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Comparative Analysis of Sequences Flanking *tet*(W) Resistance Genes in Multiple Species of Gut Bacteria

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tet(W) is one of the most abundant tetracycline resistance genes found in bacteria from the mammalian gut and was first identified in the rumen anaerobe _Butyrivibrio fibrisolvens_ 1.230, where it is highly mobile and its transfer is associated with the transposable chromosomal element Tn_B1230_. In order to compare the genetic basis for _tet_(W) carriage in different bacteria, we studied sequences flanking _tet_(W) in representatives of seven bacterial genera originating in diverse gut environments. The sequences 657 bp upstream and 43 bp downstream of _tet_(W) were 96 to 100% similar in all strains examined. A common open reading frame (ORF) was identified downstream of _tet_(W) in five different bacteria, while another conserved ORF that flanked _tet_(W) in _B. fibrisolvens_ 1.230 was also present upstream of _tet_(W) in a human colonic _Roseburia_ isolate and in another rumen _B. fibrisolvens_ isolate. In one species, _Bifidobacterium longum_ (strain F8), a novel transposase was located within the conserved 657-bp region upstream of _tet_(W) and was flanked by imperfect direct repeats. Additional direct repeats 6 bp long were identified on each end of a chromosomal ORF interrupted by the insertion of the putative transposase and the _tet_(W) gene. This _tet_(W) gene was transferable at low frequencies between _Bifidobacterium_ strains. A putative minielement carrying a copy of _tet_(W) was identified in _B. fibrisolvens_ transconjugants that had acquired the _tet_(W) gene on Tn_B1230_. Several different mechanisms, including mechanisms involving plasmids and conjugative transposons, appear to be involved in the horizontal transfer of _tet_(W) genes, but small core regions that may function as minielements are conserved.

Tetracyclines are broad-spectrum antimicrobial agents that are active not only against a wide range of gram-positive and gram-negative bacteria but also against chlamydiae, mycoplasmas, rikettsiae, and protozoan parasites (8, 27, 28). Tetracyclines are widely used in both human and veterinary therapy, as prophylactics, and in many countries in animal feed as growth promoters. Resistance to tetracyclines is the most common bacterial antibiotic resistance found in nature, and tetracycline resistance (Tc^r) genes are present in a variety of bacteria isolated from animal and human feces and from the environment. The majority of tetracycline resistance genes are located on mobilizable or conjugative elements, which may partially explain their wide distribution among bacterial species (8, 27).

Tetracyclines bind to the ribosome and inhibit the elongation phase of protein synthesis by interfering with the binding of the aminoacyl-tRNA to the ribosomal A site (8, 9, 27). Resistance to this antibiotic is commonly associated with tetracycline efflux proteins and ribosomal protection proteins (8, 27). In rare situations, resistance is mediated through direct inactivation of the antibiotic (34) or by 16S rRNA mutation (29). Most studies on bacterial resistance have concerned clinical pathogens, opportunistic pathogens, or antibiotic-producing bacteria. In recent years, however, there has been interest in the carriage of antibiotic resistance genes by commensal bacteria in the human and animal gut (8). In general it was observed that most bacteria causing disease carry the same tetracycline resistance genes as environmental or commensal bacteria. This supports the suggestion that environmental and commensal bacteria act as a reservoir for tetracycline and other antibiotic resistance genes found in pathogens. It is therefore very important to elucidate how antibiotic resistance genes are maintained and spread through commensal bacterial communities.

The ribosome protection-type tetracycline resistance gene *tet*(W) (3, 33) is one of the most widespread tetracycline resistance genes in environmental samples (1, 38). This 1.9-kb gene was originally identified in the rumen commensal anaerobe *Butyrivibrio fibrisolvens*, where it was present on a large mobile chromosomal element (3, 32). The partial sequence of this element, designated Tn*B1230*, was published recently (21) (GenBank accession number AJ222769). Copies of *tet*(W) have also been found in other isolates of *B. fibrisolvens* and in isolates of *Selenomonas* spp., *Mitsuokella* spp., *Clostridium* spp., *Roseburia* spp., *Bifidobacterium longum*, and *Megasphaera elsdenii* from bovine and sheep rumens as well as porcine and human feces (3, 33, 35, 37). The conservation of the *tet*(W) gene sequences from different isolates is remarkably high (3, 33). The *tet*(W) gene was also reported to occur in isolates of the animal pathogen *Arcanobacterium pyogenes*, where it is also carried on a mobile genetic element (5).

The aim of the present study was to determine whether the widespread distribution of *tet*(W) genes can be ascribed to one or a small number of mobile genetic elements or transfer cassettes. We conclude that the genetic context of *tet*(W) varies widely between different bacteria, but the immediate flanking regions reveal several conserved features, suggesting that they might function as a minielement or transfer cassette.

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TABLE 1. Bacterial strains used in this study

Strain(s)	Source	Drug resistance	Reference and/or provider
Bifidobacterium longum F8[a]	Human feces; RRI[b]	Tc^r	33
Butyrivibrio fibrisolvens JK51[a]	Sheep rumen; Australia	Tc^r	Keith Gregg and Jan Kopečný
Butyrivibrio fibrisolvens 1.230[a]	Bovine rumen 1; RRI	Tc^r	32
Clostridium sp. strain K10[a]	Human feces 1; RRI	Tc^r	33
Mitsuokella multiacida P208-58[a]	Pig feces; Japan	Tc^r	23
Roseburia sp. strain A2-183[a]	Human feces 2; RRI	Tc^r	4
Selenomonas ruminantium FB32, FB34, and FB322[a]	Bovine rumen 2; RRI	Tc^r	12
Bifidobacterium adolescentis L2-32	Human feces 3; RRI	Tc^s	4
Bifidobacterium adolescentis L2-32^{Rc}	Spontaneous Rif^r mutant of strain L2-32	Tc^s	This work
Butyrivibrio fibrisolvens 2221^{Rc}	Spontaneous Rif^r mutant of type strain 2221	Tc^s	21
Mitsuokella multiacida F120^{Rc}	Spontaneous Rif^r mutant of strain A405-1	Tc^s	Jennifer Martin
Roseburia inulinivorans A2-194^{Rc}	Human feces 2; RRI; spontaneous Rif^r mutant	Tc^s	4; Jennifer Martin
Selenomonas ruminantium HD4^{Rc}	Sheep rumen; United States; spontaneous Rif^r mutant	Tc^s	19
Butyrivibrio fibrisolvens Tc8 to Tc12, Tc21	Transconjugants from matings between 1.230 and 2221^R	Tc^r	32
Bifidobacterium adolescentis TcKK1 to TcKK5	Transconjugants from matings between F8 and L2-32^R	Tc^r	This work
Bifidobacterium adolescentis LMG11579[d]	Bovine rumen; Gent, Belgium	Tc^r	Liesbeth Masco
Bifidobacterium animalis subsp. *lactis* LMG11580[d]	Chicken feces; Gent, Belgium	Tc^r	Liesbeth Masco
Bifidobacterium bifidum LM588[d]	Probiotic product; Gent, Belgium	Tc^r	Liesbeth Masco
Bifidobacterium pseudocatenulatum LMG11593[d]	Sewage; Gent, Belgium	Tc^r	Liesbeth Masco

[a] Isolate for which flanking sequences were studied in detail in this work.
[b] RRI, Rowett Research Institute.
[c] Strain which was used as a recipient in mating experiments.
[d] Only DNA isolated from the strain was used in this study.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The unrelated bacterial strains, isolated from various human and animal hosts over a period of years (33), that were used in this study are described in Table 1. All strains were routinely grown at 37°C in anaerobic M2SGC broth (24) containing tetracycline (10 μg/ml) or rifampin (100 μg/ml) (antibiotics obtained from Sigma). For conjugation experiments, M2GSC agar plates (2.5% [wt/vol] agar) supplemented with appropriate antibiotics were used. Strains were cultured in Bellco tubes under 100% CO_2 or in an anaerobic cabinet Concept Plus (Ruskin Technology Limited) in an atmosphere of 10% CO_2, 10% H_2, and 80% N_2.

Conjugation experiments. Bacterial matings were based on the method of Hespell and Whitehead (16). Cells were grown overnight anaerobically in M2GSC medium (24) containing appropriate antibiotics. The following morning bacteria were subcultured into fresh medium without antibiotic selection, grown to mid-exponential phase (optical density at 600 nm of 0.4), and pelleted at 1000 × *g* for 15 min at 18°C. The pellet was washed twice anaerobically in 1/4 of the original volume of RGM broth lacking a carbohydrate source (RGM-C) (17) and finally resuspended in 1/10 of the original volume of RGM-C broth. The donor and recipient cultures were then mixed (1:1 ratio, assuming the same cell density) and centrifuged gently. Most of the supernatant was decanted, and the cells were resuspended in the remainder and placed on the center of the 0.2-μm-pore-size Millipore filter disc on an M2GSC agar plate. After incubation on the filter for 16 h, cells were washed in RGM-C buffer and dilutions were plated onto selective M2GSC agar plates containing the appropriate double-antibiotic selection. For matings between *B. longum* F8 and *Butyrivibrio adolescentis* L2-32^R, basal M2 medium with 0.5% starch as a sole carbon source was used. Potential transconjugants grew in 2 days, and their resistance phenotype was confirmed by replating selected colonies on antibiotic plates containing tetracycline and rifampin.

DNA isolation and molecular techniques. Total genomic DNA was isolated from overnight cultures by using the Wizard Genomic DNA purification kit (Promega, Southampton, United Kingdom). Southern blotting and nucleic acid hybridizations were performed following standard procedures (30).

Genome walking of the regions flanking *tet*(W) was carried out using a Universal GenomeWalker kit (BD Biosciences Clontech), following the manufacturer's recommendations. Total genomic DNA digested with a blunt-cutting enzyme was purified and concentrated using Centri-Sep spin columns (Cambio, United Kingdom) prior to ligation to the GenomeWalker adaptor. The ligation was carried out overnight at 16°C, followed by heat inactivation (70°C for 5 min) of the ligase enzyme. The ligation mix was used as the template in a PCR. PCR products for sequencing flanking regions were obtained using BD Advantage polymerase mix (BD Biosciences Clontech). Primer AP1 and nested primer AP2 (provided in the Universal GenomeWalker kit) were used in combination with primer 5′tetW or nested primer 5′GSPtet, and with primer 3′tetW and nested primer 3′GPtet, (Table 2) to amplify regions upstream and downstream of the gene, respectively.

Standard PCR products for sequencing were obtained using BioTaq DNA polymerase (BIOLINE, United Kingdom), optimizing the annealing temperature and extension time according to the specific primers and the size of the expected product. PCR products were sequenced using a *Taq* ABI Prism kit (Perkin-Elmer, Warrington, United Kingdom) and separated on an ABI377 automated sequencer. Sequences were assembled using UWGCG software (10), which was available through the HGMP facility (Human Genome Mapping Project, Cambridge, United Kingdom).

The primer combinations tetW_out-3′GPtet and tetW_out-meth_out (Table 2) were used to amplify any circular intermediates containing *tet*(W).

Nucleotide sequence accession numbers. The nucleotide sequences described in this paper have been deposited in the GenBank database with the following accession numbers: *Roseburia* sp. strain A2-183, AJ421625; *B. fibrisolvens* JK51, AJ427421; *Clostridium* sp. strain K10, AY601650; *Mitsuokella multiacida* P208-58, AY603069; *Selenomonas ruminantium* FB322, DQ294295; *S. ruminantium* FB32, DQ294296; *S. ruminantium* FB34, DQ294297; and *B. longum* F8, DQ294299.

TABLE 2. Primers used in this study

Primer	Sequence (5′→3′)
5′tetW	AGGGCATAAAAATCCCCAGCAGTAAA
5′GSPtet	CCTGTTTGTGATTGCTGTTTTTGGG
3′tetW	TACCTTTCCAGGGCTTATCATGATGC
3′GPtet	TACAGAGCTGAAAGGATATCAGGCCG
tetW_out	GGCATATAGCAGGCTCTCC
meth_out	GGCAATTATGGATATTACGG

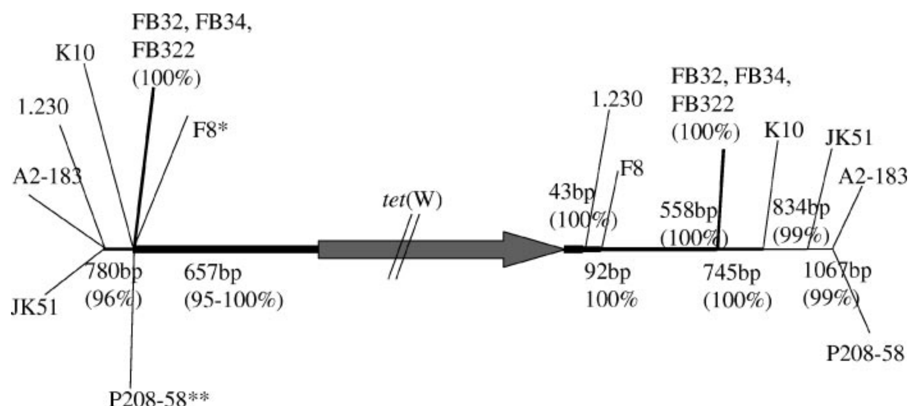

FIG. 1. Diagram showing the points at which the sequences upstream and downstream of *tet*(W) in the nine analyzed isolates diverge. The *tet*(W) ORF is indicated by an arrow. The thickness of the line represents the number of species for which sequence is conserved. As the sequence diverges, the line becomes thinner. This figure is not drawn to scale. *, *B. longum* F8 has insertion of 1,047 bp into the conserved region upstream of *tet*(W). **, *M. multiacida* P208-58 contains an insertion of a 23-bp directly repeated sequence 291 bp upstream from the *tet*(W) start codon.

RESULTS

Sequence conservation in *tet*(W) flanking regions. The regions flanking *tet*(W) in representatives from six different genera of gut commensal bacteria (Table 1) were sequenced, and these sequences were compared to the recently published partial sequence of the *B. fibrisolvens* 1.230 transposon Tn*B1230* (21) (GenBank accession number AJ222769). A 657-bp region upstream from the start codon showed 95 to 100% sequence conservation in all analyzed strains and was also present upstream of the Tn*B1230* *tet*(W) gene (21) (Fig. 1). Putative regulatory regions, including a short, 14-amino-acid (aa) open reading frame (ORF) possibly involved in transcriptional attenuation, were present in the first 330 bp upstream of *tet*(W) (21). *Clostridium* sp. strain K10 was the most divergent, with 22 to 25 randomly spaced base pair differences in the upstream region compared to other strains. In *B. longum* F8 an inserted sequence of 1,047 bp was present 354 bp upstream of the *tet*(W) start codon. The conserved upstream sequence continued to 790 bp in *Roseburia* sp. strain A2-183, *B. fibrisolvens* JK51, and *B. fibrisolvens* 1.230, while the three *S. ruminantium* strains (FB32, FB324, and FB322) were 100% identical throughout the available sequence (5.4 kb) (Fig. 1).

Downstream of *tet*(W) the sequences diverged more rapidly, and only 43 bp was 100% conserved between all analyzed isolates (Fig. 1). Thus, it was possible to distinguish a conserved core region of 2.6 kb, including the *tet*(W) gene, in all strains studied (Fig. 1).

Analysis of ORFs in regions flanking *tet*(W). Potential ORFs were identified in the sequences flanking *tet*(W) (Fig. 2). The deduced amino acid sequences were compared to those present in the nonredundant protein database at the National Center for Biotechnology Information website by using the TBLASTN and BLASTP programs.

(i) MAFF protein-encoding ORFs. It was previously reported that in Tn*B1230*, *tet*(W) is flanked by two identical direct-repeat (DR) DNA sequences (707 bp in length) that encode proteins with significant similarity (38% identity) to bacterial nitroreductases (Nrd) (Fig. 2) (21). An alternative start codon present upstream of DR1 potentially encodes a larger nitroreductase, while the shorter *nrd2* gene within DR2 is truncated at the 5′ end.

Sequences upstream of *tet*(W) in *Roseburia* sp. strain A2-183, *B. fibrisolvens* JK51, and *B. fibrisolvens* 1.230 all contained a short ORF (here designated MAFF to represent the first four amino acids) capable of encoding a 46-aa protein (Fig. 2). This protein showed 87 to 93% amino acid sequence conservation between these three strains and also had 61 to 63% identity to a protein of unknown function found in *Enterococcus faecalis* V583 (GenBank accession number AE016954).

The 46-aa MAFF protein was encoded at the 3′ end of the Tn*B1230* DR. The MAFF ORF downstream of the *nrd2* gene continued outside the DR to encode a 70-aa protein (designated MAFF2) (Fig. 2). The closest database match for this longer protein (79% amino acid sequence identity) was a previously unidentified ORF encoding a hypothetical protein of 71 aa located on the *E. faecalis* conjugative transposon Tn*1549* (14) (GenBank accession number AF192329). In summary, the sequences upstream of *tet*(W) in Tn*B1230* encoded a full-length Nrd and a C-terminally truncated MAFF protein, while the sequences downstream encoded an N-terminally truncated Nrd and a full-length MAFF.

(ii) ORFY-type sequences. A second conserved ORF was found immediately downstream of *tet*(W) in five of the analyzed species from different genera. This ORF encoded a protein of 184 aa (*S. ruminantium* FB32, FB34, and FB322), 246 aa (*Clostridium* sp. strain K10), or 255 aa (*B. fibrisolvens* JK51, *Roseburia* sp. strain A2-183, and *M. multiacida* P208-58), depending on the location of the stop codon (Fig. 2). This ORF was 100% conserved at the amino acid level between most of the analyzed isolates except the *S. ruminantium* strains, where the last 13 residues differed from those in other species. The translated product of the ORF had 28 to 30% identity to a methyltransferase protein (and contained the conserved methyltransferase domain) and to the hypothetical protein OrfY. The *orfY* gene is present in many mobile plasmids and transposons and is often associated with erythromycin resistance genes (6) (GenBank accession number AF516335). *orfY* was also found

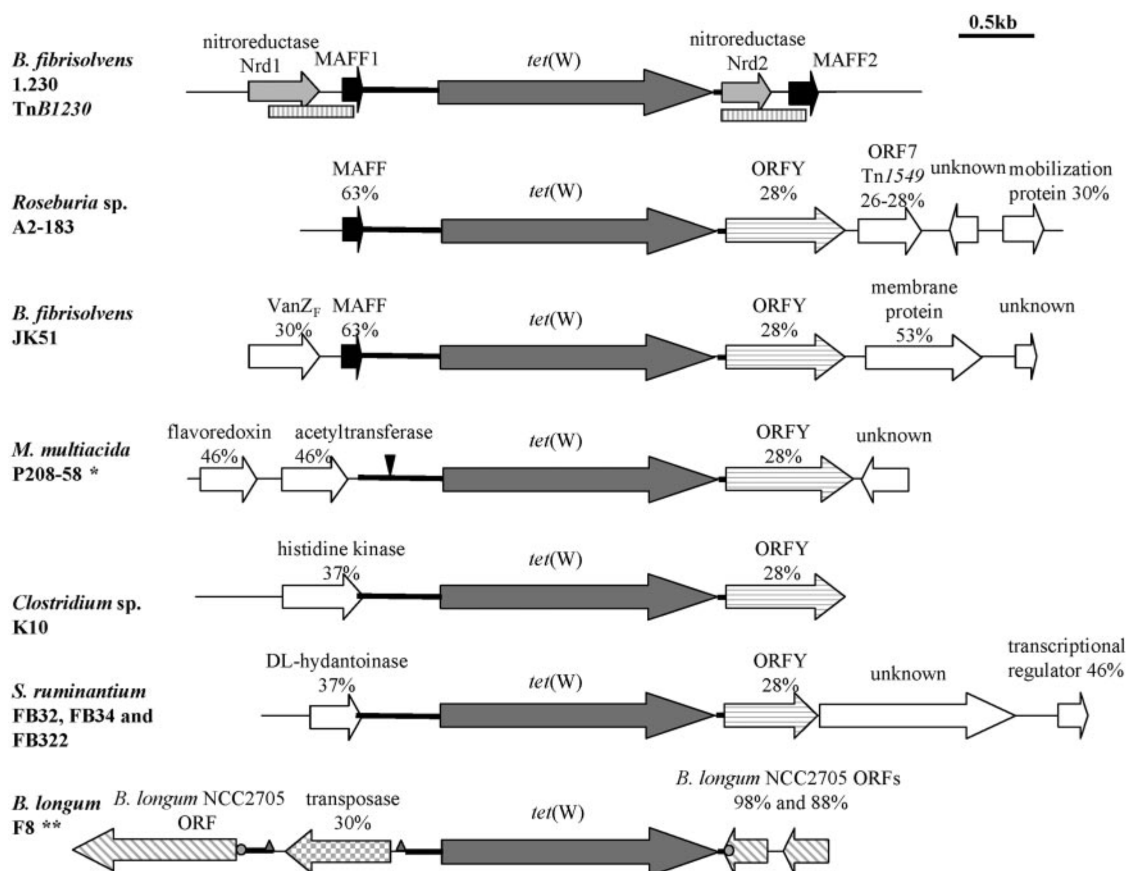

FIG. 2. Organization of the regions upstream and downstream of *tet*(W). ORFs are pattern coded and those conserved between different species are presented in the same shading. The percent identities to the closest match in the database are given above the ORFs. The rectangular boxes underneath the *B. fibrisolvens* diagram indicate the positions of the DRs. *, *M. multiacida* P208-58 contains an inserted 23-bp directly repeated sequence 291 bp upstream from the *tet*(W) start codon (indicated by the arrowhead). **, *B. longum* F8 has tandem repeats flanking the transposase inserted into the conserved core of 657 bp (represented by triangles) and duplicated 6 nucleotides flanking the insertion of the *tet*(W) and transposase genes (represented by circles).

in the *A. pyogenes* erm(B)-like element, which does not encode tetracycline resistance (18).

(iii) Diversity of the ORFs identified in sequences flanking *tet*(W). All other ORFs identified differed between the analyzed species (Fig. 2). Those further downstream of *tet*(W) in *Roseburia* sp. strain A2-183 had identities to other ORFs associated with mobile genetic elements, including one with 26 to 28% amino acid identity to ORF7 on Tn*5832*, Tn*1549*, Tn*5397*, and Tn*916* (Fig. 2), which itself encodes a hypothetical protein.

The product of the second gene upstream of *tet*(W) in *B. fibrisolvens* JK51 had 30% amino acid identity to $VanZ_F$, a transmembrane protein from *Paenibacillus popilliae* that is associated with a vancomycin resistance gene cluster (25).

In *S. ruminantium* FB32, FB34, and FB322, *tet*(W) was preceded by an ORF with 37% amino acid identity to *Pseudomonas* sp. DL-hydantoinase, an enzyme often encoded on plasmids (39) (Fig. 2). Hybridization profiles of DNA extracted following either plasmid or chromosomal purification methods indicated that the *tet*(W) genes in *S. ruminantium* FB32 and FB34 were plasmid encoded, while that in strain FB322 was chromosomally encoded (data not shown) (2).

Transposase adjacent to *tet*(W) in *B. longum* F8. The upstream conserved region in *B. longum* F8 was interrupted by an inserted sequence of 1,047 bp (Fig. 2), as noted earlier. Terminal direct repeats with a single-nucleotide deletion of 29 bp (5′-TACAATAAGGGGAAGAAAAATTTCTTTTA-3′, right arm) and 30 bp (5′-TACA$\underline{T}$ATAAGGGGAAGAAAAATTT CTTTTA-3′, left arm) defined the ends of this insertion sequence (IS). The left-arm sequence repeat was present within the conserved 657-bp sequence.

This insertion sequence contained an 818-bp ORF capable of encoding a 272-aa protein with up to 30% identity to transposases found on different mobile elements, including IS*1002* from *Bordetella* sp., the *Vibrio cholerae* pathogenicity island, and the *Micrococcus* sp. strain 28 plasmid pSD10. The C-terminal end of the F8 transposase, from residue P_{135}, contained a conserved integrase and transposase catalytic core domain (Fig. 3), including the triad of amino acids with characteristic spacing known as the DDE motif and the associated conserved residues. The spacing between each residue in the triad was $D_{142}(X_{65})D_{208}(X_{45})E_{254}$ (Fig. 3). Each of the amino acids in the triad together with the conserved surrounding

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1     MSKSITQDMAYRQSLMKYAEKYGVSRASRKYNKSRSYIYFWKARWNGSIESLACQSRRPH 60
61    RHPNQHTEAELKLIQNMRRRNPSLGMVELWHKLRQRGYTRRPESLFRVMRKLGLFPPAKK 120
121   KNTYKPKPYEQMTYPGQRVQVDVKVVPRRCITDPELRLFQYTAIDEFTRLRFLGAYTEQS 180
                        N2
181   TYSSADFLKKLFKWYARRGIRVECVQTDNGFEFTNRFSNSKKGLPTLFEATAVSLGIQHK 240
                                   N3
241   LIRPYTPRHNGKVERSHREDRNTFTPATAFTH 272
              C1
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FIG. 3. Amino acid sequence of the *B. longum* F8 transposase. The conserved catalytic domain starts at residue P_{135}. Amino acids creating the DDE motif are indicated in boldface, characteristic residues upstream and/or downstream of the triad are in boldface italic, and regions N2, N3, and C1 are underlined.

residues created three characteristic regions, N2, N3, and C1 (Fig. 3), which are conserved motifs in transposases of the IS*4* family of insertion sequences (15, 26).

The sequences immediately downstream of *tet*(W) and immediately upstream of the transposase had 98% identity to the same ORF of unknown function from *B. longum* NCC2705 (GenBank accession number AE014714). Two short directly repeated sequences flanked the insertion of the transposase and *tet*(W) genes (Fig. 2). These short duplicated sequences were six nucleotides long, CAATGC. Thus, it appeared that a single *B. longum* ORF had been interrupted by the insertion of a DNA fragment carrying the putative transposase and the *tet*(W) gene and that duplication of the 6-bp sequence had occurred during the insertion event. The last ORF downstream of *tet*(W) had 88% identity to another hypothetical ORF from *B. longum* NCC2705. The relative orientation of these ORFs in *B. longum* NCC2705 and *B. longum* F8 is the same.

PCR-based analysis of four additional *Bifidobacterium* strains known to carry *tet*(W) (Table 1) indicated that none of them contained the transposase.

Mobility of the *tet*(W) genes. The mobility of the *tet*(W) genes was investigated in filter mating experiments with tetracycline-susceptible (Tc^s) recipient strains. We used selected isolates carrying *tet*(W) as donors, and in each case *B. fibrisolvens* 2221^R and a Tc^s isolate belonging to the same species as the donor strain were used as recipients. No transconjugants were obtained in matings involving *Roseburia* sp. strain A2-183, *M. multiacida*, or, *S. ruminantium* as the donor strain. In previous matings, transfer of *tet*(W) from *Clostridium* sp. strain K10 to *B. fibrisolvens* 2221^R was not detected, although a second tetracycline resistance gene [*tet*(O/32/O)] was transferable (22, 36).

Colonies were obtained at a low frequency ($<2 \times 10^{-7}$ per recipient cell) in matings between *B. longum* F8 and *B. adolescentis* L2-32^R. Total genomic DNA was isolated from these transconjugants, and 16S rRNA sequences confirmed that they were indeed derived from *B. adolescentis* L2-32^R. PCR amplifications using specific primer sets confirmed that *tet*(W) and the upstream region including the transposase gene were present in all transconjugants analyzed. Furthermore, the same ORF was interrupted by the *tet*(W) insertion in *B. adolescentis* L2-32^R transconjugants as in the donor strain (Fig. 2). This ORF was again 100% identical to the homologue in *B. longum* NCC2705. Attempts to transform *B. adolescentis* L2-32^R with genomic DNA extracted from *B. longum* F8 were unsuccessful (results not shown).

Identification of a *tet*(W) minielement. The presence of conserved sequences and of identical ORFs upstream or downstream of the *tet*(W) gene suggested that there might be a common element(s) involved in the spread of the gene. A PCR-based approach was used in order to detect circular forms of any possible mobile minielements. Primer pairs reading out from the ends of *tet*(W) (primers tetW_out and 3′GPtet) and also reading out from the 5′ end of *tet(*W) (tetW_out) and the 3′ end of the conserved ORF located downstream of the *tet*(W) gene (ORFY; primer meth_out) (Fig. 4a) were tested in all isolates as well as in *B. fibrisolvens* 1.230 and in six *B. fibrisolvens* transconjugants containing the transposon Tn*B1230* (Table 1) (32). A specific PCR product was obtained only when DNAs isolated from *B. fibrisolvens* transconjugants Tc8 to Tc12 and Tc21 were used (Fig. 4b). Sequencing of the 1.5-kb product revealed that it contained the conserved regions upstream (657 bp) and downstream (43 bp) of the *tet*(W) gene and truncated versions of the *nrd* and MAFF genes (Fig. 4c). We postulate that this may be part of a transferable circular intermediate involved in the spread of *tet*(W) in certain hosts (Fig. 4d). Interestingly no product was obtained for the donor strain (*B. fibrisolvens* 1.230) used in conjugal matings producing these transconjugants.

DNAs from the transconjugants and the donor and recipient strains were analyzed after restriction with EcoRI or HindIII in Southern blot hybridization with the *tet*(W)-specific probe. A single band of the expected size (6.9 kb when cut with EcoRI) was obtained for the donor strain *B. fibrisolvens* 1.230 (Fig. 5). In all the transconjugants an additional band of 3.3 kb was observed (Fig. 5), which corresponds in size to the postulated circular minielement carrying *tet*(W) (Fig. 4). Similarly, the HindIII digest gave hybridizing bands of the expected sizes in the donor and all transconjugants, with an additional band visible only in the transconjugants (data not shown). These results confirmed the presence of a second copy of the *tet*(W) gene in transconjugants Tc8 to Tc12 and Tc21, which can apparently exist as a circular intermediate.

DISCUSSION

Mobile genetic elements, bacteriophage-mediated transduction, and natural transformation are all recognized as potential mechanisms for gene transfer between bacteria that inhabit the complex gut communities of the rumen and large intestine in mammals. There is, however, very little information on the mechanisms responsible for natural gene transfer in the groups of predominant anaerobic gut bacteria considered here. Plasmids are known to be abundant in *Butyrivibrio* and *Selenomonas* (11, 13), but little is known about the prevalence of chromosomal conjugative elements or natural transformation in these bacteria (31–33).

Direct evidence for *tet*(W) transfer, in laboratory matings, has been obtained from two of the bacterial strains examined here, *B. fibrisolvens* 1.230 and *B. longum* F8. *tet*(W) transfer has also been detected at low frequencies between *A. pyogenes* strains (5). *tet*(W) is transferred at high frequencies from *B. fibrisolvens* 1.230 to other *B. fibrisolvens* strains, in association with a 45- to 50-kb conjugative element designated Tn*B1230* (3, 21, 32).

The circular minielement detected here in *B. fibrisolvens*

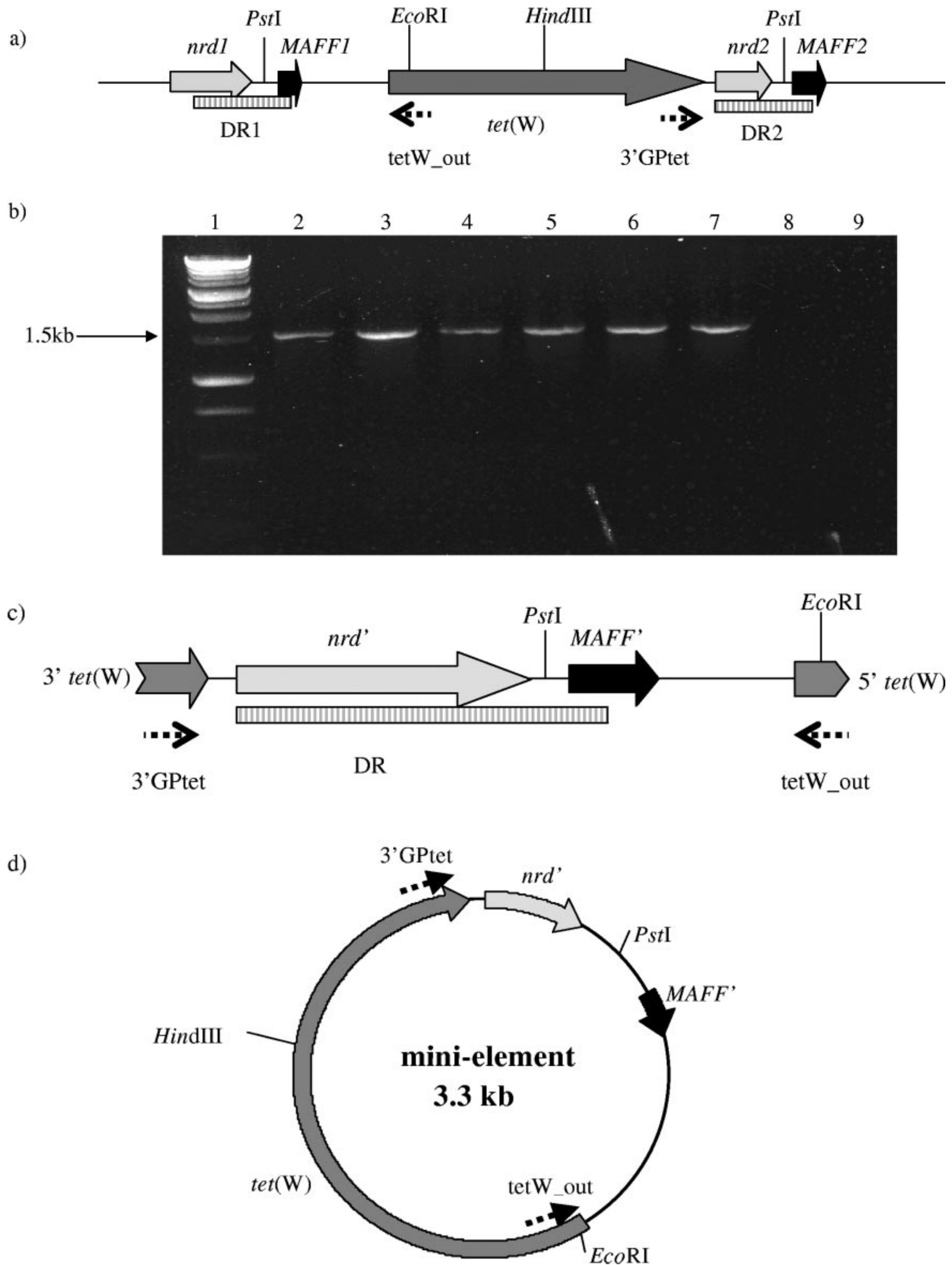

FIG. 4. Evidence for the presence of the circular minielement. ORFs are represented as solid arrows, and the locations of the primers used in PCR are shown by dotted arrows. Restriction sites are indicated, and DRs are represented by hatched boxes. (a) Organization of the *tet*(W) gene and its flanking regions in Tn*B1230* from *B. fibrisolvens*. (b) PCR products obtained using primers tetW_out and 3′GPtet reading outwards from the 5′ and 3′ ends of *tet*(W), respectively. Lane 1, 1-kb ladder (Promega, United Kingdom); lanes 2 to 7, *B. fibrisolvens* transconjugants Tc8 to Tc12 and Tc21, respectively; lane 8, *B. fibrisolvens* 1.230 (donor); lane 9, *B. fibrisolvens* 2221^R (recipient). (c) Organization of the PCR product shown in panel b following sequence analysis. *MAFF′* and *nrd′* are the truncated forms of the respective genes. (d) Diagram representing the circular form of the minielement carrying *tet*(W) identified in *B. fibrisolvens* transconjugants.

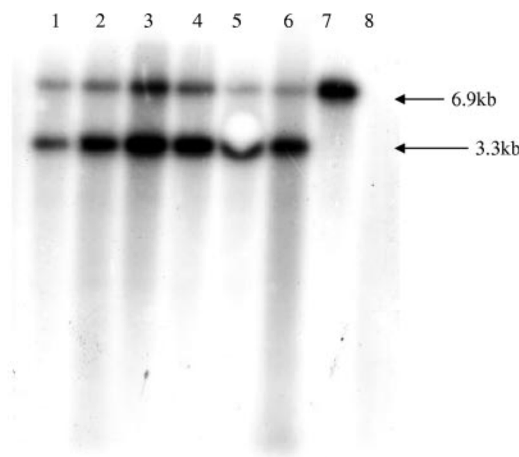

FIG. 5. Southern blot of EcoRI-digested chromosomal DNAs from *B. fibrisolvens* strains hybridized to the 1-kb *tet*(W)-specific probe. Lanes 1 to 6, transconjugants Tc8 to Tc12 and Tc21, respectively; lane 7, Donor strain 1.230; lane 8, recipient strain 2221^R. The sizes of the bands are indicated.

2221^R transconjugants that had acquired Tn*B1230* was not detected in the donor strain *B. fibrisolvens* 1.230 or in other *B. fibrisolvens* strains examined. The minielement contains truncated *nrd* and MAFF genes, found in Tn*B1230*, as well as the *tet*(W) gene itself, strongly suggesting that the DRs that flank *tet*(W) in Tn*B1230* give rise to the circular intermediate via homologous recombination. The appearance of the minielement may depend on a *trans*-acting factor from the recipient strain, thus explaining its absence in the donor strain. While there is no evidence that formation of the minielement is essential for *tet*(W) transfer between strains, it may help to explain secondary *tet*(W) insertions observed in the transconjugants (3).

We previously reported that proteins encoded by the DRs show significant amino acid sequence identity (38%) with bacterial nitroreductases (21). The proteins encoded by both the *nrd* genes also contain residues ($D_8D_{38}D_{78}$) that could represent the catalytic core of an IS-associated transposase (20). Although the third core residue is more typically glutamic acid, the spacing between these acidic residues, and the key flanking amino acids, appears to be consistent with previously characterized IS transposases. Functional studies will be required to clarify the true role and origin of the DR ORFs. The small ORF (MAFF) that occurs in the *B. fibrisolvens* DR regions, both upstream and downstream of *tet*(W), was also found upstream of *tet*(W) in two other strains, but the significance of this is unknown.

We have also shown here that *tet*(W) transfers at low frequencies in laboratory matings between *B. longum* F8 and *B. adolescentis* L2-32^R. The putative transposase gene inserted into the region upstream of *tet*(W) in *B. longum* F8 is assumed to be responsible for its mobilization in this strain. The catalytic core domain characteristic of IS-encoded transposases, which coordinates the divalent cations required for catalysis and excision of the minielement (15, 20), is present in the putative transposase of *B. longum* F8. The site for chromosomal insertion of the *tet*(W)/transposase element in *B. adolescentis* L2-32 transconjugants was identical to that in the donor strain *B. longum* F8, consistent with a site-specific insertion event. On the other hand, we cannot rule out the possibility of a larger mobile element or that this transfer occurred by transformation followed by homologous recombination.

No transfer of *tet*(W) was observed from any of the other strains tested (*Roseburia* sp. strain A2-183, *B. fibrisolvens*, *M. multiacida*, *S. ruminantium*, and *Clostridium* sp. strain K10). In each of these strains *tet*(W) was followed by a highly conserved (although truncated in the *S. ruminantium* strains) ORFY sequence. We hypothesize that the conserved *tet*(W)/ORFY core unit might constitute some form of mobile cassette, but the absence of *tet*(W) transfer from these strains suggests that other factors may be required for mobilization. It has been demonstrated that some bacteria that are able to act as recipients for conjugative transposons cannot themselves act as donors (7). In some cases (e.g., *Roseburia* sp. strain A2-183 and *B. fibrisolvens* JK51), sequences of the variable flanking sequences outside this core region suggest that the cassette is, or has been, embedded in a larger mobile element. In other cases (e.g., *M. multiacida*), the flanking regions may represent chromosomal genes.

Evidence for plasmid carriage of the *tet*(W) gene has been found so far only in *S. ruminantium* FB32 and FB34. The related strain of *S. ruminantium* (FB322) carried an identical *tet*(W)/ORFY sequence that appeared to be chromosomally located. The similarity in sequence of the flanking regions of these three strains, together with the hybridization results, implies that this plasmid, or part of it, is capable of chromosomal integration.

In conclusion, this analysis demonstrates the transfer of *tet*(W) by distinct mechanisms in *B. fibrisolvens* 1.230 and *B. longum* F8. The possible small transposable element in *B. longum* consists only of *tet*(W) and an adjacent transposase, whereas the transposable element in *B. fibrisolvens* 1.230 is about 50 kb in size. In the other strains studied here transfer was not detected in the laboratory, and flanking regions varied, but with strong conservation of a core sequence including *tet*(W) and ORFY. This implies the presence of a cassette in which ORFY might contribute to the acquisition of *tet*(W) through as-yet-unknown mechanisms. The highly abundant *tet*(W) gene has evidently become distributed via many different mechanisms, with one or more small core cassettes becoming incorporated into larger mobile elements that include both conjugative transposons and plasmids.

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