ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2008, p. 4001–4009 Vol. 52, No. 11
0066-4804/08/$08.00+0 doi:10.1128/AAC.00308-08

A New Tetracycline Efflux Gene, *tet*(40), Is Located in Tandem with *tet*(O/32/O) in a Human Gut Firmicute Bacterium and in Metagenomic Library Clones▽†

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Received 5 March 2008/Returned for modification 6 May 2008/Accepted 10 July 2008

The bacterium *Clostridium saccharolyticum* K10, isolated from a fecal sample obtained from a healthy donor who had received long-term tetracycline therapy, was found to carry three tetracycline resistance genes: *tet*(W) and the mosaic *tet*(O/32/O), both conferring ribosome protection-type resistance, and a novel, closely linked efflux-type resistance gene designated *tet*(40). *tet*(40) encodes a predicted membrane-associated protein with 42% amino acid identity to *tetA*(P). Tetracycline did not accumulate in *Escherichia coli* cells expressing the Tet(40) efflux protein, and resistance to tetracycline was reduced when cells were incubated with an efflux pump inhibitor. *E. coli* cells carrying *tet*(40) had a 50% inhibitory concentration of tetracycline of 60 μg/ml. Analysis of a transconjugant from a mating between donor strain *C. saccharolyticum* K10 and the recipient human gut commensal bacterium *Roseburia inulinivorans* suggested that *tet*(O/32/O) and *tet*(40) were cotransferred on a mobile element. Sequence analysis of a 37-kb insert identified on the basis of tetracycline resistance from a metagenomic fosmid library again revealed a tandem arrangement of *tet*(O/32/O) and *tet*(40), flanked by regions with homology to parts of the VanG operon previously identified in *Enterococcus faecalis*. At least 10 of the metagenomic inserts that carried *tet*(O/32/O) also carried *tet*(40), suggesting that *tet*(40), although previously undetected, may be an abundant efflux gene.

Tetracycline has been used extensively for more than 50 years, both therapeutically and prophylactically, to combat bacterial infections in humans and animals (34). The estimated use of antimicrobial agents in animal husbandry and agriculture far outweighs the total use in humans (19), and there is scientific evidence linking the use of antibiotics in agriculture and the emergence of bacterial antimicrobial resistance in humans (16, 36) and in the environment (5). Tetracycline resistance (Tc^r) is one of the most common bacterial antibiotic resistances.

Tc^r genes that are less than 80% identical fall into different classes (15), and to date 39 distinct Tc^r genes have been described, conferring resistance by four different mechanisms: ribosomal protection (RP) mechanism, tetracycline efflux, enzymatic inactivation of tetracycline, and modification of the ribosomal target (28). Mosaic derivatives of RP-type genes, in which part of the gene is recombined between two or more different classes of RP-type Tc^r genes, have been described recently (26, 38, 39, 40). This is thought to be a recent event in the evolution of Tc^r genes, driven by intense selection pressure and the presence of multiple resistance genes in the same bacterium (39). Multiple RP-type Tc^r genes have been identified in several bacterial species, including *Butyrivibrio fibrisolvens* [*tet*(O) and *tet*(W) (2)] and *Streptococcus pyogenes* [*tet*(O) and *tet*(M) (10)], while both RP- and efflux-type Tc^r genes have been identified in *Enterococcus* and *Streptococcus* spp. [*tet*(M) and *tet*(L) (12, 29)] and *Clostridium perfringens* [*tetA408*(P) linked to *tet*(M) (18) and *tetA*(P) with *tetB*(P) (37)].

Many Tc^r genes are found on mobile genetic elements, such as plasmids and conjugative transposons (CTn) (28), contributing to their widespread distribution. The type of mobile element with which a specific Tc^r determinant is associated influences the ability to spread horizontally to new bacterial genera. CTn are found in both gram-positive and gram-negative bacteria (24, 31). The RP-type genes *tet*(M), *tet*(W), and *tet*(Q) are the most widespread Tc^r genes (27) and are often associated with CTn.

The human gut contains a dense microbial population of more than 500 bacterial species and may represent an ideal situation for horizontal gene transfer. Commensal gut bacteria carry a variety of plasmids and mobile genetic elements that can be transferred by conjugation (35). Furthermore, bacterial adaptation to the presence of antibiotic resistance genes can largely abolish the selective disadvantages incurred by possessing resistance genes in the absence of antibiotic selection (30). Isolation of antibiotic-resistant bacteria from healthy individuals (4, 25, 33, 42) proves that the human gut microbiota is an important reservoir of antimicrobial resistance, and this can explain the resurgence of antimicrobial resistance after administration of antibiotics (16, 21).

Clostridium saccharolyticum K10 was previously reported to carry two RP-type resistance genes, *tet*(W) and the mosaic *tet*(O/32/O) (21, 26, 40). We report here the discovery of a new Tc^r gene, herein named *tet*(40), that is located in tandem with

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† Supplemental material for this article may be found at http://aac.asm.org/.

▽ Published ahead of print on 8 September 2008.

TABLE 1. Oligonucleotide primers used in this work

Procedure	Primer name	Primer sequence	Reference or source
Genome walking	Tet32-3′	TCATTCTGAAAGGAGAAATCCCTGCTAG	This work
	TetExt	ACCGCAGGAATATCTCTCACGGGCGTA	This work
Tc^r gene amplification	TetOFF2	TTGTTTTGGGGCTATTGGAG	26
	TetOFR3	TATATGACTTTTGCAAGCTG	26
	TetWarray-for	GGAGGAAAATACCGACATA	27
	TetWarray-rev	AATCTTACAGTCCGTTACG	27
	Tet32(2)array-rev	CTCTTTCATAGCCAGGCC	27
	TetQarray-for	CAAGATGTCCTGTTTATGC	27
	TetQarray-rev	GAATCCCTTCAAAAACGC	27
Sequencing	519r	GWATTACCGCGGCKGCTG	
Fosmid subcloning	adapt-I	AATTCGGCACGAGG	This work
	adapt-II[a]	O_3P-CCTCGTGCCG	This work

[a] This primer is phosphorylated at the 5′ end.

the *tet*(O/32/O) gene in *C. saccharolyticum* K10. Metagenomic libraries provided a powerful approach for analyzing Tc^r genes in human gut bacteria and indicated that these two genes commonly occur in tandem and are likely to be associated with mobile genetic elements.

MATERIALS AND METHODS

Bacterial isolates and growth conditions. Anaerobic strains were routinely grown at 37°C in anaerobic M2GSC broth (23) containing tetracycline (10 μg/ml). Strains were cultured in Bellco tubes under 100% CO_2 or in an anaerobic cabinet in an atmosphere of 10% CO_2, 10% H_2, 80% N_2. *Escherichia coli* TransforMax EPI300 (Epicentre, Madison, WI) and OneShot Top10 (DH10B, a derivative of DH101; Invitrogen, Paisley, United Kingdom) were grown in LB medium (32) at 37°C. *E. coli* containing fosmid clones was screened for Tc^r by positive selection on LB agar plates supplemented with 5 or 10 μg/ml tetracycline. All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich.

Analysis of IC of tetracycline. The gene encoding *tet*(40) was cloned into the T vector (pGEM-T Easy vector; Promega, Southampton, United Kingdom) and electroporated into TransforMax EPI300 *E. coli* cells (Epicentre). The inhibitory concentration (IC) of tetracycline was determined by inoculating 0.1 ml of an overnight culture into 5 ml fresh LB medium containing serial dilutions of tetracycline (0 to 140 μg/ml), in triplicate. Tubes were incubated at 37°C for 16 h, and the optical density at 650 nm (OD_{650}) was subsequently read (LKB Novaspec II; Pharmacia). The lowest concentration of tetracycline reducing the growth of the bacterial cells by 50% (illustrated by a 50% reduction in the OD_{650} compared to that of a control culture grown in the absence of tetracycline) was defined as the 50% inhibitory concentration (IC_{50}). The effect of the efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on the growth of *E. coli* cells containing either *tet*(40) or *tet*(W) in the presence of tetracycline was established. Triplicate tubes containing three concentrations of tetracycline (40, 60, and 80 μg/ml) were incubated in the presence or absence of 2.5 μg/ml CCCP, and the OD_{650} was measured after 18 h of growth.

Detection of tetracycline in samples. Triplicate cultures were set up by inoculating 5 ml LB containing 5 μg/ml tetracycline with clones containing pGEM-T Easy (negative control), *tet*(40), *tet*(W), *tet*(X), or *tetA*(P). After overnight growth, cultures were spun for 10 min at 5,400 × *g*. The cleared supernatant was removed and 100 μl mixed with 900 μl acetonitrile (3) in a 1.5-ml Eppendorf tube. The remaining pellets were resuspended in 300 μl of water and sonicated for 10 to 15 s (amplitude, 9 μm). The resulting cell lysates were centrifuged at 4°C for 15 min at maximum speed to remove the cell debris, and 100 μl of the supernatant was mixed with 900 μl acetonitrile. Samples were then mixed vigorously on a Whirlimixer for 15 s, incubated for 10 min at room temperature, and spun down for 10 min at maximum speed. Final supernatants were transferred to fresh 1.5-ml Eppendorf tubes. The acetonitrile contained the internal standard demeclocycline hydrochloride at a final concentration of 500 pg/μl to allow quantification of the tetracycline.

The concentration of tetracycline in each fraction was analyzed by liquid chromatography-tandem mass spectrometry (LC–MS-MS) at room temperature, basically as described previously (3), using an Agilent 1100 high-performance liquid chromatography system (Agilent Technologies, Wokingham, United Kingdom) with a Jupiter 5-μm, C_{18} column (Phenomenex, Macclesfield, United Kingdom) and an organic mobile phase. Mobile-phase solvents were a mixture of solutions A and B, where solution A was water containing 0.1% formic acid and solution B was acetonitrile containing 0.1% formic acid. The gradient program started at 95% of solution A held for 5 min, followed by 3% of solution A held for 5 min and then 95% of solution A held for 4 min in preparation for the next injection. The flow rate was 300 μl/min, and the injection volume was 5 μl. The LC eluent was directed into, without splitting, a Q-Trap triple quadrupole mass spectrometer (Applied Biosystems, Warrington, United Kingdom) with a Turbo ion spray source fitted in positive ion mode for the detection and quantitation of the antibiotics. Tetracycline and demeclocycline hydrochloride were detected using multiple reaction monitoring transitions, which were calculated by infusing standards directly into the mass spectrometer, via a syringe pump, at a concentration of 5 ng/μl. Data were normalized according to the detection of antibiotics in the pGEM-T control and tabulated as the means of six replicates from two independent growth experiments.

Genome walking and PCR. DNA preparation and genome walking were carried out as previously described (13). PCR amplification was conducted using forward primer AP1 and nested primer AP2 (provided in a Universal GenomeWalker kit; Clontech) in combination with reverse primer Tet32-3′ or nested primer TetExt (Table 1) to amplify regions downstream of the gene.

Extraction of high-molecular-weight DNA (for metagenomic library). A stool sample from donor Ab1 was resuspended in an equal volume (wt/vol) of buffer A (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 500 mM NaCl), and 2-ml fractions were frozen in liquid nitrogen and crushed using a mortar and pestle precooled in liquid nitrogen. The crushed sample was then pulverized using a motorized hammer mill (Spex 6700 freezer/mill; Glen Creston Ltd., Middlesex, United Kingdom) for 2 min at full speed. Proteinase K (100 μg/ml) was added and the mixture incubated for 1 h at 50°C. Sodium dodecyl sulfate was added to the sample (1% final concentration), and incubation was continued for 1 h at 55°C. The sample was briefly pelleted (2,000 rpm for 2 min) and the supernatant collected in a 50-ml Falcon tube. Equal volumes of molten (70°C) 2% agarose type VI-A dissolved in buffer A were mixed with the sample, and the mix was poured into 5-ml petri dishes. Agarose slabs approximately 0.5 cm thick were transferred into 50-ml Falcon tubes and washed six times in TE50 buffer (20 mM Tris-HCl, 50 mM EDTA, pH 8.0) at 4°C, with changes of buffer approximately every 12 h. Finally, the slabs were washed overnight in storage buffer (50% glycerol, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at −20°C until required.

Fosmid metagenomic library construction. DNA embedded in agarose was size fractionated using pulsed-field gel electrophoresis. Slabs were placed in a 1% pulse field-certified agarose gel (SeaKem gold; Cambrex, United Kingdom) dissolved in 0.5× TAE buffer (32) and subjected to contour-clamped homogeneous electric-field gel electrophoresis (CHEF-DR II; Bio-Rad) at 6 V/cm for 1 h at 14°C with a current switch of 10 to 1 s, using 0.5× TAE as running buffer. The size range of electrophoresed DNA was estimated by comparison with DNA Hyperladder VI (Bioline). Agarose containing low-molecular-mass DNA (<10

kb) was excised and discarded, and electrophoresis continued for 20 h, changing the pulse field current switch to 0.4 to 1.5 s. The DNA fraction of 30 to 50 kb was excised from the gel, and the DNA was electroeluted from the agarose into dialysis tubing (32). The electroeluted DNA was dialyzed against 1× Tris-EDTA buffer for 2 h prior to ethanol precipitation and resuspension in 40 μl of 10 mM Tris-HCl buffer, pH 8.0. The DNA was end repaired by incubation for 2 h at 37°C with T4 DNA polymerase, T4 polynucleotide kinase, and Klenow enzyme following the manufacturer's guidelines (Roche). Enzymes were heat inactivated at 70°C for 15 min, and the DNA was purified by gel exclusion chromatography using Chromaspin 1000-TE columns (Clontech) following the manufacturer's guidance.

Finally, 250 ng of concentrated, size-fractionated, end-repaired DNA was ligated with 500 ng of blunt-ended fosmid vector (pcc1FOS; Epicentre) at 16°C for 16 h and the ligation mix was packaged using MaxPack packaging extracts (Epicentre) and transformed into *E. coli* TransforMax EPI300 cells (Epicentre) following the manufacturer's guidelines. Colonies were selected using a BioRobotics BioPick colony picker (Genomic Solutions, Ann Arbor, MI) and arrayed into 384-well microtiter plates containing 70 μl of freezing mix (2× LB medium supplemented with 10% glycerol), grown overnight at 37°C, and stored at −70°C.

Fosmid DNA extraction and PCR screening of Tc^r fosmid clones. The copy number of fosmids contained within Tc^r colonies was increased using 1× copy number induction solution (Epicentre) following the manufacturer's protocol. Fosmid DNA was purified from these cultures using a QIAprep miniprep kit (Qiagen) following the manufacturer's instructions, with appropriate modifications for recovery of large plasmids from larger culture volumes. DNA was finally eluted using 50 μl of 5 mM prewarmed (70°C) Tris-HCl, pH 8.0, and concentrated to ~10 μl in a vacuum concentrator before being stored at −20°C until further use.

DNA purified from Tc^r fosmids was screened by PCR amplification for known Tc^r genes, using conditions described previously (26, 27). Primers used were specific for *tet*(O), *tet*(W), *tet*(O/32/O), and *tet*(Q) (Table 1).

Bacterial 16S rRNA gene analysis. Approximately 100 ng of metagenomic DNA, purified as described above, was used as a template for PCR amplification of 16S rRNA with eubacterial universal primers fD1 and rP2 (43). Touchdown PCR amplification was done under standard conditions with a Bio-Rad MyCycler thermal cycler, using an initial annealing temperature 5°C higher than optimal. Cycling conditions were an initial cycle of 94°C for 3 min followed by 10 cycles of 94°C for 45 s, 62°C for 45 s (with a decrement of 0.5°C for each subsequent cycle), and 72°C for 2 min. A further 10 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 2 min were followed by a final cycle step at 72°C for 10 min. This method ensured amplification of specific products by combining stringent conditions for primer hybridization and simultaneously allowing underrepresented bacterial species to be amplified during successive cycles and minimizing the PCR bias associated with a higher number of cycles. Resulting PCR products were ligated into the pGEM-T Easy vector (Promega). A total of 96 transformed colonies were selected and arrayed in a 96-well microtiter plate. PCR products amplified from these colonies using vector-specific primers M13 Forward and pGEM-R were sequenced using the eubacterial universal primer 519r, and the partial sequence was analyzed by a BLAST search at the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp). Sequences of single representative clones in the array were extended using fD1 and rP2 primers and assembled using the program CAP integrated to work under the program BioEdit (11). Phylogenetic analysis was completed using ClustalX (41), and the resulting phylogenetic tree was edited using the program Molecular Evolutionary Genetic Analysis, version 3.1 (MEGA 3.1, http://www.megasoftware.net/).

Sequencing of fosmid clones. Two different approaches were used to sequence the selected fosmid clone T45, which carries multiple Tc^r genes. First, in vitro random insertion transposon mutagenesis using transposon EZ-Tn5<kan> (Epicentre) was carried out following the manufacturer's guidelines. Fosmid DNA was then extracted from 384 clones selected on kanamycin LB agar plates and sequenced using transposon EZ-Tn5-specific primers. Second, a shotgun plasmid library of fosmid clone T45 was prepared using DNA purified from 1 liter LB medium by CsCl gradient centrifugation (32) to eliminate *E. coli* chromosomal DNA. Purified DNA, resuspended in 750 μl of shearing buffer (Tris-EDTA supplemented with 10% glycerol), was randomly sheared using a nebulizer (Invitrogen, Paisley, United Kingdom) for 1 min at a pressure of ~100 kPa g^{-1} in a single-head diaphragm pump (Laboport; KNF Neuberger, Denmark). Sheared DNA was ethanol precipitated, resuspended in 40 μl sterile water, and end repaired. DNA was then ligated with 2.0 μM of specific adaptor primers (adapt-I and adapt-II [Table 1]) and finally size fractionated in a size exclusion column (chromaspin 1000-TE; Clontech). This DNA was used as a template for standard PCR amplification with the adapt-I primer in a 50-μl reaction mix using a 35-cycle protocol with an annealing temperature of 47°C. The PCR products were ligated into the pGEM-T Easy vector and colonies in the shotgun library arrayed in six 96-well microtiter plates containing 150 μl LB medium. Following overnight growth, colony PCR amplification using the M13 Forward and pGEM-R primer set was carried out, and the PCR products were sequenced using nested primers T7 promoter and M13 Reverse.

Sequences at each end of the fosmid clones carrying Tc^r genes were determined using vector-specific primers, following the manufacturer's guidance (Epicentre). Sequence assembly was performed using the package Phred/Phrap/Consed (http://www.phrap.org/phredphrapconsed.html) in the Rowett Research Institute's in-house computer facility (openMosix Beowulf cluster), and genome analysis and annotation were carried out using release 8 of the Artemis program (http://www.sanger.ac.uk/Software/Artemis/). Identification of open reading frames (ORFs) was conducted using the heuristic-model option for gene prediction of GeneMarkK (http://exon.biology.gatech.edu/GeneMark/heuristic_hmm2.cgi). Computer-assisted analysis of ORFs was carried out using the SIP BLAST network service hosted by the Swiss Institute of Bioinformatics BlastP (http://us.expasy.org/tools/blast/). Conserved regions were further analyzed using Pfam, Prosite, Interpro, and Print databases.

Nucleotide sequence accession numbers. The partial DNA sequence of fosmid clone T45, including the *tet*(40) gene, has been deposited in the EMBL database under the accession number AM419751. The accession number for the 16S rRNA sequence of *C. saccharolyticum* K10 is EU305624, and that for the tandem *tet*(O/32/O) and *tet*(40) genes in this bacterial strain is AJ295238.

RESULTS

Identification of a new Tc^r gene, *tet*(40), in *C. saccharolyticum* K10. The mosaic *tet*(O/32/O) gene was identified previously in *C. saccharolyticum* K10 isolated from a healthy human fecal sample received from an individual (Ab1) undergoing long-term tetracycline therapy (21). The full 16S rRNA sequence of *C. saccharolyticum* K10 was assembled and had 99% sequence identity to the butyrate-producing human gut isolates M62/1 and SM4/1, which were themselves most closely related to *C. saccharolyticum* (17).

The sequence of the *tet*(O/32/O) gene (20, 39) was extended by genome walking. The 150-nucleotide (nt) sequence upstream of the start codon had 100% identity to a similar region in *tet*(O). This included the regulatory regions for *tet*(O), which differ from those of *tet*(W) and *tet*(M) in lacking a leader peptide sequence (22). Immediately downstream of the *tet*(O/32/O) gene there was an ORF of 1,220 nt potentially encoding a 406-amino-acid protein. The gene, which has been designated *tet*(40) (15), was located 50 nt downstream of the *tet*(O/32/O) stop codon and had its own ribosome binding site (AGGAG) as well as the canonical Pribnow-Gilbert box (−10 TATAA and −35 TTAACA). The DNA percent G+C content of the coding region for *tet*(O/32/O) was 41%, compared to 56.5% for *tet*(40), indicating that the two genes probably originated from different donor microorganisms.

The protein encoded by *tet*(40) had 42% amino acid sequence identity to the TetA(P) Tc^r efflux protein from *C. perfringens* (37) and 43% identity to TetA408(P) (18). Alignment of the novel protein Tet(40) with TetA(P) and TetA408(P) showed that the efflux protein motif $E_{60}xP_{62}xxxxxD_{68}xxx R_{72}R_{73}$ was strongly conserved and very similar in sequence to the consensus motif ExPxxxxxDxxxRK (Fig. 1). Other amino acids of putative functional importance were also conserved among the proteins P_{62}, T_{63}, A_{119}, A_{122}, G_{137}, E_{233}, D_{236}, and S_{361}, including three glutamic acid residues shown to be functionally important in TetA(P) (1, 14), specifically, E_{53}, E_{60}, and E_{90} (Fig. 1) in Tet(40). Secondary-structure analysis of Tet(40) revealed that there were 12 transmembrane segments, indicative of membrane localization.

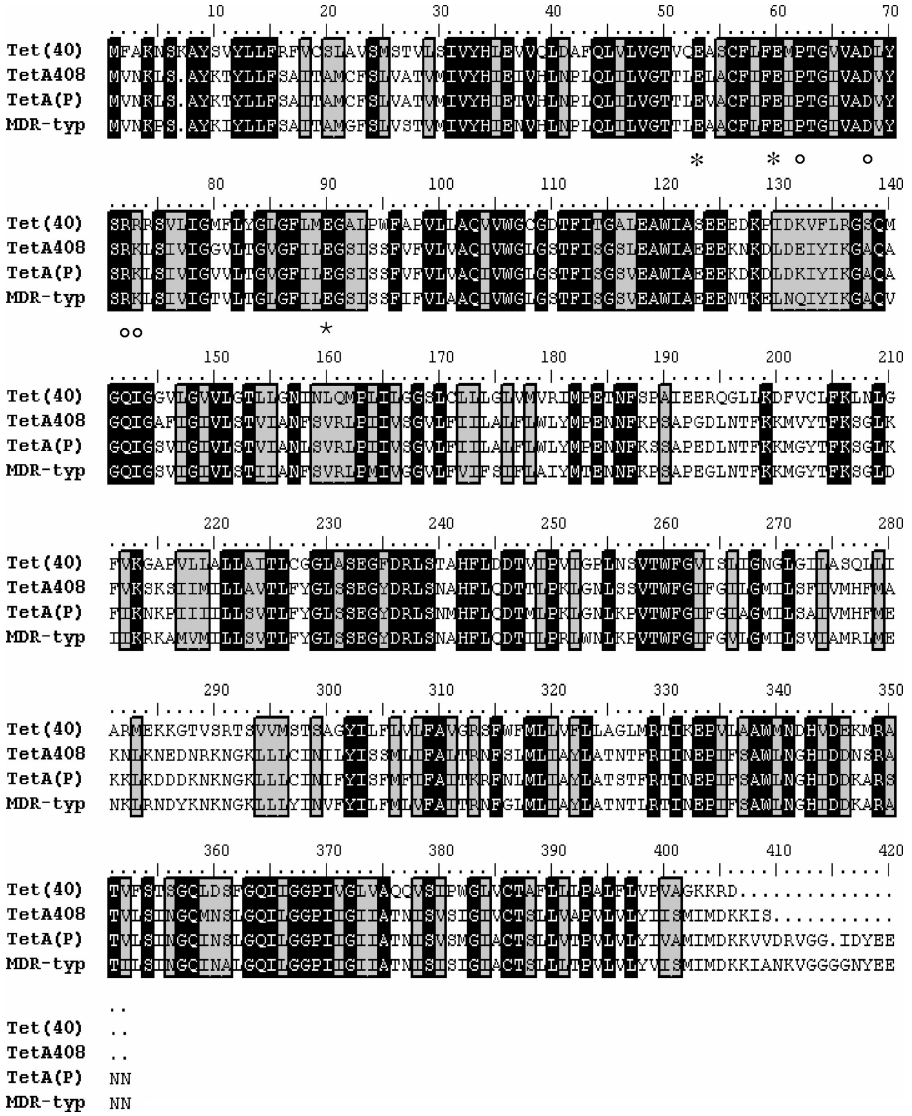

FIG. 1. Multiple alignment of the new Tc^r protein Tet(40) against efflux proteins from *Clostridium* species: TetA408(P) from *C. perfringens* (BAB71965.1), TetA(P) from *C. septicum* (BAB71966.1), and a multiple drug resistance type (MDR-typ) from *C. acetobutylicum* (AAK79415.1) (accession numbers are from the EMBL database). The alignment was carried out using the program ClustalW. Functional glutamate residues E_{53}, E_{60}, and E_{90} are indicated with an asterisk, and residues comprising the conserved efflux protein motif are indicated with a circle.

Functional analysis of the Tet(40) protein. The full ORF of the *tet*(40) gene together with the upstream regions containing predicted ribosomal binding site and promoter sequences were specifically amplified by PCR and cloned into the pGEM-T Easy vector. Expression of the gene in *E. coli* indicated that the presence of *tet*(40) itself, not in tandem with *tet*(O/32/O), was sufficient for expression of the Tc^r phenotype. The IC_{50} of tetracycline for sensitive *E. coli* EPI300 cells (IC_{50} of ~2 μg/ml) containing the *tet*(40) gene cloned in the pGEM-T vector was 60 μg/ml.

The cloned *tet*(40) gene was also assessed for activity as an efflux pump by determination of the relative amounts of tetracycline in the supernatant and cell lysate following overnight bacterial incubation (3). The LC–MS-MS results obtained for the *tet*(40) clones were compared with those obtained for clones containing genes conferring Tc^r by alternative mechanisms: *tet*(W), ribosome protection; *tet*(X), tetracycline inactivation; and *tetA*(P), an efflux pump. Clones containing *tet*(40) or *tetA*(P) accumulated less tetracycline than those containing the RP gene *tet*(W) (Fig. 2), confirming that *tet*(40) confers Tc^r by actively pumping tetracycline out of the bacterial cell. Tetracycline was virtually undetectable in fractions of samples containing *tet*(X), due to modification of the tetracycline molecule into an undetectable form.

The efflux pump inhibitors phenyl-arginine-β-naphthylamide and CCCP both reduced the growth of *E. coli* cells containing *tet*(40) in the presence of tetracycline but had no effect on cells containing *tet*(W). In the presence of 40 to 60 μg/ml tetracycline and CCCP, the growth of cells containing *tet*(40) was reduced by ~50% compared to the growth of cells

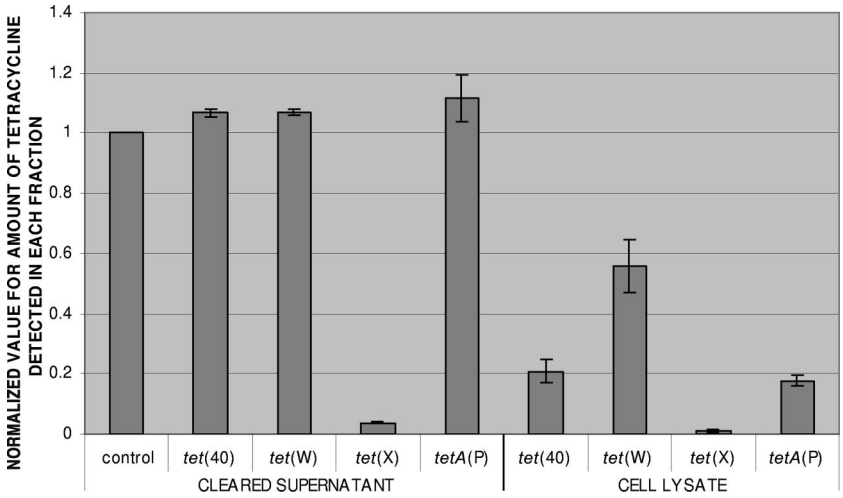

FIG. 2. LC–MS-MS analysis of efflux pump activity compared to other Tc^r mechanisms. The control culture was a broth of *E. coli* EPI300 cells transformed with the native pGEM-T vector. The Tc^r genes were all full-length PCR amplicons cloned into the pGEM-T vector and transformed into competent *E. coli* host cells. The amount of tetracycline detected in the control sample is assumed to represent 100% recovery of the introduced tetracycline, and all other values are normalized against this. Slightly more tetracycline was recovered in the supernatants from all of the test cultures than from the control sample, resulting in values of >1 for the cleared supernatants. No tetracycline was detected in the final cellular pellet fraction of any of the samples tested. The results show the average normalized data from two biological replicates, each carried out in triplicate; error bars represent standard deviations.

containing tetracycline only, while the effect on cells containing *tet*(W) was negligible (<1% reduction in growth) (Table 2). A growth reduction of 32% was observed for *tetA*(P) under the same conditions (data not shown).

Human colon metagenomic library. At the time of isolation of *C. saccharolyticum* K10, almost 100% of the cultivable anaerobes in feces from individual Ab1 were tetracycline resistant. More than 90% of anaerobes remained resistant 3 years later, although there had been no tetracycline therapy in the intervening period. DNA extracted from the second Ab1 fecal sample was screened for the presence of multiple Tc^r genes by use of a macroarray (27). This confirmed the presence of four of the most prevalent RP-type Tc^r genes [*tet*(W), *tet*(O), *tet*(Q), and *tet*(O/32/O)], but known efflux genes, including *tetA*(P), were not detected (data not shown). This DNA was used to construct a metagenomic library in the *E. coli* fosmid vector pcc1Fos. The average insert size was estimated to be 35 kb, following restriction analysis (data not shown). Eighty out of approximately 4,000 fosmid clones grew on 10 μg/ml tetracycline, and of these, 33 randomly selected Tc^r clones were screened by PCR amplification using specific primers targeting the four RP Tc^r genes known to be present in the Ab1 sample. Two clones were found to contain *tet*(O) and *tet*(W), 17 carried *tet*(O/32/O) and *tet*(W), and one *tet*(O/32/O) and *tet*(Q). In nine further clones, *tet*(O/32/O) was the only resistance gene detected, and one clone contained only *tet*(W). The three remaining clones failed to amplify with *tet*(O)-, *tet*(W)-, or *tet*(Q)-specific primer pairs.

TABLE 2. Effects of tetracycline with or without CCCP on growth of *E. coli* cells containing *tet*(40) or *tet*(W)

Gene	Tetracycline concn (μg/ml)	Avg (±SD) OD_{650}[a]		% Growth[b]
		Plus tetracycline	Plus tetracycline and CCCP	
tet(40)	40	0.47 ± 0.04	0.26 ± 0.03	56.5
	60	0.33 ± 0.07	0.17 ± 0.03	53.4
	80	0.20 ± 0.005	0.03 ± 0.004	16.5
tet(W)	40	0.91 ± 0.04	0.87 ± 0.02	96.4
	60	0.80 ± 0.09	0.79 ± 0.09	99.2
	80	0.73 ± 0.06	0.55 ± 0.03	75.4

[a] The OD_{650} measurements given are the averages (±standard deviations) from triplicate tubes for a single experiment, except those quoted in the presence of 60 μg/ml tetracycline, which are the averages from triplicate tubes for three separate experiments.

[b] Percentage of growth in the presence of CCCP and tetracycline compared to that in the presence of tetracycline alone.

Bacterial diversity of the Ab1 DNA sample was examined by direct amplification and sequencing of 16S rRNA genes. Phylogenetic analysis indicated a distribution of bacterial species in sample Ab1 that is fairly typical of that for human fecal samples, with approximately 90% of sequences related to *Firmicutes* (including 50% in clostridial cluster XIVa and 29% in clostridial cluster IV) and 9% to *Bacteroidetes* (Fig. 3). Sequencing the ends of the 80 fosmid inserts conferring Tc^r suggested that these metagenomic inserts were also derived from diverse bacterial groupings, with the largest number (approximately 50%) related to sequences from *Firmicutes*, based on BlastX searches (Fig. 3).

Sequencing of fosmid clones carrying tandem Tc^r genes. The sequences of 10 selected clones harboring *tet*(O/32/O) were extended downstream and in all cases revealed the presence of the *tet*(40) gene. The sequences of both the *tet*(40) and *tet*(O/32/O) genes were >99.9% identical to those identified in *C. saccharolyticum* K10. The T45 clone contains an insert of ~37 kb, and sequences of ~21 kb and ~8 kb were assembled from each end by creating and sequencing a small-insert shotgun library. Despite repeated efforts using different methods, we were unable to sequence across the central gap, estimated to be 7 to 9 kb, and *orf20* and *orf21*, flanking the gap, were truncated

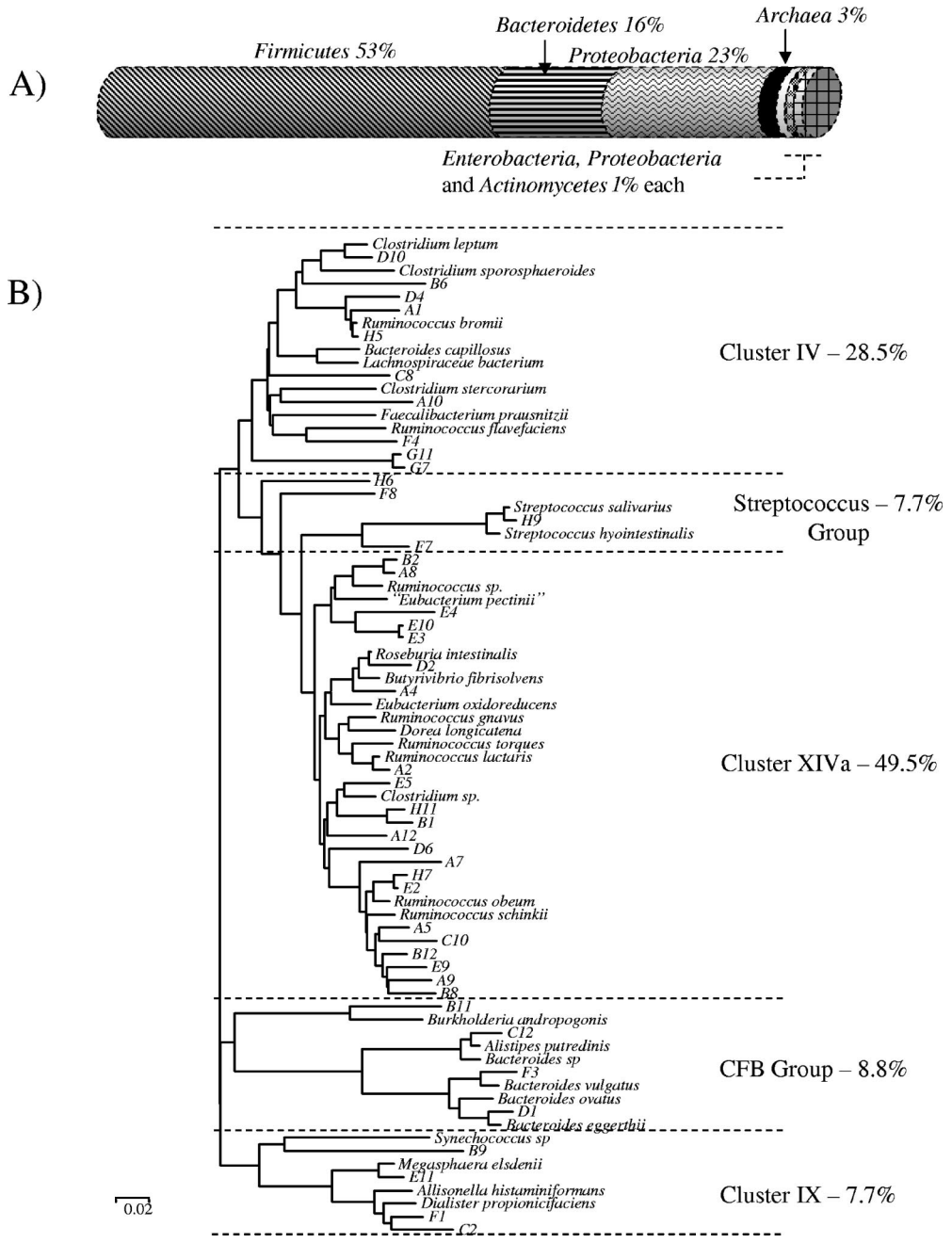

FIG. 3. (A) Diagram depicting the results of sequencing each end of some of the Tc^r fosmid clones isolated from the Ab1 metagenomic fosmid library by use of vector-specific primers. Sequences were analyzed using the program BlastX at the network service hosted by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Results were distributed within one of the bacterial phyla shown and expressed as percentages. For comparison, bacteria included in phylogroups IV, IX, and XIVa and the streptococcus group (below) all belong to the phylum *Firmicutes*. (B) Phylogenetic analysis of 96 cloned 16S rRNA sequences amplified using eubacterial universal primers from DNA extracted from the Ab1 fecal sample. The phylogenetic tree (rooted phylogram) was created using the ClustalX neighbor-joining method and edited using MEGA 3.1. The number of bootstrap trials was set to 1,000. The proportion of bacterial species falling into each of the five phylogroups detected is shown. CFB, *Cytophaga-Flavobacterium-Bacteroidetes* group, corresponds to *Bacteroidetes* in panel A.

(did not include start/stop codons, respectively). Sequences upstream of the tandem Tc^r genes *tet*(O/32/O) and *tet*(40) were highly homologous (with between 31 and 83% amino acid identity) to the region of the VanG genetic element between *orfG11* and *orfG20* (Fig. 4; also see Table S1 in the supplemental material). The VanG element identified in *Enterococcus faecalis* is a mobile element carrying a set of genes conferring vancomycin resistance (the VanG operon) downstream of *orfG22* (6, 19). In clone T45, the tandem Tc^r genes are located after *orf10*, which has 41% identity to *orfG22* in the VanG element in *E. faecalis* (7, 20) (Fig. 4). Downstream of the tandem Tc^r genes, clone T45 *orf18* encodes a protein with 52% identity to a RecA DNA repair

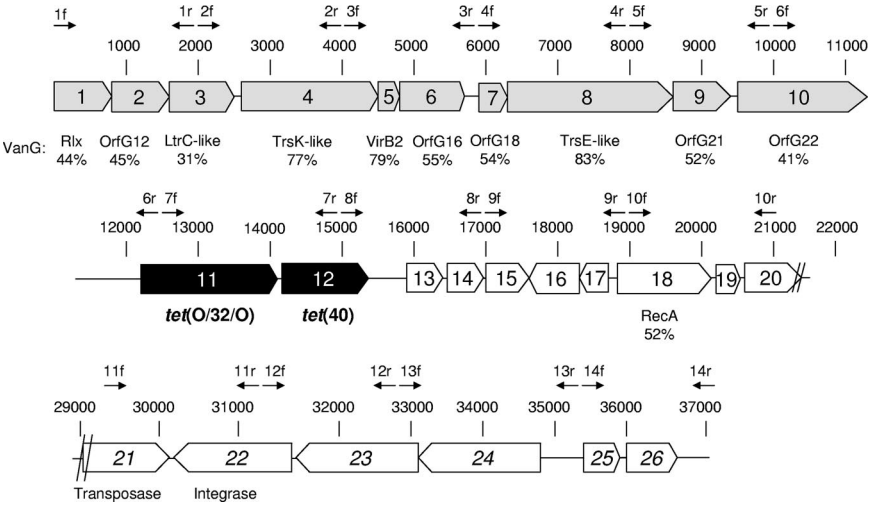

FIG. 4. Diagram showing genome structure of DNA insert in fosmid clone T45. Sequence analysis and editing were carried out using the program Artemis (http://www.sanger.ac.uk/Software/Artemis/), and the locations of genes were predicted using the heuristic approach for prokaryotic-gene predictions under the program GeneMark (http://exon.biology.gatech.edu/GeneMark/). Numbers inside arrows represent *orf*s in T45. Amino acid sequence identities to equivalent ORFs in the VanG operon in *E. faecalis* (shown in gray) are shown underneath the arrows. Tandem Tc^r genes are highlighted in black. The locations of primer pairs used for assessing the presence of the transposon-like element and tandem Tc^r genes are shown. BlastP results for all ORFs are summarized in Table S1 in the supplemental material. The gap in the sequence between *orf*s 20 and 21 is indicated (//).

protein from *Clostridium thermocellum* (see Table S1 in the supplemental material). The VanG element of *E. faecalis* does not contain *xis* or *int* genes that are typical of the Tn*916*-like CTn, and a RecA protein is postulated to perform this function (7). The DNA percent G+C contents of ORFs after the sequence gap in T45 range from 46 to 58%, whereas those of the ORFs in the first 21-kb sequence range from 35 to 46%, with the exception of *tet*(40), which has a higher G+C content (~56%). The closest relationships of the ORFs in T45 are summarized in Table S1 in the supplemental material.

PCR screening to identify the transposon-like element carrying tandem Tc^r genes. A PCR-based screen was conducted, using primers designed from the sequence of fosmid clone T45, on DNA from the other metagenomic clones and *C. saccharolyticum* K10 (detailed in Fig. 4). Fourteen primer sets were specifically designed, each amplifying a region of 2 kb, contiguously in both parts of the T45 sequence. Amplicons of the *tet*(O/32/O) and *tet*(40) genes were designed to be approximately 2.5 kb for ease of identification (Fig. 5).

In addition to T45, six fosmid clones containing the *tet*(O/32/O) gene carried the linked *tet*(40) gene and many of the same flanking ORFs. Certain flanking ORFs were not amplified in three inserts, indicating variability in the organization of the putative transposable element in one case (Fig. 5, T14) and variation in the cloned sequences in clones T10 and T18. *C. saccharolyticum* K10 and a transconjugant derived from a mating between *C. saccharolyticum* K10 and *Roseburia inulinivorans* (K. Scott, unpublished data) were also screened. Products were obtained for all amplicons upstream of *tet*(O/32/O) and *tet*(40) and for those based on the first 6 kb downstream of *tet*(40) in fosmid clone T45 in these bacteria (Fig. 5), indicating that the sequences diverge sometime after this point. This suggests strongly that the tandem Tc^r genes are transferred via a transposon-like mobile genetic element which finishes more than 6 kb after the end of *tet*(40), corresponding to the gap in the T45 sequence. Clone T45 and related clones T2, T9, and T21 do not appear to originate from *C. saccharolyticum* K10, since they possess different sequences downstream of *orf20*.

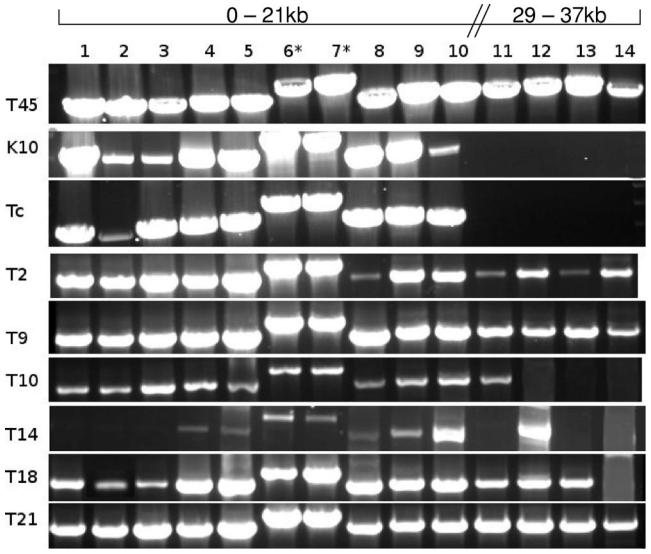

FIG. 5. PCR screening of new mobile element carrying the tandem Tc^r genes in other clones in the metagenomic library (T2 to T21), *C. saccharolyticum* K10, and a representative *R. inulinivorans*-K10 transconjugant (Tc) genomic DNA. Primers were designed from the sequence of T45 in such a way that the PCR products were contiguous along the length of the clone, as shown in Fig. 4. The sizes of the PCR products were predicted to be 2 kb in all cases, except for *tet*(O/32/O) and *tet*(40), which were approximately 2.5 kb each (marked by asterisk). The gap in the sequence of fosmid clone T45 between primer pairs 10 and 11 is indicated (//).

DISCUSSION

The new *tet*(40) gene described here encodes an efflux pump with similarities to *tetA*(P) isolated in some *Clostridium* species. Tetracycline efflux proteins are membrane-associated efflux pumps belonging to the major facilitator superfamily (MFS) that actively export tetracycline from bacterial cells against the concentration gradient (6). The efflux pump activity of Tet(40) was inhibited by CCCP. This compound acts as a proton uncoupler and inhibits MFS efflux pumps that use proton-motive force antiport systems to pump tetracycline out of bacterial cells. There are six groups of Tet efflux proteins based on amino acid sequence identity. TetA(P) and presumably also Tet(40) belong to group 4, characterized by the presence of 12 transmembrane α-helices and their prevalence in gram-positive *Clostridium* species (6). Key functional residues and domains were conserved between the amino acid sequences of Tet(40) and TetA(P) (1). *tetA*(P) is part of the Tet P determinant consisting of two genes overlapping by 17 bp. *tetA*(P) encodes a transmembrane protein that mediates active efflux of tetracycline, whereas *tetB*(P) encodes an RP type of tetracycline resistance. Although the RP *tet*(O/32/O) and the efflux *tet*(40) genes present in *C. saccharolyticum* K10 do not overlap and have distinct ribosome binding sites, they were cotransferred on the same transferable element, Tn*K10*. Presumably the tandem presence of RP and efflux genes confers a greater level of tetracycline resistance on the host cell. All of the metagenomic clones analyzed in detail in this study contained the tandem arrangement of *tet*(O/32/O) and *tet*(40). Since *tet*(40) has not been described previously, its distribution has not been assessed, and we do not know whether either gene exists independently.

Overall, 2% of clones in the metagenomic library constructed from the Ab1 sample conferred resistance to 10 μg/ml of tetracycline. If we assume an average bacterial genome size of 5 Mb and an average insert size of 35 kb, then the 4,000 clones screened correspond to approximately 28 bacterial genomes. If each bacterial genome harbored one Tc^r gene, we would expect 28 resistant clones out of 4,000 screened. The recovery of 80 resistant clones therefore implies the recovery of more than one chromosomal region conferring resistance from each genome. Furthermore, individual positive clones contained up to three Tc^r genes. Thus, there was an extraordinarily high incidence of Tc^r genes in the metagenomic library. The fecal sample used to prepare the library was obtained from donor Ab1, who had received repeated therapeutic doses of tetracycline for many years and for whom more than 90% of fecal bacteria were tetracycline resistant (21). The most abundant Tc^r gene in the metagenomic library was *tet*(O/32/O), detected in 27 out of 33 inserts expressing resistance to 10 μg tetracycline/ml. *tet*(W) was present in 19 inserts, *tet*(O) in 2 inserts, and *tet*(Q) in 1 insert, while 17 inserts contained both *tet*(O/32/O) and *tet*(W). More clones contained the *tet*(O/32/O) and *tet*(W) combination than single genes, and these genes were both also present in *C. saccharolyticum* K10. Whereas *tet*(O/32/O) and the novel *tet*(40) gene were cotransmissible in matings from *C. saccharolyticum* K10, *tet*(W) was not cotransferred. Thus, despite the fact that *tet*(W) and *tet*(O/32/O) are close enough to be recovered in the same inserts in several clones in the metagenomic library, they did not reside on the same transmissible element.

Novel mobile elements carrying the tandem Tc^r genes were detected in *C. saccharolyticum* K10 (21) and also by sequencing in several clones from the metagenomic library. Recent findings indicate that *tet*(O/32/O) is one of the most abundant genes in fecal samples from pigs and humans (26), and it seems likely that the new transposon-like element Tn*K10* has been responsible for at least some of the spread of this Tc^r gene. It is also probable that the closely linked *tet*(40) gene may prove to be as abundant as *tet*(O/32/O). Efflux genes have previously been found mainly in gram-negative bacteria (28). The sequence of the putative transposon in clone T45 contained regions with strong identity to the VanG transposon (7), which itself is homologous to Tn*1549*, another CTn conferring vancomycin resistance (9). The mobile conjugative element identified previously in the related cluster XIVa anaerobe *B. fibrisolvens* 1.230, Tn*B1230*, is also similar to Tn*1549* (22). Thus, it appears that both of these transposons containing transferable Tc^r genes are related to enterococcal transposons conferring vancomycin resistance and that the Tc^r genes replace the vancomycin resistance gene cassette. The two transposons from the commensal anaerobes are, however, more similar to their respective enterococcal homologues than to each other.

Based on the information available from sequencing and PCR amplification, it appears that at least part of the CTn present in *C. saccharolyticum* K10 (Tn*K10*) and clone T45 are very similar. The lack of amplification of any of the ORFs downstream of the gap in clone T45 indicates that the 3′ end of the Tn*K10* transposon occurs within this sequence gap. The successful amplification of T45 *orf1* (PCR 1) in all of the fosmid clones, *C. saccharolyticum* K10, and the *R. inulinivorans* transconjugant implies that the 5′ end of the transposon is not contained in clone T45. It is possible that the element in clone T45 is a composite transposon with the central part homologous to Tn*K10*. Thus, the *recA* gene (*orf18*) could be instrumental in the conjugative transfer of Tn*K10*, whereas the transposase and integrase encoded on *orf21* and *orf22* could be part of a larger composite transposon present in fosmid clones T45, T2, T9, and T21. The differences in DNA %G+C strongly indicate different origins for the two parts of the sequence.

In conclusion, the *tet*(40) gene reported here represents a new efflux-type resistance determinant, found to be present in tandem with an RP-type resistance gene. The potential abundance of this new gene among gut bacteria, at least in individuals with a history of oral tetracycline therapy, is suggested by its recovery in many inserts conferring Tc^r from a human fecal metagenomic library. This is the first report of the use of a metagenomic approach for the analysis of tetracycline resistance in bacteria associated with the human colon, although a new Tc^r gene encoding a novel NADPH-dependent oxidoreductase that enzymatically inactivates tetracycline, *tet*(37), was identified in a human oral metagenomic library (8). Metagenomic approaches are therefore potentially valuable for investigating the occurrence of antibiotic resistance genes and for the recovery of novel genes, especially from microorganisms that cannot easily be cultivated under laboratory conditions and that may represent an important reservoir of antibiotic resistance in the environment.

ACKNOWLEDGMENTS

We thank Donna Henderson for automated DNA sequencing and Gill Campbell for automatic colony picking. Many thanks are also given to Anthony Travis for helpful assistance in sequence assembly using the RRI computer facility and to Gary Duncan for the LC–MS-MS analysis.

K.A.K. is currently supported by the Department for Environment, Food and Rural Affairs (DEFRA), and M.T.R. was supported by European Union grant GEMINI (QLRT-2001-02056). The Rowett Research Institute is supported by SG-RERAD (Scottish Government Rural and Environment Research and Analysis Directorate).

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