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Identification and Sequence of a *tet*(M) Tetracycline Resistance Determinant Homologue in Clinical Isolates of *Escherichia coli*

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The presence of the tetracycline resistance determinant *tet*(M) in human clinical isolates of *Escherichia coli* is described for the first time in this report. The homologue was >99% identical to the *tet*(M) genes reported to occur in *Lactobacillus plantarum*, *Neisseria meningitidis*, and *Streptococcus agalactiae*, and 3% of the residues in its deduced amino acid sequence diverge from *tet*(M) of *Staphylococcus aureus*. Sequence analysis of the regions immediately flanking the gene revealed that sequences upstream of *tet*(M) in *E. coli* have homology to Tn*916*; however, a complete IS*26* insertion element was present immediately upstream of the promoter element. Downstream from the termination codon is an insertion sequence that was homologous to the IS*Vs1* element reported to occur in a plasmid from *Vibrio salmonicida* that has been associated with another tetracycline resistance determinant, *tet*(E). Results of mating experiments demonstrated that the *E. coli tet*(M) gene was on a mobile element so that resistance to tetracycline and minocycline could be transferred to a susceptible strain by conjugation. Expression of the cloned *tet*(M) gene, under the control of its own promoter, provided tetracycline and minocycline resistance to the *E. coli* host.

Tetracyclines are a family of broad-spectrum antibiotics with an excellent safety profile that, some 6 decades after their discovery, still have clinical utility, albeit somewhat limited (12, 32). Tetracyclines have been found to be effective for the treatment of human parasitic diseases and are, in fact, the drug class of choice for treating mefloquine-resistant *Plasmodium falciparum* infection. Tetracyclines also have a number of non-antibacterial effects that are presumed to play a role in the utility of the drug family for the treatment of periodontal disease and acne (12, 32). The continued spread of tetracycline resistance determinants among clinically important pathogens, aided by nonclinical uses of the compound, has severely limited the clinical utility of the drug class (12, 31, 32).

Tetracycline resistance in bacteria is mediated by four mechanisms: efflux, ribosomal protection, enzymatic inactivation, and target modification (12, 33). Efflux and ribosomal protection are widely distributed among both gram-negative and gram-positive organisms, whereas the other two mechanisms have each been described for only a few bacterial genera (12, 33, 35, 40). Since the first report of transferable tetracycline resistance in *Shigella dysenteriae* in 1960 (2), 23 genes encoding efflux pumps and 11 genes encoding ribosomal protection proteins have been described for bacteria (12, 31, 33). Ribosomal protection as a mechanism of tetracycline resistance was first reported for streptococci in 1986 (9). The mediators of this resistance mechanism are proteins (e.g., TetM, TetO, TetQ, and TetS) that have the ability to block the binding of and/or displace tetracycline from the 30S subunit of the ribosome (12).

The broad distribution of the ribosomal protection gene *tet*(M) is due to its association with highly permissive conjugative transposons, such as Tn*916* in *Streptococcus* spp. and Tn*1545* in *Enterococcus* spp. (13). These elements have been identified in over 50 different bacterial genera, both gram negative and gram positive, and have played a major role in the spread of antibiotic resistance determinants between and across genera (12, 13).

Although *tet*(M) is widely distributed and provides functional resistance to tetracyclines when expressed in *Escherichia coli*, a *tet*(M) homologue had not been identified in an environmental or clinical isolate of *E. coli* until 2004, when it was identified in strains isolated from pigs and chickens (8). The analysis by Bryan et al. (8) examined the presence of 12 tetracycline resistance determinants in natural, non-selected, nonclinical *E. coli* strains from humans and animal sources and was the first report of the *tet*(M) determinant in an isolate of *E. coli* (8).

Tigecycline is the novel 9-*t*-butyl glycylamido derivative of minocycline that has been approved for clinical use in the treatment of complicated skin and skin structure infections and complicated intra-abdominal infections worldwide (39). During the course of the tigecycline clinical studies, all bacterial isolates were screened for susceptibility to tetracycline and minocycline among a panel of other antibiotics. Isolates of *E. coli* that were resistant to minocycline and/or tetracycline (MIC $\geq$ 8 μg/ml) were screened by PCR for the presence of tetracycline resistance determinants, including *tet*(M). Three isolates from two different patients were found to carry *tet*(M). This is the first report of the identification of the *tet*(M) tetracycline resistance determinant originating in a human clinical isolate of *E. coli*.

MATERIALS AND METHODS

Bacterial strains. Clinical isolates of *E. coli* (Table 1) were from the phase 3 double-blind clinical trials comparing the safety and efficacy of tigecycline to those of an active comparator for the treatment of complicated intra-abdominal infections (2002 to 2004) (6). Patient specimens were processed and bacterial pathogens were cultured by each site laboratory according to local practices. Individual

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TABLE 1. *E. coli* strains used in study

Strain	Characteristic(s)	Source or reference[a]
NCTC 50268	*tet*(A)	NCTC
NCTC 50365	*tet*(B)	NCTC
NCTC 50270	*tet*(C)	NCTC
NCTC 50271	*tet*(D)	NCTC
NCTC 50272	*tet*(E)	NCTC
GC2270	*tet*(M)[b]	Wyeth Collection
DH5α	Cloning strain	18
GAR3139	*tet*(M) *tet*(A)	7
GAR3141	*tet*(M) *tet*(A)	7
GAR3142	*tet*(M) *tet*(A)	7
GAR7071	Tetracycline susceptible, levofloxacin resistant	7
GAR7090	Tetracycline susceptible, levofloxacin resistant	7
GC7939	*tet*(M), *tet*(A) transconjugant; GAR3139 donor, GAR7071 recipient	This study
GC7940	*tet*(M) *tet*(A) transconjugant; GAR3139 donor, GAR7090 recipient	This study
GC7941	*tet*(M) *tet*(A) transconjugant; GAR3141 donor, GAR7071 recipient	This study
GC7942	*tet*(M) *tet*(A) transconjugant; GAR3141 donor, GAR7071 recipient	This study
GC7949	*tet*(M) cloned into pCR-XL-TOPO	This study

[a] NCTC, National Collection of Type Cultures (www.hpa.org.uk).
[b] *S. aureus tet*(M) cloned into pUC19 (Wyeth, unpublished data).

investigators sent all bacterial isolates to a central laboratory for identification and susceptibility testing.

Susceptibility determination. Susceptibility tests were performed by broth microdilution with Mueller-Hinton II broth (MHB) as recommended by the Clinical Laboratory Standards Institute (CLSI [formerly the NCCLS]) (14, 24). A screening test for resistance to tetracycline was also performed using the disk diffusion method according to standard protocols (23).

Amplification of tetracycline resistance determinants. In order to monitor the presence of resistance determinants for tetracyclines (minocycline and tetracycline), PCR assays were developed and optimized using published sequence information (3, 4, 21, 27, 34) and sequence information directly deposited in GenBank (www.ncbi.nlm.nih.gov) for primer design. Primer sequences, primer location (starting base pair), and expected amplicon size are shown in Table 2. In addition to the resistance determinant, a primer pair specific for 16S rRNA was also included for internal standardization and quality control of the assay (30). Control strains that were previously characterized with respect to antibiotic resistance and the presence of specific determinants were utilized for assay development (Table 1). DNA was obtained from whole-cell lysates as follows. *E. coli* isolates were plated on Luria agar (Becton Dickenson and Company, Cockeysville, MD), and following overnight incubation, several colonies were collected with a 10-μl loop, resuspended in 500 μl of distilled water, and incubated for 5 min in a boiling water bath. The lysate was subjected to brief centrifugation (Savant SFA13K; Savant, Farmingdale, NY) at 13,000 × *g*, and 1 μl of the supernatant was used as the template for amplification.

The FAILSAFE PCR system (Epicenter Technologies, Madison, WI) was used for amplification. Appropriate buffers were experimentally identified for each primer set. One microliter of the whole-cell lysate was used in the PCR assay in a 25-μl volume reaction mixture. Cycling conditions were as follows: the initial denaturation step was for 5 min at 94°C; amplification was 30 cycles of 1 min at 56°C, 1 min at 72°C, 1 min at 94°C, and a final 1 min at 45°C; and extension was for 5 min at 75°C. The reaction products were resolved on a 0.8% agarose gel containing 0.5 μg/ml ethidium bromide. A 1-kb ladder (Fermentas, Hanover, MD) was run on each gel as a size reference.

Sequence determination. PCR amplicons were TA cloned into pCR-XL-TOPO (Invitrogen, Carlsbad, CA) for sequence analysis using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit mix (version 3.1; Applied Biosystems, Foster City, CA). Amplification reactions were performed on a model PTC-225 cycler (Bio-Rad, Hercules, CA) for 40 cycles (96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 2 min), and the excess dye was removed by gel filtration on a 96-well Performa DTR plate (from EdgeBiosystems, Gaithersburg, MD). The samples were heat denatured for 2 min at 90 to 95°C and separated by electrophoresis on a model ABI3730 genetic analyzer (Applied Biosystems, Foster City, CA) under conditions recommended by the manufacturer. Manual sequence editing was performed using Sequencher 4.2 (Gene Codes, Ann Arbor, MI).

Genomic analysis. Sequences were identified by BLAST analysis (5) against sequences in the GenBank database (www.ncbi.nlm.nih.gov).

Conjugation studies. Conjugation experiments were performed using filter mating (1). Overnight cultures of tetracycline-resistant, levofloxacin-susceptible donor (GAR3139 and GAR3141) and tetracycline-susceptible, levofloxacin-resistant recipient (GAR7071 and GAR7090) strains, grown in Luria broth, were seeded at a 1:50 dilution in separate flasks of brain heart infusion broth (BD, Sparks, MD). Following growth to early log phase (approximately 2 h) with shaking at 37°C, 5 ml each of the donor and the recipient cultures were mixed and pelleted by centrifugation (1,500 × *g*). The supernatant was decanted, and the pellet was carefully spread onto a nitrocellulose filter (type HA, 45-μm pore size; Millipore, Billerica, MA) and placed on a brain heart infusion broth plate. The plate was incubated for 4 hours at 37°C, after which time the filter was removed from the plate and placed in a tube with 5 ml sterile saline. The tube was vortexed in order to dislodge the cells from the filter, and serial 10-fold dilutions were prepared in sterile saline. Controls containing only the donor or the recipient were similarly processed. One hundred microliters of each dilution was spread on LB plates supplemented with 10 μg/ml tetracycline and 10 μg/ml levofloxacin. Controls were also spread onto LB agar plates containing 10 μg/ml tetracycline or 10 μg/ml levofloxacin or were not subjected to selection. Plates were incubated overnight at 37°C, and colonies were picked onto LB plates containing 10 μg/ml tetracycline and 10 μg/ml levofloxacin for further characterization. RiboPrinting was performed according to the manufacturer's instructions (Dupont-Qualicon, Wilmington, DE) to confirm the transfer of the resistance determinant from donor to recipient.

Southern blotting. Detection of *tet*(M) by hybridization was carried out using a PCR digoxigenin probe and hybridization kit (Roche Molecular Systems, Summerville, NJ) according to the manufacturer's instructions. Chromosomal DNA preparations digested with AccI [single site in *tet*(M)] were transferred to a nylon membrane (Roche Molecular Systems). The digoxigenin-labeled ~1.0-kb *tet*(M) PCR fragment (Table 2) was used as the probe. Membrane-bound DNA was hybridized at 30°C overnight, washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 68°C, developed with the alkaline phosphatase substrate CDP-Star, and visualized on X-ray film.

Cloning. *E. coli tet*(M) was cloned, along with its resident promoter sequences, by using the ec*tet*(M) clone PCR primer pair (Table 2). The amplicon was TA cloned into pCR-XL-TOPO according to manufacturer's instructions (Invitrogen, Carlsbad, CA).

Nucleotide sequence accession number. The complete sequence of the *E. coli tet*(M) gene and the flanking sequences has been deposited in GenBank (accession number DQ534550).

RESULTS

Identification and cloning of *E. coli tet*(M). Of the 1,462 *E. coli* clinical isolates screened from the tigecycline phase 3 clinical trials, 333 (23%) were minocycline resistant (MIC ≥ 8 μg/ml) and 234 (16%) were tetracycline resistant (MIC ≥ 8 μg/ml) but susceptible to minocycline (MIC ≤ 4 μg/ml). Three isolates (GAR3139, GAR3141, and GAR3142), isolated from two patients from Taiwan, of the 567 screened with the *tet*(M) primer set resulted in products of the appropriate size (~1 kb) (Fig. 1 and data not shown). All three strains were also positive for the *tet*(A) determinant.

One strain from each patient was chosen for further study. In order to recover the 5′ and 3′ ends of the *E. coli tet*(M) gene, internal primers were designed from the cloned sequence and paired with corresponding upstream [ec*tet*(M)up] and downstream [ec*tet*(M)dwn] primers derived from the reference

TABLE 2. Primers used in this study

Target	Sequence (5′–3′)[a]	Start point (bp)[b]	Amplicon size (bp)	Reference sequence accession no.[c]
tet(A)	F: 5′ GTA ATT CTG AGC ACT GTC GC R: 5′ CTG CCT GGA CAA AAT TGC TT	25 981	956	AJ313332
tet(B)	F: 5′ GTT ACT CGA TGC CAT GGG GA R: 5′ GAA GGT CAT CGA TAG CAG GA	36 1157	1,121	AB089594
tet(C)	F: 5′ GCG CTR TAT GCR DTG ATG C R: 5′ TGG TCG TCA TCT ACC TGC	148 996	748	J01749
tet(D)	F: 5′ GCG CTR TAT GCR DTG ATG C R: 5′ CAT CCG GAA GTG ATA GC	142 890	748	X65876
tet(E)	F: 5′ GCG CTR TAT GCR DTG ATG C R: 5′ CTA CCT GAC CGA CAC G	162 1011	849	L06940
tet(M)[d]	F: 5′ ATA GAY ACG CCA GGM CAT A R: 5′ GGA GCC CAG AAA GGA TTY GG	698 1768	1,070	M21136
ec*tet*(M)up	F: 5′ GTG ATT CTA AAG TAT CC R: 5′ TAG GAT ACA GTT CTA CC	342 928	586	M21136
ec*tet*(M)dwn	F: 5′ CAA GAA AAG TAT CAT GTG G R: 5′ TTT CAT CTT ATT TAA CAA GAA ACC	1666 2403	737	M21136
ec*tet*(M)up1000	F: 5′ CAA CTA TCA TAG AAA AGG AAT ACG R: 5′ TTC CCA CTG AAA AGA GGT TAT TCC	11016 630	1,004	U09422 M21136
ec*tet*(M)up500	F: 5′ TCA AGC TCT ATC CTA CAG C R: 5′ TTC CCA CTG AAA AGA GGT TAT TCC	11502 630	532	U09422 M21136
ec*tet*(M)dwn500	F: 5′ ATA GTC GGA TAG ATA AAG TAC G R: 5′ AAC TTG GTA AAA AGC ACC C	2353 14495	475	M21136 U09422
ec*tet*(M)dwn1000	F: 5′ ATA GTC GGA TAG ATA AAG TAC G R: 5′ GAC AAG AAC CCA ATG TAA GG	2353 14973	955	M21136 U09422
ec*tet*(M)clone	F: 5′ TTA CAA ATA TGC TCT TAC GTG C R: 5′ TTT CAT CTT ATT TAA CAA GAA ACC	152 2403	2,251	M21136
16S rRNA	F: 5′ GCCAGCAGCGCGGTAATACG R: 5′ GGACTACCAGGGTATCTAATCC	537 808	271	M87484

[a] IUB codes (underlined) are as follows: R, A + G; Y, C + T; D, G + A + T; and M, A + C. F, forward; R, reverse.
[b] Numbering is per the referenced sequence.
[c] GenBank accession numbers (www.ncbi.nlm.nih.gov).
[d] Primers were modified from reference 34.

Staphylococcus aureus (M21136) sequence (25) (Table 2; Fig. 2). PCR resulted in products of the appropriate size, which were cloned and sequenced to complete and reconstruct the sequence of the *E. coli tet*(M) homologue in silico (Fig. 3). The DNA sequences from the two isolates (GAR3139 and GAR3141) analyzed differed by a single nucleotide; however, the substitution was silent (data not shown).

Although highly similar (approximately 90% or greater at the amino acid level) to the 23 full-length *tet*(M) sequences present in GenBank (www.ncbi.nlm.nih.gov), the *E. coli tet*(M) coding sequence was found to be most similar, ≥99.5% at the amino acid level, to the published sequences from *Streptococcus agalactiae* (accession no. AAM99809), *Lactobacillus plantarum* (AAN40886), and *Neisseria meningitidis* (CAA52967), differing by 1, 2, and 3 residues, respectively (15, 17, 38). The results of a phylogenetic analysis of the 23 full-length *tet*(M) genes and the sequence from *E. coli* GAR3141 are presented in Fig. 4. An amino acid alignment of the *E. coli* Tet(M) protein and the Tet(M) proteins from *N. meningitidis* (accession no. CAA52967), *S. agalactiae* (AAM99809), *L. plantarum* (AAN40886), and the *S. aureus* (M21136) and *Enterococcus faecalis* (X56353) strains are shown in Fig. 5. Compared to the *S. aureus* sequence used in the design of the PCR primers (25), the encoded *E. coli* protein differed at 20 amino acid residues.

Upstream sequence determination. In order to capture upstream sequence information, we designed forward-facing primers based on Tn*916* sequences located 500 and 1,000 base pairs upstream of the *S. aureus* coding sequence [ec*tet*(M)up500 and ec*tet*(M)up1000, respectively] and a reverse primer internal to the *E. coli* gene (Table 1; Fig. 2). Attempts to use the primer pair to clone 1,000 base pairs of upstream sequence were unsuccessful; however, the primer pair designed to clone 500 base pairs of upstream sequence resulted in a product. Interestingly, the PCR product was approximately

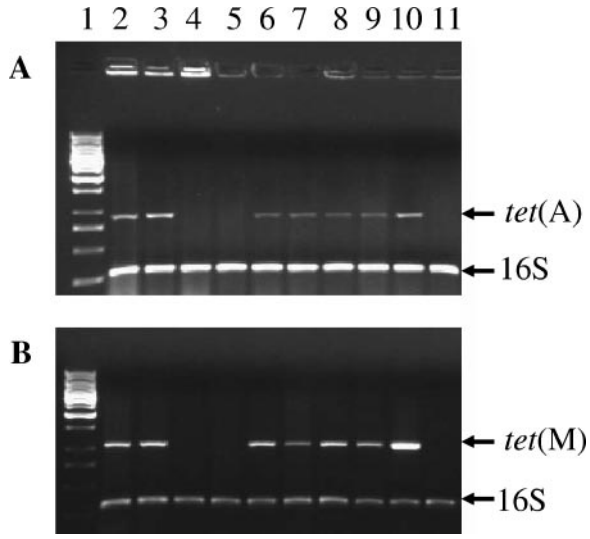

FIG. 1. PCR detection of *tet* resistance markers in *E. coli* clinical isolates. The PCR primer pairs for the detection of *tet*(A) (A) and *tet*(M) (B) are presented in Table 1. Templates were prepared from GAR3139 (lane 2), GAR3141 (lane 3), GAR7071 (lane 4), GAR7090 (lane 5), GC7939 (lane 6), GC7940 (lane 7), GC7941 (lane 8), GC7942 (lane 9), GC2270 as a positive control (lane 10), and DH5α (lane 11). As a control for lysate preparation, gel loading, and the PCR conditions, primers for 16S rRNA were included in the assay. Molecular weight standards were loaded in lane 1 for reference.

1.3 kb instead of the expected 500 bp, suggesting that the region upstream from the *E. coli tet*(M) coding sequence diverged from the published *S. aureus* sequence. Sequence analysis revealed that the inserted sequence was identical to the IS*26* sequence encoding the transposase gene *tnpA*, initially described by Mollet et al. (22). The IS*26* element inserted 113 bp upstream of the −35 element of the *tet*(M) promoter (25, 37). The promoter elements (−10, −35, +1 start site) of the *tet*(M) gene as well as the translational start site and putative regulatory control region within the leader peptide are completely conserved in *E. coli*, by comparison to the sequence published by Nesin et al. (25) (Fig. 3).

Downstream sequence determination. Downstream sequence information was obtained using reverse-facing primers based on Tn*916* sequences 500 and 1,000 base pairs downstream of the *S. aureus tet*(M) coding sequence [ec*tet*(M)dwn500 and ec*tet*(M) dwn1000, respectively] and a forward-facing primer internal to the *E. coli* gene (Table 1; Fig. 2). Again, the attempt to clone the larger downstream fragment using the Tn*916* primer 1 kb from the end of the coding sequence was unsuccessful. However, the use of the ec*tet*(M)dwn500 primer resulted in a fragment approximately 700 bp in length that, upon sequencing, revealed a 200-bp insertion sequence that was identical to a sequence previously reported to occur in the *V. salmonicida* plasmid pRVS1 and referred to as IS*Vs1* (36). The IS*Vs1*

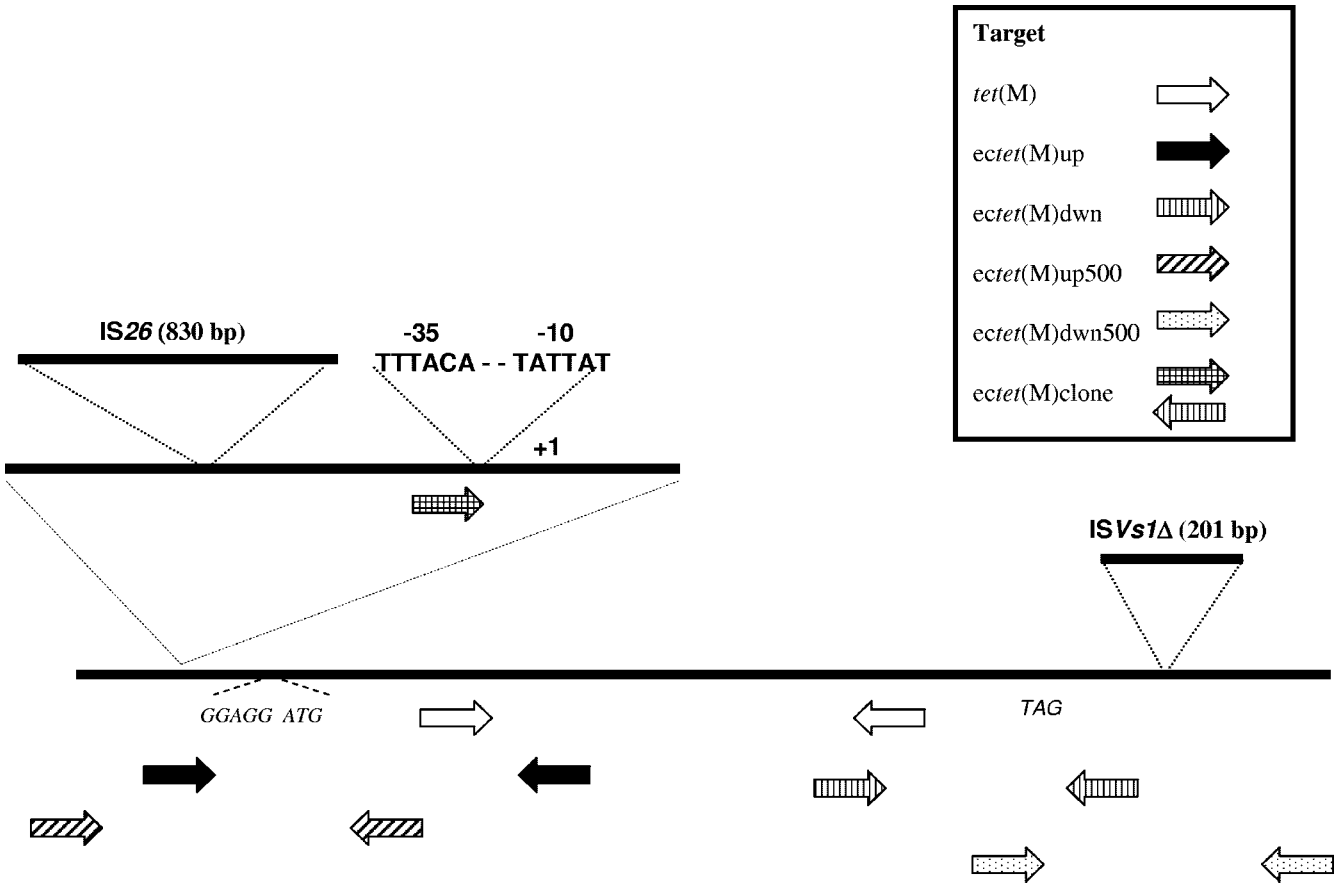

FIG. 2. Cloning strategy and schematic diagram of the *E. coli tet*(M) gene and flanking regions. Locations of primers are indicated with arrows. The IS*26* and IS*Vs1* insertions are also shown diagrammatically along with the *tet*(M) promoter region.

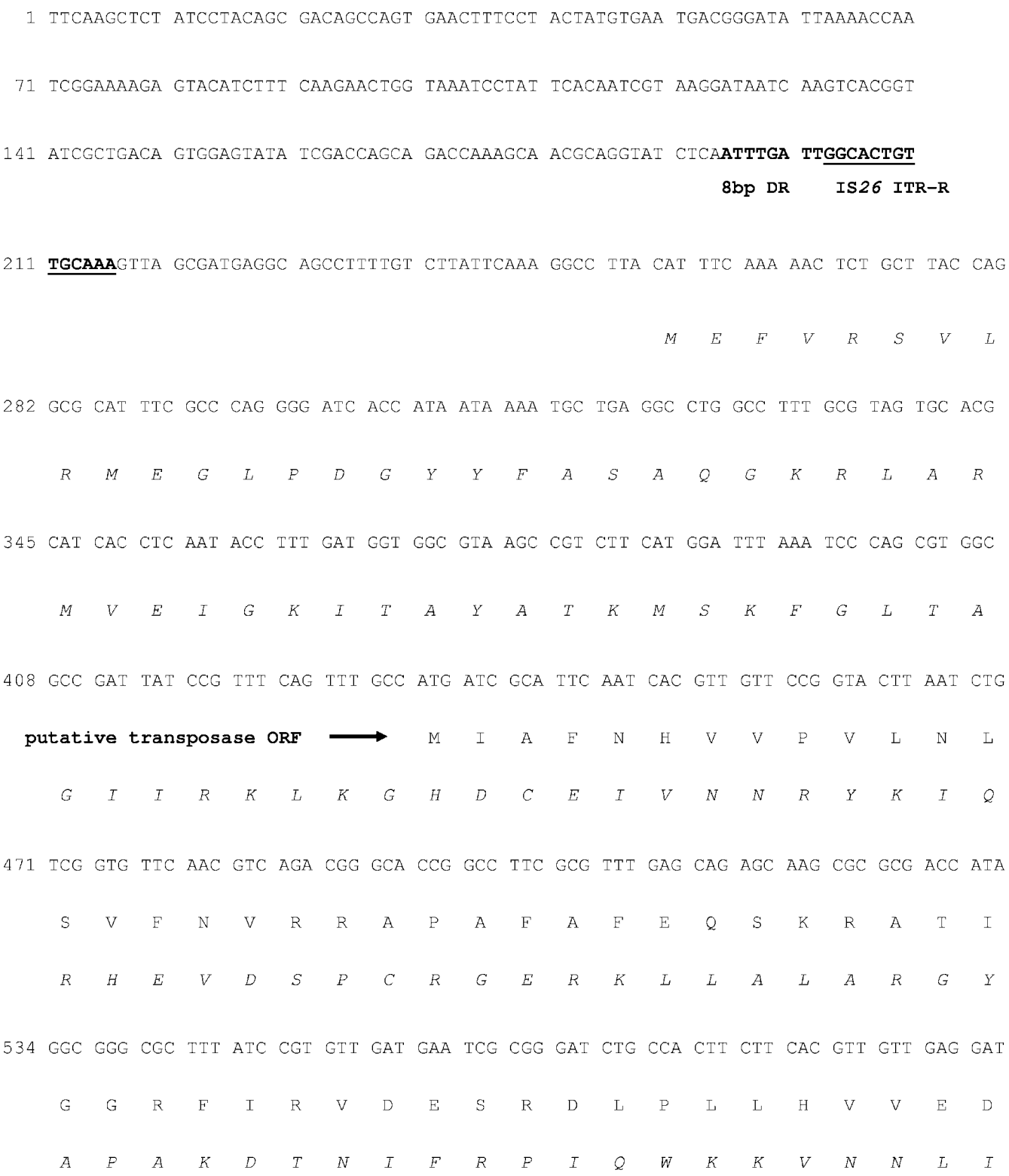

FIG. 3. Nucleotide sequence of the *E. coli tet*(M) gene and flanking regions. The encoded amino acid sequence of *tet*(M) and the putative transposase are presented by the single-letter code under the respective nucleotide sequence. The deduced amino acid sequence of the IS*26* transposase *tnpA* encoded on the noncoding strand of IS*26* is shown in italics for clarity. Transcriptional control elements for *tet*(M) and the start point for the Tet(M) leader peptide are indicated in boldface. The terminal repeat features for IS*26* and IS*Vs1* are also shown in bold and underlined, and the 8-bp direct repeat sequence for IS*26* is presented in bold. The silent point mutation at position 2570 that differentiates the *tet*(M) sequence from those of strains GAR3139 and GAR3141 is indicated in bold and underlined. DR, direct repeat; ITR-R, right inverted terminal repeat; ORF, open reading frame; ITR-L, left inverted terminal repeat; IRL, inverted repeat left.

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597 TTT ACC CAG AAA CCG GTA TGC AGC TTT GCT GTT ACG ACG GGA GGA GAG ATA AAA ATC GAC AGT
     F   T   Q   K   P   V   C   S   F   A   V   T   T   G   G   E   I   K   I   D   S
     K   G   L   F   R   Y   A   A   K   S   N   R   R   S   S   L   Y   F   D   V   T

660 GCG GCC CCG GCT GTC GAC GGC CCG GTA CAG ATA CGC CCA GCG GCC ATT GAC CTT CAC GTA GGT
     A   A   P   A   V   D   G   P   V   Q   I   R   P   A   A   I   D   L   H   V   G
     R   G   R   S   D   V   A   R   Y   L   Y   A   W   R   G   N   V   K   V   Y   T

723 TTC ATC CAT GTG CCA CGG GCA AAG ATC GGA AGG GTT ACG CCA GTA CCA GCG CAG CCG TTT TTC
     F   I   H   V   P   R   A   K   I   G   R   V   T   P   V   P   A   Q   P   F   F
     E   D   M   H   W   P   C   L   D   S   P   N   R   W   Y   W   R   L   R   K   E

786 CAT TTC AGG CGC ATA ACG CTG AAC CCA GCG GTA AAT CGT GGA GTG ATC GAC ATT CAC TCC GCG
     H   F   R   R   I   T   L   N   P   A   V   N   R   G   V   I   D   I   H   S   A
     M   E   P   A   Y   R   Q   V   W   R   Y   I   T   S   H   D   V   N   V   G   R

849 TTC AGC CAG CAT CTC CTG CAG CTC ACG GTA ACT GAT GCC GTA TTT GCA GTA CCA GCG TAC GGC
     F   S   Q   H   L   L   Q   L   T   V   T   D   A   V   F   A   V   P   A   Y   G
     E   A   L   M   E   Q   L   E   R   Y   S   I   G   Y   K   C   Y   W   R   V   A

912 CCA CAG AAT GAT GTC ACG CTG AAA ATG CCG GCC TTT GAA TGG GTT CAT GTG CAG CTC CAT CAG
     P   Q   N   D   V   T   L   K   M   P   A   F   E   W   V   H   V   Q   L   H   Q
     W   L   I   I   D   R   Q   F   H   R   G   K   F   P   N   M   H   L   E   M

                                                                  <-- IS26 TnpA

975 CAA AAG GGG ATG ATA AGT TTA TCA CCA CCG ACT ATT GCC AAC AGT GCC ATT TGA TTTGGTACTT
     Q   K   G   M   I   S   L   S   P   P   T   I   C   N   S   A   I

                                                  IS26 ITR-L      8bp DR
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FIG. 3—*Continued.*

1039 GAAAAGAACG GGAGTAATTG GAAGATTGTA AAATAACAAA TATTGGTACA TGATTACAGA TACTTTGTAA

1109 TCATGTACTC TTTTTGATAA AAAATTGGAG ATTCC**TTTAC A**AATATGCTC TTACGTGC**TA TTA**TTTAAGT

-35 **-10**

1179 G**A**CTATTTAA AAGGAGTTAA TAAATATGCG GCAAGGTATT CTTAAATAAA CTGTCAATTT GATAGCGGGA

+1

1249 ACAAATAATT AGATGTCCTT TTTTAGGAGG GCTTAGTTTT TTGTACCCAG TTTAAGAATA CCTTTATCAT

1319 GTGATTCTAA AGTATCCAGA GAATATCTGT **ATG**CTTTGTA TACCTATGGT TATGCATAAA AATCCCAGTG

Leader peptide ⟶

1389 ATAAAAGTAT TTATCACTGG GATTTTTATG CCCTTTTGGG TTTTTGAATG **GAGGA**AAATCAC ATG AAA ATT

TetM ⟶ M K I

1460 ATT AAT ATT GGA GTT TTA GCT CAT GTT GAT GCA GGA AAA ACT ACC TTA ACA GAA AGC TTA TTA

I N I G V L A H V D A G K T T L T E S L L

1523 TAT AAC AGT GGA GCG ATT ACA GAA TTA GGA AGC GTG GAC AAA GGT ACA ACG AGG ACG GAT AAT

Y N S G A I T E L G S V D K G T T R T D N

1586 ACG CTT TTA GAA CGT CAG AGA GGA ATT ACA ATT CAG ACA GGA ATA ACC TCT TTT CAG TGG GAA

T L L E R Q R G I T I Q T G I T S F Q W E

1649 AAT ACG AAG GTG AAC ATC ATA GAC ACG CCA GGA CAT ATG GAT TTC TTA GCA GAA GTA TAT CGT

N T K V N I I D T P G H M D F L A E V Y R

1712 TCA TTA TCA GTT TTA GAT GGG GCA ATT CTA CTG ATT TCT GCA AAA GAT GGC GTA CAA GCA CAA

S L S V L D G A I L L I S A K D G V Q A Q

FIG. 3—*Continued.*

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1775 ACT CGT ATA TTA TTT CAT GCA CTT AGG AAA ATG GGG ATT CCC ACA ATC TTT TTT ATC AAT AAG
       T   R   I   L   F   H   A   L   R   K   M   G   I   P   T   I   F   F   I   N   K

1838 ATT GAC CAA AAT GGA ATT GAT TTA TCA ACG GTT TAT CAG GAT ATT AAA GAG AAA CTT TCT GAC
       I   D   Q   N   G   I   D   L   S   T   V   Y   Q   D   I   K   E   K   L   S   D

1901 GAA ATT GTA ATC AAA CAG AAG GTA GAA CTG TAT CCT AAT ATG TGT GTG ACG AAC TTT ACC GAA
       E   I   V   I   K   Q   K   V   E   L   Y   P   N   M   C   V   T   N   F   T   E

1964 TCT GAA CAA TGG GAT ACG GTA ATA GAG GGA AAC GAT GAC CTT TTA GAG AAA TAT ATG TCC GGT
       S   E   Q   W   D   T   V   I   E   G   N   D   D   L   L   E   K   Y   M   S   G

2027 AAA TCA TTA GAA GCA TTG GAA CTC GAA CAA GAG GAA AGC ATA AGA TTT CAT AAT TGT TCC CTG
       K   S   L   E   A   L   E   L   E   Q   E   E   S   I   R   F   H   N   C   S   L

2090 TTC CCT GTT TAT CAC GGA AGT GCA AAA AAC AAT ATA GGG ATT GAT AAC CTT ATA GAA GTG ATT
       F   P   V   Y   H   G   S   A   K   N   N   I   G   I   D   N   L   I   E   V   I

2153 ACG AAT AAA TTT TAT TCA TCA ACA CAT CGA GGT CCG TCT GAA CTT TGC GGA AAT GTT TTC AAA
       T   N   K   F   Y   S   S   T   H   R   G   P   S   E   L   C   G   N   V   F   K

2216 ATT GAA TAT ACA AAA AAA AGA CAA CGT CTT GCA TAT ATA CGC CTT TAT AGT GGA GTA CTA CAT
       I   E   Y   T   K   K   R   Q   R   L   A   Y   I   R   L   Y   S   G   V   L   H

2279 TTA CGA GAT TCG GTT AGA GTA TCA GAA AAA GAA AAA ATA AAA GTT ACA GAA ATG TAT ACT TCA
       L   R   D   S   V   R   V   S   E   K   E   K   I   K   V   T   E   M   Y   T   S

2342 ATA AAT GGT GAA TTA TGT AAG ATT GAT AGA GCT TAT TCT GGA GAA ATT GTT ATT TTG CAA AAT
       I   N   G   E   L   C   K   I   D   R   A   Y   S   G   E   I   V   I   L   Q   N
```

FIG. 3—*Continued.*

2405 GAG TTT TTG AAG TTA AAT AGT GTT CTT GGA GAT ACA AAA CTA TTG CCA CAG AGA AAA AAG ATT

E F L K L N S V L G D T K L L P Q R K K I

2468 GAA AAT CCG CAC CCT CTA CTA CAA ACA ACT GTT GAA CCG AGT AAA CCT GAA CAG AGA GAA ATG

E N P H P L L Q T T V E P S K P E Q R E M

2531 TTG CTT GAT GCC CTT TTG GAA ATC TCA GAT AGT GAT CC**G** CTT CTA CGA TAT TAC GTG GAT TCT

L L D A L L E I S D S D P L L R Y Y V D S

2594 ACG ACA CAT GAA ATT ATA CTT TCT TTC TTA GGG AAA GTA CAA ATG GAA GTG ATT AGT GCA CTG

T T H E I I L S F L G K V Q M E V I S A L

2657 TTG CAA GAA AAG TAT CAT GTG GAG ATA GAA CTA AAA GAG CCT ACA GTC ATT TAT ATG GAG AGA

L Q E K Y H V E I E L K E P T V I Y M E R

2720 CCG TTA AAA AAT GCA GAA TAT ACC ATT CAC ATC GAA GTG CCG CCA AAT CCT TTC TGG GCT TCC

P L K N A E Y T I H I E V P P N P F W A S

2783 ATT GGT TTA TCT GTA TCA CCG CTT CCG TTG GGA AGT GGA ATG CAG TAT GAG AGC TCG GTT TCT

I G L S V S P L P L G S G M Q Y E S S V S

2846 CTT GGA TAC TTA AAT CAA TCA TTT CAA AAT GCA GTT ATG GAA GGG ATA CGC TAT GGT TGC GAA

L G Y L N Q S F Q N A V M E G I R Y G C E

2909 CAA GGA TTA TAT GGT TGG AAT GTG ACG GAT TGT AAA ATC TGT TTT AAG TAT GGC TTA TAC TAT

Q G L Y G W N V T D C K I C F K Y G L Y Y

2972 AGC CCT GTT AGT ACC CCA GCA GAT TTT CGG ATG CTT GCT CCT ATT GTA TTG GAA CAA GTC TTA

S P V S T P A D F R M L A P I V L E Q V L

3035 AAA AAA GCT GGA ACA GAA TTG TTA GAG CCA TAT CTT AGT TTT AAA ATT TAT GCG CCA CAG GAA

K K A G T E L L E P Y L S F K I Y A P Q E

FIG. 3—*Continued.*

```
3098 TAT CTT TCA CGA GCA TAC AAC GAT GCT CCT AAA TAT TGT GCG AAC ATC GTA GAC ACT CAA TTG
      Y   L   S   R   A   Y   N   D   A   P   K   Y   C   A   N   I   V   D   T   Q   L
3161 AAA AAT AAT GAG GTC ATT CTT AGT GGA GAA ATC CCT GCT CGG TGT ATT CAA GAA TAT CGT AGT
      K   N   N   E   V   I   L   S   G   E   I   P   A   R   C   I   Q   E   Y   R   S
3224 GAT TTA ACT TTC TTT ACA AAT GGA CGT AGT GTT TGT TTA ACA GAG TTA AAA GGG TAC CAT GTT
      D   L   T   F   F   T   N   G   R   S   V   C   L   T   E   L   K   G   Y   H   V
3287 ACT ACC GGT GAA CCT GTT TGC CAG CCC CGT CGT CCA AAT AGT CGG ATA GAT AAA GTA CGA TAT
      T   T   G   E   P   V   C   Q   P   R   R   P   N   S   R   I   D   K   V   R   Y
3350 ATG TTC AAT AAA ATA ACT TAG TGTATTTTAT GTTGTTATAT AAATATGGTT TCTTGTTAAA TAAGATGAAA
      M   F   N   K   I   T
3421 TATTTTATAA TAAAGATTTG AATTAAAGTG TAAAGGAGGA GATAGTTATT ATAAACTACA AGTGGATATT
3491 GTGTCCTGTA TGTGGAAATA AAACACGATT AAAGATAAGG GAAGATACTG GGGTTCTAGG GATTTTCCCC
                                                           ISVs1 IRL
3561 TCTAAAGTAA CATAAATCGC CACAACCCTC GCAGTTAATG GCTTGTGGAG CTGGGTTACC TTTCATTTTA
3631 GACTTCATTT TCAACAATAG GCCTAGTTAT TTCCTGTAAA TCAGGTAGTT TGCGGTTACT AGCGAACTGA
3701 TAAGTGCCGC TCAGATTAAT GTTCTGCCAT GCAACGGGTG CTTTTTACCA AGTT
```

FIG. 3—*Continued.*

sequence inserted 170 bp downstream of the *tet*(M) stop codon (Fig. 3). The downstream sequence encoding the putative *tet*(M) transcriptional terminator was not captured for analysis.

Conjugation studies. It was of some interest to determine if the *E. coli tet*(M) gene was associated with a mobile element, as the sequence analysis revealed that at least two insertion sequences were located proximal to the gene. As shown in Table 3, resistance to both tetracycline and minocycline was successfully transferred by conjugation from both GAR3139 and GAR3141 to two tetracycline-susceptible recipient strains (GAR7071 and GAR7090). Ribotyping was used to confirm that the resistance determinants moved from the donor to the recipient (data not shown). In both cases, PCR analysis revealed that both the *E. coli tet*(M) and the *tet*(A) gene were mobilized into the donor. Analysis by direct PCR and Southern blotting using a PCR amplicon [*tet*(M) forward and reverse primers (Table 2)] as the probe demonstrated the presence of the *E. coli tet*(M) gene in the recipient strains (data not shown). MIC analysis of the recipient strains (Table 3) indicated that the *tet*(M) gene is functional in the transconjugants, as *tet*(A) does not efficiently efflux minocycline (28).

Cloning and expression of *E. coli tet*(M). The *E. coli tet*(M) gene was cloned, along with its resident promoter, from the clinical isolate and expressed in *E. coli*. As shown in Table 3, the cloned *E. coli*-derived *tet*(M) gene conferred resistance to

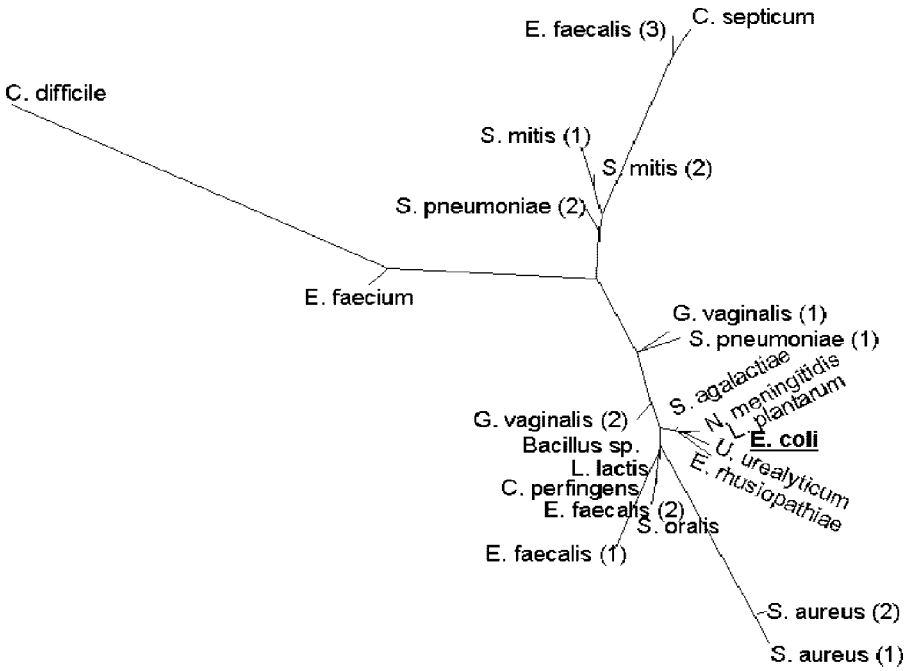

FIG. 4. Phylogenetic tree. An unrooted phylogenetic tree was created from a ClustalW (10) alignment of 24 unique Tet(M) protein sequences. A neighbor-joining tree was drawn by PhyloDraw 0.8 (11). The bar indicates an evolutionary distance of 0.01 amino acid substitution per position. Sequences are derived from *Bacillus* sp. (GenBank accession no. AAM19211), *Clostridium difficile* (accession no. AAO24820), *Clostridium perfringens* (accession no. AAK17952), *Clostridium septicum* (accession no. BAB71968), *E. coli* (this paper), *E. faecalis* 1 (accession no. CAA63530), *E. faecalis* 2 (accession no. CAA39796), *E. faecalis* 3 (accession no. CAA27977), *Enterococcus faecium* (accession no. EAN10521), *Erysipelothrix rhusiopathiae* (accession no. BAB82500), *Gardnerella vaginalis* 1 (accession no. AAB05245), *Gardnerella vaginalis* 2 (accession no. AAB05246), *Lactococcus lactis* (accession no. AAY62599), *L. plantarum* (accession no. AAN40886), *Neisseria meningitidis* (accession no. CAA52967) *S. agalactiae* (accession no. AAM99809), *S. aureus* 1 (accession no. AAA26678), *S. aureus* 2 (accession no. BAB56560), *Streptococcus mitis* 1 (accession no. CAE46077), *S. mitis* 2 (accession no. CAE46076), *Streptococcus oralis* (accession no. CAE46078), *Streptococcus pneumoniae* 1 (accession no. AAS45561), *S. pneumoniae* 2 (accession no. AAR22397), and *Ureaplasma urealyticum* (accession no. AAA73978).

tetracycline and minocycline on the recombinant *E. coli* host strain.

DISCUSSION

The first tetracycline, chlortetracycline (Aureomycin), was identified and developed through the ingenuity and dedication of Benjamin Duggar and Lederle Laboratories in the late1940s (16). Tetracycline resistance mechanisms appeared soon after this antibiotic class was introduced into the marketplace in 1948. Although the efflux mechanism of tetracycline resistance quickly appeared in enteric pathogens (2), the ribosomal protection mechanism of resistance had not been detected in enteric organisms until 2004 (8). This is curious as both resistance mechanisms are associated with mobile elements and the *tet*(M) gene has been detected in other gram-negative hosts, such as *Neisseria*, *Haemophilus* spp., and *Campylobacter* spp.(12).

We report the identification *tet*(M) in clinical isolates of *E. coli* from two patients enrolled in phase 3 clinical trials for the recently approved broad-spectrum antibiotic tigecycline (6, 26). Both patients were undergoing treatment for complicated intra-abdominal infections at the same hospital in Taiwan. The *tet*(M) genes from the clinical isolates were most similar to *tet*(M) genes from *S. agalactiae*, *L. plantarum*, and *N. meningitidis*.

Based on the premise that the source of the *E. coli tet*(M) gene was the Tn*916*/Tn*1545* family of conjugative transposons (13), PCR primers were designed based on Tn*916* sequences located upstream and downstream of the *tet*(M) open reading frame in order to recover the entire gene and flanking regions. The fact that this approach worked indicated that Tn*916* sequences were contained in the element that mobilized into *E. coli*. However, the identification of additional insertion sequences identical to IS*26* and to IS*Vs1* suggested that there may have been one or more intermediate hosts between the original host and the clinical *E. coli* strains. Preliminary experiments using PCR primers 1,000 bp upstream and downstream of the *tet*(M) structural gene to recover additional sequences were unsuccessful, suggesting that only limited Tn*916*-derived sequences remain associated with the *tet*(M) gene in *E. coli*.

The IS*26* insertion sequence was first described in association with Tn*2680*, which encodes kanamycin resistance on the R plasmid, Rts1, from *Proteus vulgaris* (22). IS*26* is 820 bp long and carries 14-bp perfect inverted terminal repeats flanking the *tnpA* gene (20). Upon integration, IS*26* creates an 8-bp duplication at the insertion site; the sequence ATTTGATT is found as a direct repeat flanking the IS*26* inverted terminal repeat at the insertion site of the element upstream of the *tet*(M) promoter in *E. coli*. The IS*Vs1* insertion sequence has been described only in association with a 170-MDa resistance plasmid, pRVS1, which encodes a Tet(E) homologue in *V. salmonicida*

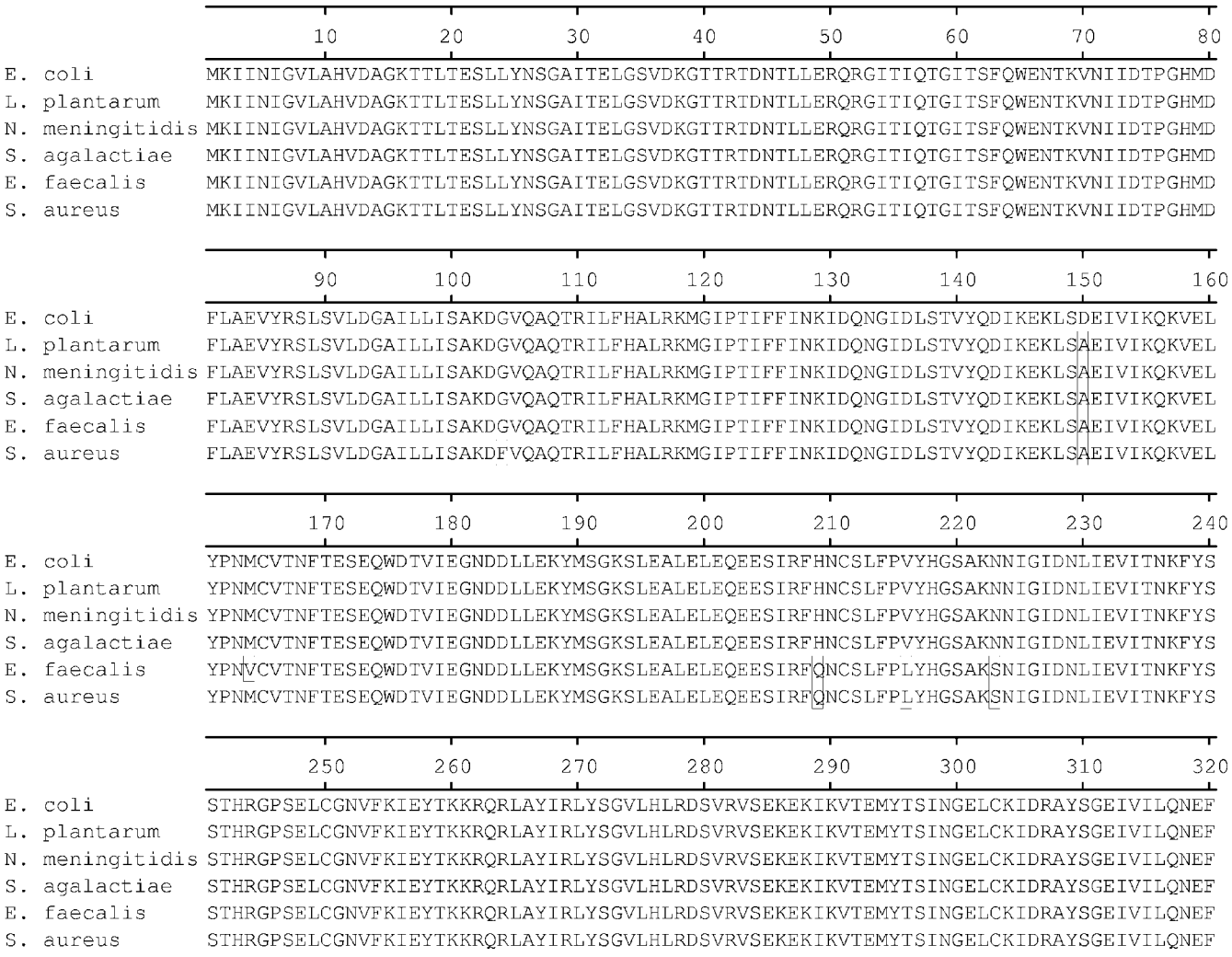

FIG. 5. Amino acid alignment. *E. coli* Tet(M) was aligned with Tet(M) from *L. plantarum* (GenBank accession no. AAN40886), *N. meningitidis* (accession no. CAA52967), *S. agalactiae* (accession no. AAM99809), *E. faecalis* (accession no. CAA39796), and *S. aureus* (accession no. AAA26678). Amino acid residues differing from those of the *E. coli* protein are shown in boxes in the alignment.

(36). The sequence of IS*Vs1* deposited in GenBank encodes a 96-residue truncated transposase gene product, whereas the sequence associated with *tet*(M) encodes the C-terminal 36 residues of the transposase. The sequence in the present report also encodes the complete left inverted terminal repeat region of 43 base pairs.

The IS*26* element inserted 113 bp upstream of the *tet*(M) promoter sequence. All upstream transcriptional and translational control sequences were maintained intact in the *E. coli* gene compared to that reported by Nesin et al. (25). Due to the inability to amplify downstream sequences beyond the IS*Vs1* insertion, we were unable to confirm the presence of the *tet*(M) terminator region reported by previous investigators (37).

We propose that the *E. coli tet*(M) gene originated in a Tn*916* host, most likely a streptococcal species, although the limited flanking sequence data indicate that most Tn*916* sequences have been lost. As IS*Vs1* insertion element sequences have not, until this report, been reported to occur in any organism except *V. salmonicida* (36), we further propose that the element passed through *V. salmonicida* and additional gram-negative hosts, acquiring IS*26* before moving into *E. coli*.

In order to get an unbiased view of the presence and types of *tet* genes in natural (nonclinical) nonselected populations of bacteria in the environment, Bryan et al. (8) screened 1,263 isolates from 12 animal sources and humans for 14 tetracycline resistance genes. The investigators report the presence of *tet*(M) in a number of isolates sourced from both pigs and chickens. A partial sequence, 386 bp, of the *tet*(M) amplicon was found to be 98% identical to the *tet*(M) gene from *E. faecalis* (8). As the complete sequence, including flanking regions, was not reported nor submitted to GenBank, a comparison to the sequences from human clinical isolates was not possible.

The work of a number of investigators (8, 19) suggests that the environmental exposure of humans and animals to tetracyclines and other antibiotics drives the development and dissemination of resistance determinants by horizontal gene transfer. Nevertheless, until recently, the *tet*(M) tetracycline

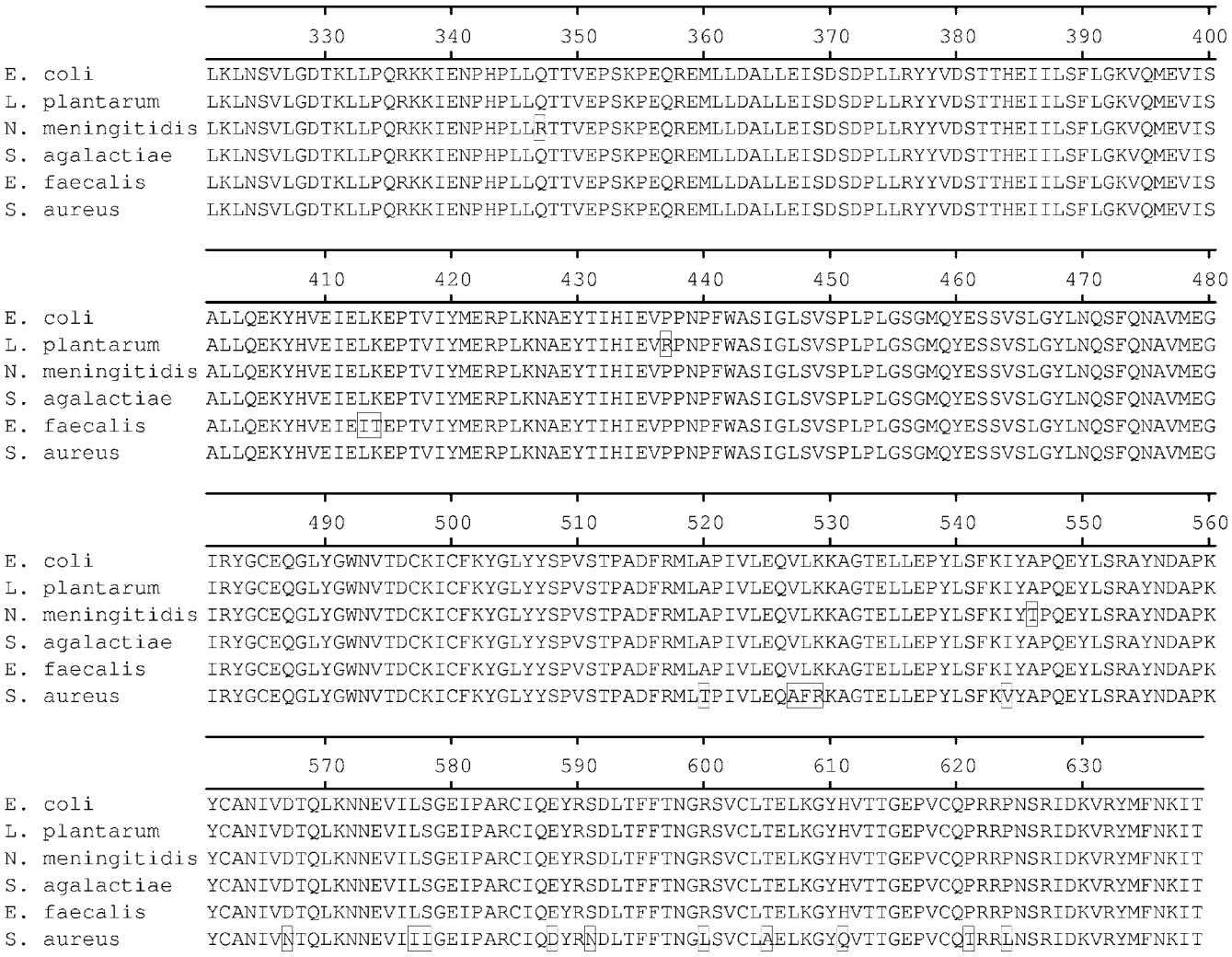

FIG. 5—*Continued.*

resistance determinant had not been reported for *E. coli*. This is despite evidence that cloned *tet*(M) genes from *S. aureus* and *E. faecalis* have been found to be expressed and functional in *E. coli* and the demonstration of the in vivo transfer of Tn*916* from *E. faecalis* to *E. coli* (29). Our data support the recent report (8) of *tet*(M) in *E. coli* isolates from farm animals and extend the finding that the ribosomal protection mechanism of tetracycline resistance, mediated by *tet*(M), has migrated into human clinical isolates of *E coli*.

These findings uphold the continued transfer of antibiotic resistance determinants among various environmental and clinical bacterial populations, which is a motivating factor for

TABLE 3. Susceptibility data

E. coli strain	Tet resistance determinant(s)[a]	MIC (μg/ml)[b]			
		Tetracycline	Minocycline	Levofloxacin	Tobramycin
GAR3139	*tet*(A), *tet*(M)	>64	8	0.25	0.5
GAR3141	*tet*(A), *tet*(M)	>64	4	0.5	0.5
GAR7071	None	2	1	>16	8
GAR7090	None	4	4	>16	1
GC7941	*tet*(A), *tet*(M)	>64	16	>16	4
GC7942	*tet*(A), *tet*(M)	>64	16	>16	0.5
GC7949	*tet*(M)	>64	32	ND	ND
PCR-XL-TOPO	None	1	0.5	ND	ND

[a] Genes were detected by PCR except for the GC7949 strain, which contains *tet*(M) cloned into pCR-XL-TOPO.
[b] ND, not done.

the ongoing search for novel antibacterial agents in the age of resistance. As a response to the diminished utility of the tetracyclines (12, 32, 33), the novel glycylcycline agent tigecycline was recently brought to the marketplace and has shown potent in vitro activity against tetracycline- and minocycline-resistant strains (7, 28).

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