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"*tet*(U)" Is Not a Tetracycline Resistance Determinant

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The enterococcal plasmid pKQ10 has been reported to carry a poorly characterized tetracycline resistance determinant designated *tet*(U). However, in a series of studies intended to further characterize this determinant, we have been unable to substantiate the claim that *tet*(U) confers resistance to tetracyclines. In line with these results, bioinformatic analysis provides compelling evidence that "*tet*(U)" is in fact the misannotated 3′ end of a gene encoding a rolling-circle replication initiator (Rep) protein.

Numerous determinants conferring resistance to the tetracycline (TET) class of antibiotics have been identified in bacteria (7). The majority of these can be assigned to one of three classes according to the mechanism by which they mediate resistance: (i) TET efflux [e.g., *tet*(K)] and (ii) ribosomal protection [e.g., *tet*(M)], which are prevalent in clinical pathogens; and (iii) TET modification [e.g., *tet*(X)], which is found in environmental bacteria. One TET resistance determinant reported in the literature which remains to be assigned to a mechanistic class is *tet*(U) (4, 5). This gene was first identified on plasmid pKQ10 in *Enterococcus faecium* by Ridenhour et al. (4) and has subsequently been detected in vancomycin-resistant *Staphylococcus aureus* (VRSA) (10). Ridenhour and colleagues showed by molecular cloning experiments that *tet*(U) confers resistance to TET in *Escherichia coli* (4), and based on low-level sequence similarity to the Tet(M) protein, proposed that Tet(U) may act to protect the ribosome from TET. However, experimental verification of this hypothesis has not yet been provided.

The present study was therefore initiated to examine whether the Tet(U) protein can protect the translation apparatus from inhibition by TET. Since the original pKQ10 plasmid was no longer available, we obtained a synthetic version of the *tet*(U) gene (open reading frame 1 [ORF1]; GenBank accession no. U01917) with codon usage optimized for expression in *E. coli* (GenScript, Piscataway, NJ). This gene was ligated into a derivative of plasmid pET28, which introduced N-terminal hexahistidine and small ubiquitin-related modifier (SUMO) tags to facilitate purification of soluble protein. Recombinant Tet(U) was successfully overexpressed in *E. coli* strain Rosetta(λDE3) (Merck, Darmstadt, Germany) by autoinduction (6) and purified by Ni^{2+}-affinity and size exclusion chromatography to >95% homogeneity, as determined by SDS-PAGE. The fusion tag was cleaved with SUMO-protease (Invitrogen, Paisley, United Kingdom) and removed using Ni^{2+}-affinity chromatography, yielding protein in the flowthrough corresponding to the expected size of Tet(U). We evaluated the ability of purified Tet(U) to protect an *E. coli*-derived *in vitro* coupled transcription-translation assay (Promega, Madison, WI) in the presence of a concentration of TET (40 μM) sufficient to bring about 80% inhibition of the system. No protection was observed even at a Tet(U) concentration (800 μM) over 100-fold greater than that required of Tet(M) to demonstrate rescue from TET-mediated inhibition in an *in vitro* translation assay (1).

During work to generate purified Tet(U), we noted that *E. coli* cells overexpressing this protein showed no reduction in susceptibility to several representatives of the TET class (tetracycline, minocycline, and doxycycline). This finding contrasted with the original study on *tet*(U), in which this determinant was reported to be functional in *E. coli* (4). We speculated that the nonphysiological nature of our experimental system [i.e., the presence of an affinity tag on Tet(U) and high intracellular concentration of the protein] might prevent detection of the TET^r phenotype. However, a pUC19-based construct expressing native (untagged) Tet(U) from the moderate-strength *lacZ* promoter also failed to confer a reduction in susceptibility to TET in *E. coli* DH5α.

To address the possibility that these constructs were in some way unsuitable for demonstrating TET resistance in *E. coli*, we recreated two *tet*(U)$^+$ plasmids reported by Ridenhour et al. (4) to confer >5-fold increases in TET MIC in *E. coli* DH5α. The entire 1.9-kb pKQ10 plasmid (accession no. U01917) was obtained by synthesis (Genscript), and used to generate constructs pKQ21 (entire pKQ10 plasmid introduced into pBluescript II KS+ via ClaI) and pKQ22 [PCR-amplified *tet*(U) inserted via EcoRI/BamHI into pBluescript II KS+]. Neither construct conferred any reduction in TET susceptibility following introduction into *E. coli* DH5α.

Our findings suggested that *tet*(U) is not a TET resistance determinant. However, it remained possible that the published nucleotide sequence of *tet*(U) contains mutations or DNA sequencing errors which would have rendered the *tet*(U) gene in our constructs inactive. We therefore sought to verify the amino acid sequence of Tet(U) encoded by pKQ10 through comparison with other examples of this protein in GenBank. A BLAST search returned several amino acid sequences exhibiting ~99% identity, all of which differed by a single residue ($E_{41}D$) from Tet(U) encoded by pKQ10. We excluded the possibility that this polymorphism was preventing detection of a TET resistance phenotype by engineering a nucleotide substitution to encode D_{41} into *tet*(U) on plasmids pKQ21 and pKQ22 by QuikChange mutagenesis (Stratagene, La Jolla, CA) and demonstrating that neither of these mutagenized constructs conferred reduced susceptibility to TET in *E. coli*.

BLAST analysis also revealed that, in contrast to that encoded by pKQ10, the Tet(U) amino acid sequence in other entries in GenBank is not a discrete polypeptide; instead, it represents the C-terminal end

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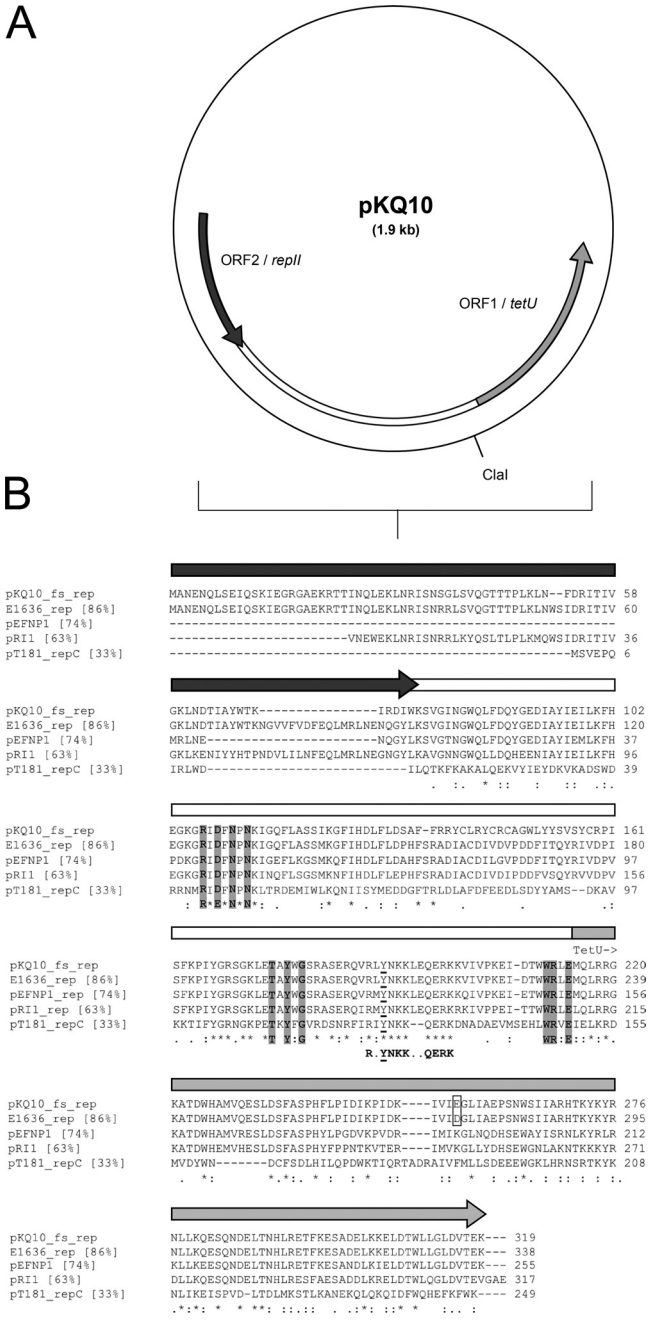

FIG 1 (A) Plasmid map of pKQ10, with annotation of open reading frames (ORFs) according to Ridenhour et al. (4). Based on the analysis presented in this study, we propose that both ORFs in fact form part of a larger, single ORF encoding a Rep protein (see below). (B) Alignment of a frameshift-corrected pKQ10 Rep sequence (pKQ10_ fs-rep) with related Rep proteins. Addition of a single nucleotide in the stop codon of ORF2 in conjunction with a single nucleotide substitution upstream of ORF1 in pKQ10 restores a contiguous gene that when translated is recognized as part of the Rep_trans superfamily (Pfam PF02486). The arrows above the alignment correspond to the ORFs indicated in panel A. The consensus sequence of the prototype pT181 RepC protein active site is indicated in boldface, with the conserved catalytic tyrosine underlined (3). Additional conserved residues important for Rep nicking activity are highlighted in gray (8). Values in square brackets indicate percentage amino acid identity over the region aligned with pKQ10 Rep.

of the rolling-circle replication initiator protein (Rep), a protein commonly encoded by small plasmids in Gram-positive bacteria. Indeed, we note that the pKQ10 nucleotide sequence comprising *tet*(U) and a portion of upstream DNA has previously been reported to share ~75% nucleotide sequence identity with the *rep* genes of the small enterococcal plasmids pRI1 and pEFNP1 (2). It is evident from alignment of the translated nucleotide sequences of plasmids pRI1, pEFNP1, and pKQ10 that the latter carries the nucleotide sequence necessary to encode the highly conserved active site residues typical of Rep initiator proteins (Fig. 1). However, mutations or DNA sequencing errors in the published pKQ10 sequence mean that, as given, it does not encode a full-length Rep protein, but instead appears to encode two smaller proteins corresponding to the N- and C-terminal portions of Rep, the latter of which is "Tet(U)" (Fig. 1).

Further corroboration of the idea that "*tet*(U)" is in fact the 3′ end of a *rep* gene and that the published nucleotide sequence of pKQ10 is probably in error was provided by a study of *E. faecium* strain E1636 (9). The draft genome sequence of this strain includes a contig (contig 159; accession no. NZ_ABRY01000147) of 1.9 kb in length that exhibits 98% DNA sequence identity across the full length of pKQ10. This nucleotide sequence therefore appears to correspond to pKQ10 or an almost identical plasmid, which we confirmed by isolation of a 1.9-kb plasmid from strain E6136, ligation into pBluescript II KS+ via ClaI, and partial DNA sequencing. In contrast to the published pKQ10 nucleotide sequence, this pKQ10-like plasmid encodes only a single protein (an intact, full-length Rep) and not a separate "Tet(U)" protein. This pKQ10-like plasmid also conferred no reduction in TET susceptibility in *E. coli*.

In summary, we have established that "*tet*(U)" is not a TET resistance determinant, but is in fact the misannotated 3′ end of a gene encoding a rolling-circle replication initiator protein.

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