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Novel Tetracycline Resistance Gene, *tet*(32), in the *Clostridium*-Related Human Colonic Anaerobe K10 and Its Transmission In Vitro to the Rumen Anaerobe *Butyrivibrio fibrisolvens*

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A novel tetracycline resistance gene, designated *tet*(32), which confers a high level of tetracycline resistance, was identified in the *Clostridium*-related human colonic anaerobe K10, which also carries *tet*(W). *tet*(32) was transmissible in vitro to the rumen anaerobe *Butyrivibrio fibrisolvens* 2221^R. The predicted gene product of *tet*(32) has 76% amino acid identity with Tet(O). PCR amplification indicated that *tet*(32) is widely distributed in the ovine rumen and in porcine feces.

The widespread use of antibiotics has resulted in the emergence of antibiotic resistance in both human and veterinary pathogens, and resistance mechanisms exist for all antibiotics currently in clinical use (1). Most resistance genes have been isolated from pathogenic bacteria or from antibiotic producers, and there has been comparatively little research on antibiotic resistance in the commensal flora of the human or animal gut. The dominant microorganisms in gut ecosystems are obligate anaerobes, specifically low-G+C-content gram-positive bacteria, bifidobacteria, and members of the gram-negative *Cytophaga-Flavobacter-Bacteroides* phylum (11, 16). It is not clear how much genetic exchange occurs between these obligate anaerobes and the smaller populations of facultative anaerobes that include major pathogenic species.

A new ribosome protection (RP) tetracycline resistance (Tc^r) gene, *tet*(W) (GenBank accession no. AJ222769), identified recently in a range of rumen anaerobes from geographically distant locations (3), was transferable in vitro between strains of *Butyrivibrio fibrisolvens* (13). *tet*(W) was also identified in a *Clostridium*-related human fecal anaerobe, K10, and in *Bifidobacterium longum* isolates (14). We report here a second novel transmissible Tc^r gene, designated *tet*(32) (8), in the commensal anaerobe K10.

The anaerobic bacterial strains *B. fibrisolvens* 2221^R, rifampin-resistant strain 2221 (13), and K10 were cultured in M2GSC broths (10). Transfer of Tc^r was investigated in anaerobic filter matings (13) in the absence of tetracycline. Transconjugants were selected on M2GSC plates containing 10 μg of tetracycline/ml and 100 μg of rifampin/ml, incubated for 2 days. The level of Tc^r was tested using 16-h cultures to inoculate fresh M2GSC broths containing various tetracycline concentrations. Minimum inhibitory tetracycline concentrations, inhibiting 90% of bacterial growth (MIC_{90}), were estimated and confirmed in broth cultures.

DNA was extracted from overnight cultures using either the Wizard Genomic purification kit or the Wizard Plasmid purification kit (Promega, Southampton, United Kingdom). DNA for genomic sequencing was extracted using the QIAGEN Genomic DNA Buffer set and 100/G tips, and for purification of total DNA from fecal and rumen fluid samples, the QIAamp DNA Stool mini-kit (Qiagen, Crawley, United Kingdom) was used. Restriction digestion, Southern blotting, and hybridization of genomic DNA followed standard procedures.

PCR amplification was done using various primer combinations: tetWfor and Tet2, specific for *tet*(W) (14); degenerate primers Tet1 and Tet2, which recognize all known RP-type genes (3); or primers specific for *tet*(32): Tet(32)For (5′ GAA CCAGATGCTGCTCTT 3′) and Tet(32)Rev (5′ CATAGCC ACGCCCACATGAT 3′). Optimizing the annealing temperature of the latter amplification to 57°C resulted in no amplification of the related RP genes, *tet*(M), *tet*(O), *tet*(Q), or *tet*(W). PCR products were sequenced using a *Taq* ABI PRISM kit (Perkin-Elmer, Warrington, United Kingdom), and for direct genomic sequencing (7), the Thermofidelase I enzyme (Fidelity Systems Inc.) was utilized. Sequences were separated on an ABI377 automated sequencer. Sequences were assembled using UWGCG software (6), which is available through the HGMP facility (Human Genome Mapping Project, Cambridge, United Kingdom).

The *tet*(32) gene was identified during investigations into the transmission of *tet*(W) from the human isolate K10 to a Rif^r mutant of the rumen anaerobe *B. fibrisolvens* 2221^R. Tc^r transferred at frequencies of 10^{-4} per donor cell. Surprisingly, although the donor K10 gave the expected *tet*(W) PCR product of 1.8 kb, the transconjugants did not. However, both donor and transconjugants gave the expected 1.3-kb product in PCR amplifications using degenerate RP primers. We concluded that the transferable Tc^r gene in K10 was not *tet*(W) but was a second Tc^r gene, *tet*(32).

The complete sequence of *tet*(32) contains an ORF whose product of 594 amino acids has 76% identity to Tet(O), 71% to Tet(M), and 68% to Tet(W) and Tet(S) (Fig. 1). *tet*(32) has a G+C content of only 40%, which is considerably lower than that of *tet*(W) (53%) but is similar to that of *tet*(M) (35%) and *tet*(O) (40%). The sequence upstream of the *tet*(32) start codon contains a GGAGG ribosome binding site (+7 nt) and two sets of inverted repeats forming secondary stem-loop structures

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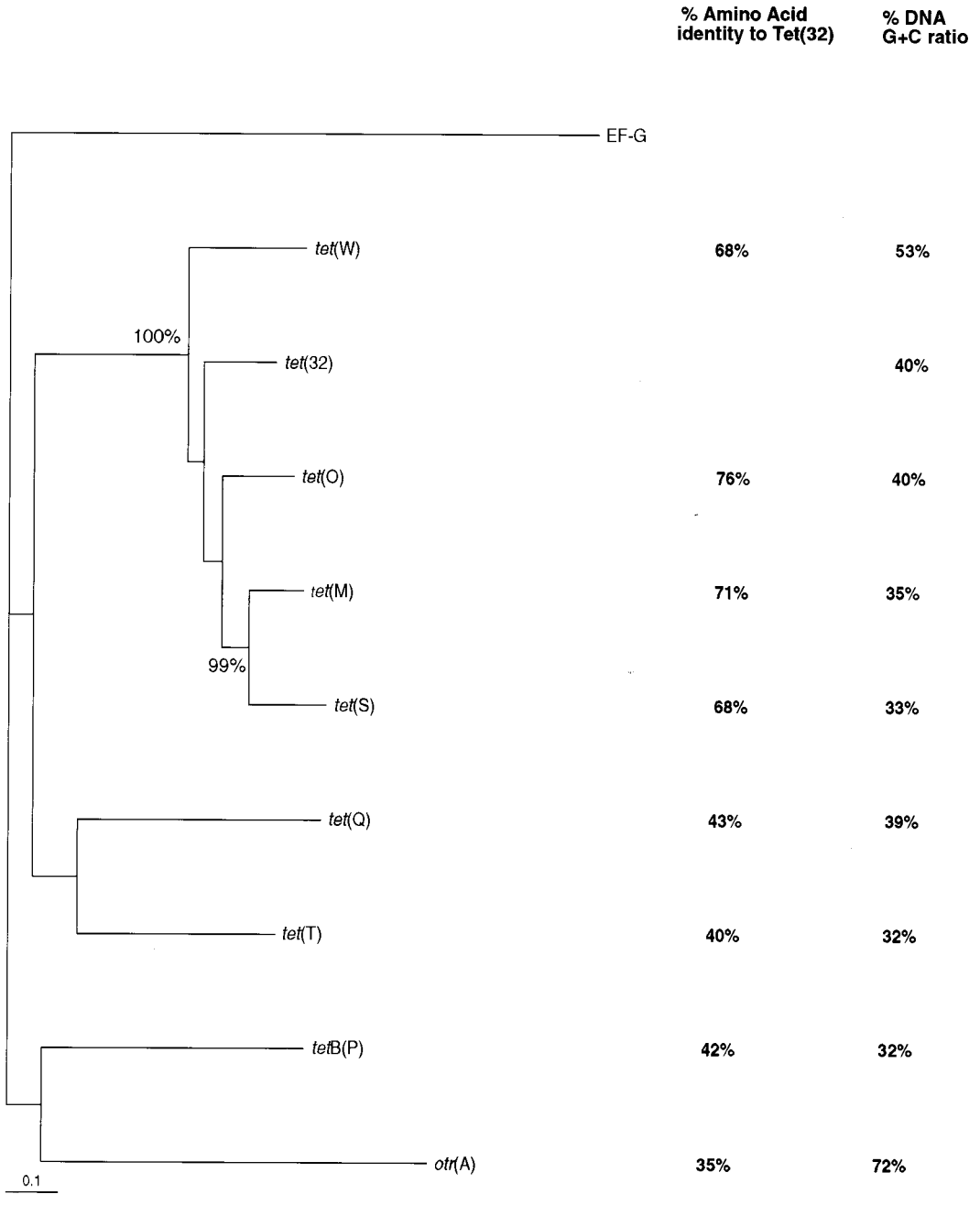

FIG. 1. Phylogenetic tree showing the evolutionary relationships of RP-type Tc^r proteins. The amino acid sequence of the *Aquifex aeolicus fus*A gene (accession no. AE000657) for translation factor EF-G was used to root the tree. GenBank accession numbers are as follows: Tet(O), Y07780; Tet(M), U58986; Tet(S), X92946; Tet(W), AJ222769; Tet(Q), X58717; Tet(T), L42544; TetB(P), L20800; and Otr(A), X53401. Figures beside nodes indicate bootstrap values when greater than 95% (based on 500 trials). Percent G+C content and percent amino acid sequence identity for each sequence relative to *tet*(32) are indicated.

with ΔG values of -20.6 and -14.0 kcal (17). The sequence of this upstream region is virtually identical to that of *Campylobacter jejuni tet*(O) (GenBank accession no. M18896; 4 in 150 nt differences), including the transcription initiation sites and promoter regions (18).

Additional plasmid DNA was not detected in transconjugants, and hybridization of genomic DNA to *tet*(32) identified bands from 9 to 12 kb in size (Fig. 2), implying that *tet*(32) is chromosomally encoded. Hybridization of the same 12-kb *Sma*I fragment in the donor and transconjugants implicated an element of at least this size in *tet*(32) transfer. There was no cross-hybridization to a *B. fibrisolvens* 2221^R transconjugant containing *tet*(W) (3). Transconjugant DNA failed to hybridize to probes specific to regions of conjugative transposons Tn*916* (15) or TnB*1230* (13), indicating that *tet*(32) transfer does not involve similar mobile elements.

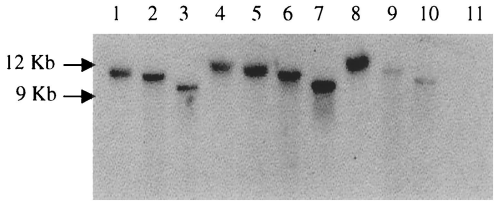

FIG. 2. Southern blot of genomic DNA from *B. fibrisolvens* 2221^R transconjugants Tcm1 (lanes 1 to 4) and Tcm8 (lanes 5 to 8) digested with *Bam*HI, *Eco*RI, *Hin*dIII, or *Sma*I, in that order, and hybridized to a PCR probe specific to *tet*(32). Controls of the *tet*(32) parent strain K10 digested with *Bam*HI (lane 9) and *Eco*RI (lane 10) and a representative *B. fibrisolvens* 2221^R transconjugant containing only *tet*(W) (lane 11) are included.

The presence of *tet*(32) in other gut environments was tested by PCR amplification of total bacterial DNA using specific *tet*(32) primers. Six out of 9 rumen samples from cannulated sheep and 8 out of 11 pig fecal samples, all from different animals, gave PCR products. Selected products were sequenced and confirmed to be *tet*(32), suggesting that *tet*(32) is abundant in farm animals. There were 25 in 584 nt differences between the porcine amplicon sequences and the K10 *tet*(32) gene, corresponding to a 4% sequence divergence.

The resistance profiles of *B. fibrisolvens* 2221^R transconjugants expressing Tet(W) or Tet(32) were compared. Tetracycline concentrations above 20 μg/ml gave a progressive reduction in growth (Fig. 3). Broth cultures based on growth data (Fig. 3) confirmed that the MIC_{90} of tetracycline for bacteria expressing Tet(W) was much lower (90 μg/ml) than that for bacteria expressing Tet(32) (200 μg/ml). For strain K10, which encodes both genes, the MIC_{90} of tetracycline was even higher (270 μg/ml). As with all RP proteins, Tet(32) also confers resistance to minocycline (10 μg/ml).

The identification of a second novel Tc^r gene, *tet*(32), from an anaerobic commensal gut bacterium following the identification of *tet*(W) (3, 14) demonstrates that the gut microflora harbors novel antibiotic resistance genes. It is important to determine the distribution of these novel resistance genes, in both obligate and facultative anaerobes from different gut and nongut habitats. We have shown that *tet*(32) is present among the gut microflora of human, ruminant, and porcine hosts and we know that *tet*(W) has a similar distribution (2, 14). These novel genes could contribute significantly to tetracycline resistance among clinical pathogens where specific resistance genes are currently unidentified (12).

This work provides a third case in which two RP-type Tc^r genes are present in the same bacterium. *B. fibrisolvens* 1.230 carries a mobile *tet*(W) gene and a nonmobile *tet*(O) gene (3); strain K10 carries a mobile *tet*(32) gene and a nonmobile *tet*(W) gene; *Streptococcus pneumoniae* contains both *tet*(M) and *tet*(O) genes (9). This phenomenon could result from intense selection pressure during the evolution of tetracycline resistance and may contribute to higher resistance levels.

The mechanism of transfer of *tet*(32) from strain K10 is unknown but may involve a mobile chromosomal element, as shown for many RP-type Tc^r genes, including *tet*(W) (3), *tet*(Q), and *tet*(M) (12). Although it was first identified as *Fusobacterium prausnitzii,* full-length 16S ribosomal DNA sequencing shows that K10 has less than 95% identity with known species but belongs to the Cluster XIVa *Clostridium* subphylum of low-G+C-content gram-positive bacteria (5). This bacterial cluster includes *B. fibrisolvens* and many other abundant colonizers of the human colon and rumen (4, 19).

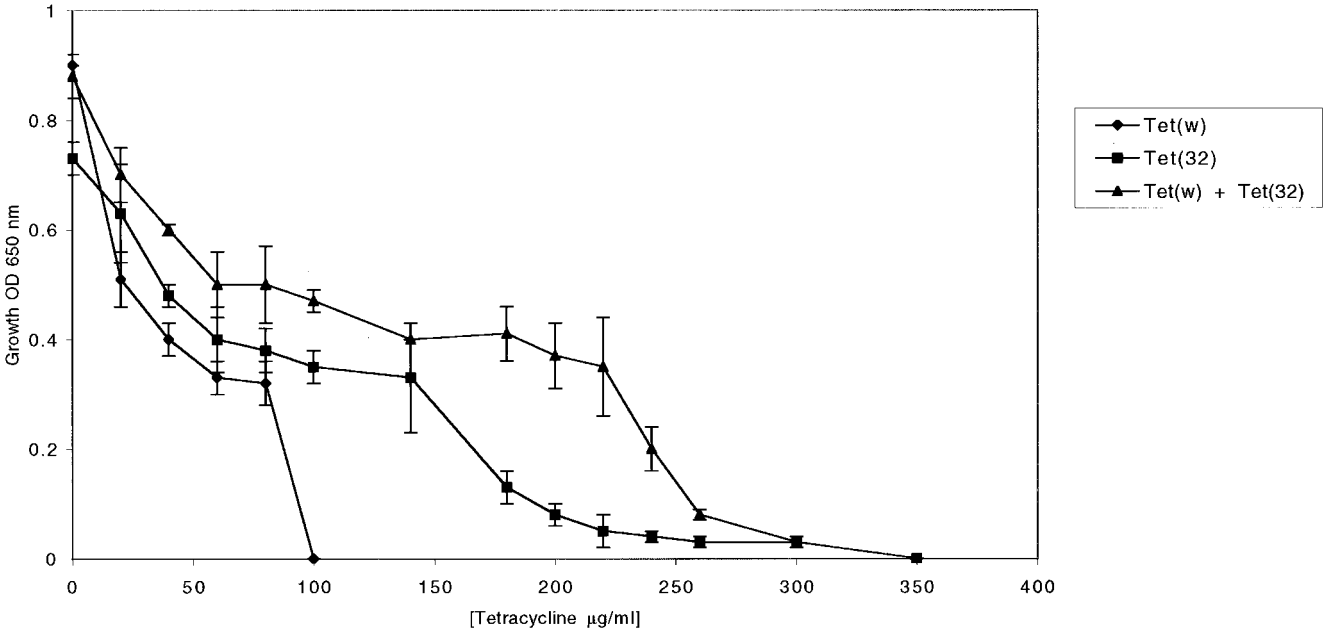

FIG. 3. Levels of tetracycline resistance expressed by Tet(W) and Tet(32) individually following transfer into the host strain *B. fibrisolvens* 2221^R and by Tet(W) and Tet(32) together in the bacterial host K10. Bacteria were grown anaerobically for 16 h in M2GSC broth at 37°C in the concentrations of tetracycline shown. The graph was used to estimate the MIC_{90} for each strain, which was then confirmed in broth culture. OD 600 nm, optical density at 600 nm.

This work provides the first direct experimental evidence that genetic exchange can occur between gram-positive obligate anaerobes from the human colon and those from the rumen. Evidence for transfer between commensal anaerobes and pathogenic gut bacteria is limited, but identical *tet*(O) genes are present in the rumen anaerobe *B. fibrisolvens* and the human pathogen *S. pneumoniae* (3). The species distribution and sequence diversity of the novel *tet*(32) and *tet*(W) genes, and of *tet*(O), should contribute significantly to our understanding of gene flow within and between gut microbial communities.

Nucleotide sequence accession number. The complete sequence of the *tet*(32) gene was submitted to the GenBank database (accession no. AJ295238).

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