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Tetracycline Resistance Gene *tet*(W) in the Pathogenic Bacterium *Clostridium difficile*▽

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In this study, the *tet*(W) gene region of a human clinical isolate of *Clostridium difficile* resistant to tetracycline was characterized. This gene was a new allele showing 99% sequence identity to the gene found in the human strain *Bifidobacterium longum* F8, and it is not transferable by "in vitro" mating experiments.

The majority of the tetracycline resistance genes coding for ribosomal protection proteins reside on mobile or conjugative elements, and this is one of the reasons for their wide distribution among bacterial genera (8, 12). *tet*(M) is the most widespread gene class, and it is usually found on conjugative elements of the Tn*916* family (7), whereas *tet*(W) has the second-largest host range and has been detected in both gram-positive and gram-negative bacteria, especially in those isolated from environmental samples (2, 3, 9–11). *tet*(W) is associated with conjugative or nonconjugative elements which may vary among different bacteria (4, 6, 16, 17). In *Clostridium difficile*, tetracycline resistance is usually mediated by a *tet*(M) gene (1, 14). Although, it has been demonstrated that *C. difficile* is able to exchange antibiotic resistance genes with other bacteria of the gastrointestinal tract and that tetracycline resistance genes can be transferred between intestinal bacteria (1, 11, 13), *tet*(W) has not been detected in *C. difficile*, so far. This paper is the first report of the presence of a *tet*(W) gene in a human clinical strain of *C. difficile* isolated from a child with *C. difficile*-associated disease.

A sample of 35 toxigenic *C. difficile* strains, isolated from symptomatic patients, was selected from our national collection as representative of the tetracycline-resistant strains isolated from 1987 to 2005. All of these strains were intermediate or resistant to tetracycline (MICs of ≥8 μg/ml), according to the Clinical and Laboratory Standards Institute (5), when analyzed by Etest (AB Biodisk, Solna, Sweden) following the manufacturer's instructions. The presence of a *tet*(M) gene was assessed by the primer couple TETMd and TETMr (12), whereas the amplification of a 457-bp internal fragment of *tet*(W) was performed using the primer couple WRC1 and WRC2, designed on the conserved region of *tet*(W) genes (Table 1). All *C. difficile* isolates were positive for *tet*(M), but one strain, *C. difficile* CD5, showing a MIC of 8 μg/ml, was also positive for *tet*(W).

To characterize the *tet*(W) region, the genomic DNA of this strain was extracted using the NucleoBond buffer set III and the NucleoBond AXG 20 (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions, except that bacterial lysis was carried out for 45 min at 37°C, using double the recommended quantity of lysozyme and proteinase K.

C. difficile CD5 DNA was then digested with HindIII and analyzed by specific hybridization assays using the *tet*(W) and

TABLE 1. Primers used in this study

Primer	Sequence (5′→3′)	Location in *C. difficile* CD5 *tet*(W) region[a]
Detection and sequencing of *tet*(W) gene		
WRC1	CATCTCTGTGATTTTCA GCTTTTCTCTCCC	1871–1897
WRC2	AGTCTGTTCGGGATAA GCTCTCCGCCG	2328–2299
W9	ATGAAAATAATCAATA TTGGAATTCT	1447–1472
W11	TTACATTACCTTCTGAA ACATATG	3366–3343
Sequencing of *tet*(W) flanking regions		
TSP1	GAGAGCTTATCCCGAA CAGA	1892–1873
TSP2	CATCTGTGCCACTGGA AGGA	1649–1630
TSP3	GCTCTCCGTCAAGGTC GTCT	1512–1493
TER1	GTGTTGTCTGCATTT TACT	979–961
AUN1	GGTGAGCCGGATTGGG	NR
TSP4	TGGAACGTAACGGACT GTAA	2920–2939
TSP5	CTCTATGCGCCCCAGG AATA	3076–3095
TSP6	GCGGAGCGTATGCCTT ACAG	3243–3262
TSP6A	TTAAGGGCTTGTCCTCT CTGCC	3642–3663
Wrev6	GCTCTGCGTCTATGC GTCT	4046–4064
Wrev4	AGATGTTCCTCGCGCA ATTTT	4497–4517
Wrev2	ATGAAACGTTTACCTA AATATAC	5059–5037
UNI3A	TCACAGAAGTATGCCA AGCGA	NR

[a] NR, the positions of AUN1 and UNI3A are not reported since these primers were used to sequence the 5′ and the 3′ ends of the *C. difficile* CD5 *tet*(W) region, respectively.

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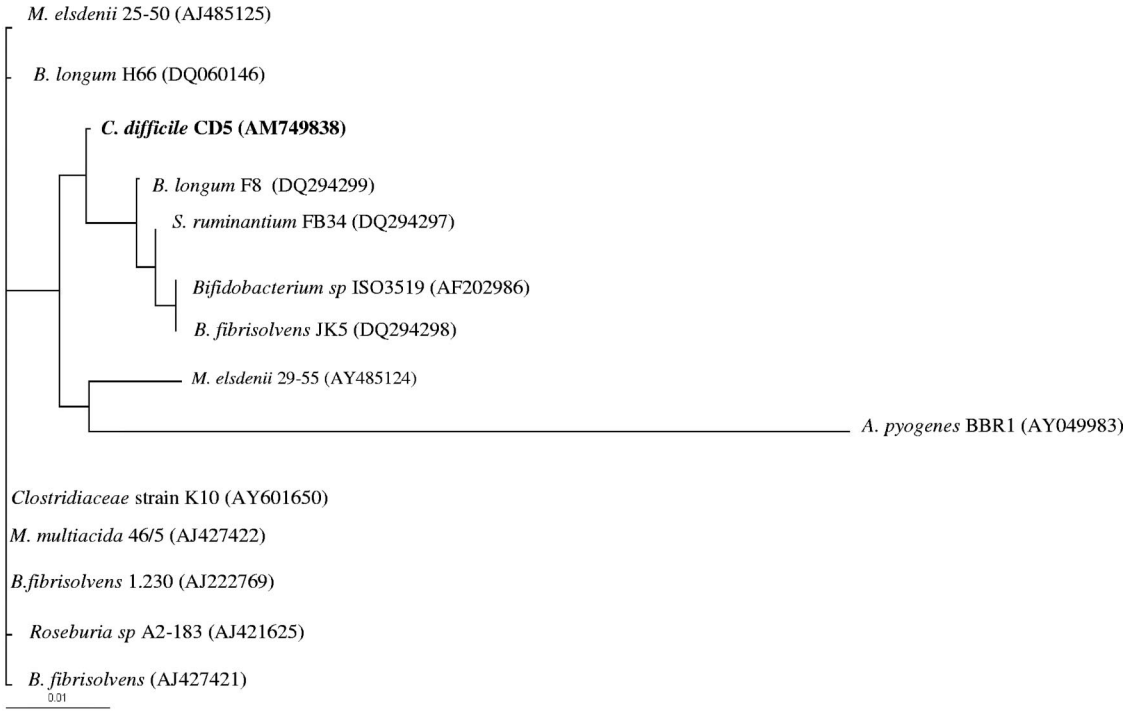

FIG. 1. Unrooted neighbor-joining phylogenetic tree obtained from the nucleotide multiple alignment of the *C. difficile tet*(W) gene identified in this study and other 13 *tet*(W) gene sequences available in GenBank. Among the species included are *Megasphaera elsdenii*, *Selenomonas ruminantium*, *Arcanobacterium pyogenes*, and *Mitsuokella multiacida*. The branch lengths are scaled in proportion to the extent of the change per position, as indicated by the scale bar. GenBank accession numbers are in parentheses.

tet(M) PCR products as probes. The PCR products were purified with the NucleoSpin extract kit (Macherey-Nagel, Düren, Germany) and labeled with the ECL enhanced chemiluminescence direct nucleic acid labeling and detection system (Amersham Biosciences, Buckinghamshire, United Kingdom). Hybridization analysis showed that strain CD5 had one copy of both *tet*(M) and *tet*(W) genes. In fact, one band of about 5.0 kb was observed with the *tet*(M) probe and one of about 2.5 kb with the *tet*(W) probe (data not shown).

The *tet*(W) gene was completely amplified using primers W9 and W11, designed on the 5′- and 3′-end conserved sequences, respectively, of many *tet*(W) genes already known (Table 1). The PCR fragment of about 1.9 kb was cloned into *Escherichia coli* using the Qiagen PCR cloning kit (Qiagen, Milden, Germany). Nucleotide and amino acid comparisons were accomplished using the European Bioinformatics Institute ClustalW server (http://www.ebi.ac.uk/Tools/clustalw/index.html), and the output was used for the construction of the phylogenetic tree by TreeView 1.4. Sequence analysis revealed that strain CD5 *tet*(W) was a new allele, showing a range of identity with the other *tet*(W) genes comprising between 85 and 99%. In particular, the sequence of *C. difficile* CD5 Tet(W) showed an identity of 99% to Tet(W) of *Bifidobacterium longum* F8. The phylogenetic tree obtained by nucleotide sequence comparison indicated that this gene belonged to the same cluster of two *Bifidobacterium* strains isolated from humans (Fig. 1). Since *B. longum* is abundant in the gastrointestinal tract of humans, a genetic transfer of *tet*(W) between these two anaerobic bacteria "in vivo" may be possible, even if further evidence and studies will be necessary to confirm this hypothesis.

The entire sequence of strain CD5 *tet*(M) was also obtained, and the sequence was 100% identical to that of the *Enterococcus faecalis* DS16 *tet*(M) gene, as already observed in other *C. difficile* strains (15).

The analysis of *tet*(W) and *tet*(M) transcription was performed with the Qiagen OneStep reverse transcription-PCR kit, using the same primers designed for *tet*(W) and *tet*(M) detection. *C. difficile* CD5 RNA was extracted using the NucleoSpin RNA II (Macherey-Nagel, Düren, Germany) from colonies grown for 24 h either in the presence or in the absence of tetracycline (10 μg/ml) and treated with recombinant DNase (DNA-*free* kit; Ambion, Austin, TX). The results confirmed that *C. difficile* CD5 *tet* genes were actively transcribed in both the presence and absence of tetracycline (data not shown).

Filter matings between *C. difficile* CD5 and the recipient strains *C. difficile* CD37^{R} and *Butyrivibrio fibrisolvens* 2221^{R} were performed as already described (13). *B. fibrisolvens* 2221^{R} is a rumen strain which, in the past, proved to be a good recipient for a *C. difficile* erythromycin resistance determinant, *erm*(B) (13). The transfer of *tet*(W) and the simultaneous transfer of both *tet*(W) and *tet*(M) were unsuccessful. On the contrary, the *tet*(M) gene was transferred to the recipient strain *C. difficile* CD37^{R} with average frequencies of 6.3×10^{-5} per donor and 3.1×10^{-4} per recipient. All transconjugants analyzed showed a MIC for tetracycline of 8 μg/ml. The conjugative element carrying the *tet*(M) gene was characterized as an element with a genetic organization similar to that of Tn*916* from *E. faecalis* DS16 (data not shown), as already described in other *C. difficile* strains (14). Any attempt to transfer *tet*(M) from *C. difficile* CD5 to *B. fibrisolvens* 2221^{R} was unsuccessful.

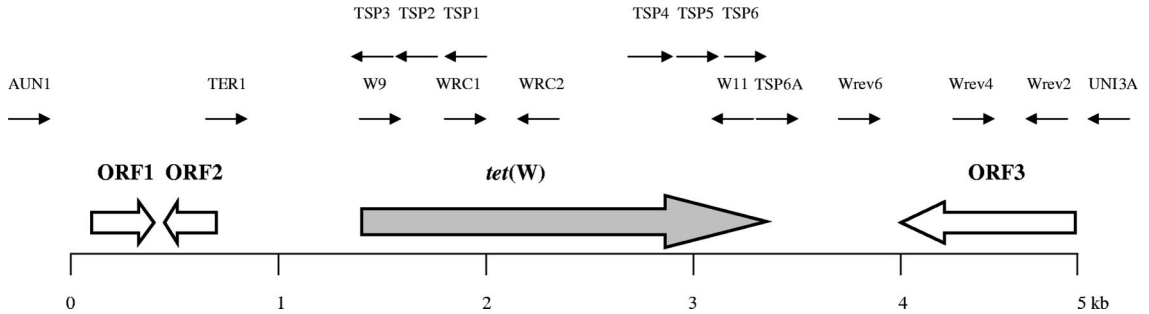

FIG. 2. Genetic organization of the *C. difficile* CD5 *tet*(W) DNA region. The approximate extent and organization of the region are not necessarily to scale. ORFs and their orientation are represented by arrows: the *tet*(W) gene is designated by a gray arrow, whereas white arrows represent the three ORFs found in the DNA regions surrounding *tet*(W). Individual primers and their orientations are indicated by small black arrows.

Genome walking of the regions flanking *tet*(W) was carried out using the DNA walking *SpeedUp* premix kit (Seegene, Seoul, South Korea), and the primers used for sequencing are reported in Table 1. Sequence analysis was performed using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) and ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The *C. difficile* CD5 *tet*(W) region of 5,059 bp is shown in Fig. 2. The sequences surrounding *tet*(W) showed three potential open reading frames (ORFs). The hypothetical protein of 115 amino acids encoded by ORF1, located upstream of *tet*(W), produced a significant alignment with the putative conserved domain DUF955. A conserved H-E-X-X-H motif of this domain is suggestive of a catalytic active site and shows similarity to that of the metallopeptidase family M48 and the cluster of orthologous groups COG2856, described as zinc metallopeptidases. This *C. difficile* CD5 protein showed high identity (99%) to that found in an uncultured bacterium from a human stool sample (GenBank protein accession no. CAM12483.1; from amino acids 63 to 177). ORF2 is oppositely oriented compared to both *tet*(W) and ORF1 and codes for a hypothetical protein of 50 amino acids that produced a significant alignment with the putative conserved domain DUF24, characteristic of transcriptional regulators related to the pfam01047 family, as observed for similar bacterial proteins identified during a sequence-based survey of members of the normal human gut microbiota (http://genome.wustl.edu/sub_genome_group.cgi?GROUP = 3&SUB_GROUP = 4) currently in progress. In particular, the first 47 amino acids of *C. difficile* CD5 ORF2 protein showed identities of 82% to that of *Coprococcus eutactus* ATCC 27759 (GenBank protein accession no. EDP 25685; from amino acids 54 to 100), 80% to that of *Clostridium leptum* DSM 753 (GenBank protein accession no. ED059633; from amino acids 63 to 107), 72% to that of *B. longum* DJO10A (GenBank protein accession no. ZP_00120895; from amino acids 60 to 106), and 70% to that of *B. longum* NCC2705 (GenBank protein accession no. NP_695657; from amino acids 70 to 116), respectively.

The region downstream of *tet*(W), from nucleotides 3550 to 5059, was 100% identical to the chromosomal region from nucleotides 5008 to 3499 of *C. difficile* QCD-32g58 (GenBank nucleotide accession no. NZ_AAML04000015). This strain was recently isolated from a patient with severe *C. difficile*-associated disease in Quebec, and the sequencing of its genome is currently in progress. In this region, strain CD5 showed an ORF (ORF3; located from nucleotides 5059 to 4076) that codes for a hypothetical protein identical to that encoded by the locus CdifQ_04003905 of *C. difficile* QCD-32g58 (GenBank protein accession no. ZP_01801544). This protein shows a putative conserved domain that characterizes the radical SAM, a protein superfamily catalyzing diverse reactions, including unusual methylations, isomerization, sulfur insertion, ring formation, anaerobic oxidation, and protein radical formation.

This paper documents for the first time the presence of *tet*(W) in *C. difficile* and, due to the high identity of *C. difficile* *tet*(W) with those of other human gastrointestinal bacteria, supports the hypothesis of horizontal gene transfer events in the rapid diffusion of this tetracycline resistance determinant.

Nucleotide sequence accession number. The nucleotide sequence described in this paper has been deposited in the EMBL database (http://www.ebi.ac.uk/embl/) under accession no. AM749838.

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