] Hyphens and numbers in parentheses indicate spaces between the three consensus sequences. X indicates any amino acid. Dots indicate amino acids identical to those in Tet(M).
[b] AAR amino acid residues in the corresponding protein aligned with Tet(M).
[c] ID and SI, percentages of aligned identical and similar amino acid residues, respectively, in the corresponding protein relative to the 131 residues of Tet(M).
[d] *B. subtilis* initiation factor 2 (36).
[e] *B. stearothermophilus* initiation factor 2G (2).
[f] *E. coli* initiation factor 2A (19, 30).
[g] *E. coli* initiation factor 2B (19, 30).
[h] The consensus sequence identifies a GTP binding protein (11).

of the expression of Tet(M) by a mechanism involving transcriptional attenuation.

The model is based on the following assumptions. First, RNA containing stem-loop 5S:6S (Fig. 3, bottom structure), formed by the inverted repeats 5-short (5S) and 6-short (6S) and followed by five U's, is a transcriptional terminator (40); a weaker terminator may be represented by stem-loop 7:8 followed by four U's. (On its own, the latter sequence would not normally be viewed as capable of causing termination [18].) Second, if a ribosome is successful in synthesizing the entire leader peptide, it is likely to continue on to initiate the synthesis of the Tet(M) protein. (The relative positions of the leader peptide stop site, the *tet*(M) Shine-Dalgarno site, and the translational initiation site are optimal for this synthesis [16].) Finally, of the first nine amino acid residues of the leader peptide, five are relatively rare among the aminoacyl-tRNA pool. These involve cysteine, methionine, and histidine. Inspection of the amino acid contents of several proteins among the enterococci [e.g., gelatinase (38), Tet(M), and proteins of Tn*917* (e.g., methylase, resolvase, and transposase; 35)] indicates agreement with observations made in *E. coli* showing that these amino acids are present at the following levels: cysteine, 3.2%; methionine, 1.8%; and histidine, 2.8% (22). The paucity of these amino acids in the aminoacyl-tRNA pool is assumed to significantly limit the rate of translation of this region and to cause the ribosome to lag behind RNA polymerase.

With the above-described assumptions, the regulation of Tet(M) synthesis is viewed as follows. Under normal (drug-free) growth conditions, transcriptional initiation occurs by use of the promoter upstream of the leader sequence. The initiation of translation of the leader peptide occurs normally, with no particular limitations on the formation of the first peptide bond (Met-Leu). However, since three of the next four codons (five of the next seven codons) call for rare aminoacyl-tRNAs, translation is retarded. RNA polymerase proceeds (see below), but transcription is terminated at the relatively strong 5S:6S termination site or, if read-through occurs, possibly at the weaker 7:8 termination site (Fig. 3). Under these conditions, the expression of Tet(M) is limited only to what can be generated as a result of transcriptional read-through of the termination site(s). Read-through of the 5S:6S site could be enhanced by translation occurring close behind transcription. That is, if the ribosome is trailing closely, it would cause disruption of the 5S:6S stem-loop and eliminate transcriptional termination. The size of the ribosome is such that it would have to incorporate the valine at position 7 before the 5S:6S stem-loop would be disrupted. (Ribosomes interact with RNA up to 13 bases ahead of the amino acid incorporation site [15, 42].) The potential 1:2 and 3:4 stem-loop structures (Fig. 3) serve as RNA polymerase pause sites (20), which would provide the ribosome additional opportunity to initiate and catch up. The effect would be that progress of the polymerase would be relatively slow, making the 5S:6S transcriptional termination signal as sensitive as possible to translation of the first six or seven codons.

When cells are exposed to tetracycline, which retards translation, there is a backup of charged tRNAs, including the rare ones needed within positions 3 through 6. The increased availability of these aminoacyl-tRNAs allows translation of the peptide to more easily keep up with transcription, disrupt 5S:6S, and prevent transcriptional termination. Transcription then proceeds into *tet*(M), and translational initiation of *tet*(M) is efficient, owing to the close proximity of the Shine-Dalgarno site to the translational stop site of the leader peptide. A key point here relates to the fact that since the leader sequence is not transcribed as quickly (because of the pause sites), the retardation of translation relative to transcription due to tetracycline is less than that for other genes in the cells.

Another facet of control may relate to the formation of 5L:6L (from 5-long and 6-long) which could occur if transcription manages to pass through 5S:6S when translation is still stalled between amino acid positions 2 and 4. Formation of this stem-loop could result in significant pausing, which would provide additional opportunity for translation to catch up. The strong 5L:6L stem-loop could represent a significant holding signal whose purpose is to ensure that if polymerase must enter *tet*(M) from here, there will be every opportunity for translation to follow. Transcription into *tet*(M) without translation of the leader peptide should still allow some synthesis of Tet(M), since free ribosomes could bind at the *tet*(M) Shine-Dalgarno site. The process may be designed to ensure a basal level of Tet(M) while also contributing to the enhancement of translational initiation under conditions of inhibition by tetracycline. It is evident that some Tet(M) is expressed in the cell in the absence of the drug, as has been reported by Burdett (3), ensuring that some translation could still occur, albeit more slowly, if cells were suddenly exposed to tetracycline.

The nucleotide sequence reported here for *tet*(M) exhibits significant homology with other *tet*(M) determinants (25, 27, 33) as well as *tet*(O) determinants (21, 23). Nesin et al. (27)

recently reported that the exposure of *S. aureus* containing *tet*(M) to tetracycline resulted in an increase in transcription; however, they did not distinguish between *tet*(M) expression and upstream sequence expression. The upstream sequence in that system was similar to the *tet*(M) sequence of Tn*916*, as was the *tet*(M) sequence of both Tn*1545* (25) and the chromosome of *U. urealyticum* (33). Thus, the *tet*(M) systems must be regulated in a similar manner.

tet(M) has been reported (5, 32) to exhibit homology with translational elongation factors EF-G and EF-Tu; we have noted homology with translational initiation factor 2 as well (Table 2). In all of these cases, homology relates to GTP binding domains. It was speculated that Tet(M) might act by substituting for an elongation factor (32); however, Burdett (5) was unable to get *tet*(M) to substitute for temperature-sensitive lesions of EF-Tu in *Bacillus subtilis* or EF-G in *E. coli*. It would be interesting to see whether Tet(M) could substitute for translational initiation factor 2.

Finally, the significance of the low level of transcriptional read-through beyond the transcriptional terminator of *tet*(M) (giving rise to a 3.2-kb transcript) is not clear. It is noteworthy that the transcription of *tet*(M) is oriented in such a way that it proceeds toward the left end of Tn*916*. The left end of the element has been shown to contain key determinants for transposition (34). Genes exhibiting homology with *int* and *xis* of lambdoid bacteriophages have been identified close to the left end of the element (8) and in the equivalent position in Tn*1545* (28). Since *tet*(M) appears to be similarly positioned with respect to the left end of several related conjugative transposons (9), the possibility that the expression of this determinant might affect the regulation of Tn*916* movement has been considered. More work will be necessary to determine whether the 3.2-kb transcript noted here is involved.

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