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Sequencing of a *tet*(Q) Gene Isolated from *Bacteroides fragilis* 1126

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Recently, Tet Q, a tetracycline resistance determinant that confers resistance by a ribosome protection mechanism, was described and added to the two previously described classes, Tet M and Tet O. The first representative of this class, *tetA*(Q)1, was isolated from *Bacteroides thetaiotaomicron* DOT. We report the sequencing of a gene isolated from *B. fragilis* 1126 which also confers tetracycline resistance. Because of its high degree of identity (97%) with the *tetA*(Q)1 gene, we defined it as *tetA*(Q)2. MIC studies revealed that *tetA*(Q)2 provides a low level of resistance to tetracycline when cloned into *Escherichia coli*. The extensive homology between *tetA*(Q)1 and *tetA*(Q)2 supports the idea of a recent horizontal transfer of *tet*(Q) genes among *Bacteroides* spp.

Widespread use of broad-spectrum tetracycline antibiotics has led to the appearance of relatively high levels of resistance to these drugs in the microbial world. Three categories of tetracycline resistance mechanisms have been described: (i) tetracycline efflux, (ii) tetracycline modification, and (iii) ribosome protection (23). In addition to mechanistic classification, genes that confer tetracycline resistance are grouped on the basis of DNA hybridization. Two genes that encode resistance to tetracycline are considered to be part of the same class if they are shown to cross-hybridize by Southern analysis. Three classes of ribosome protection mechanisms have been reported: Tet M, Tet O, and Tet Q. Tet M represents the most widespread tetracycline determinant, as it has been isolated from a wide range of gram-positive, as well as gram-negative, bacteria (2, 3, 5, 7, 8, 18, 22, 29–31). The Tet O determinant found in streptococci (21) and campylobacters (25) codes for resistance to all tetracycline antibiotics, including minocycline. Only one gene of the newly reported ribosomal protection determinant Tet Q has been described. This gene was isolated from *Bacteroides thetaiotaomicron* DOT (27) and is suggested to have spread horizontally among *Bacteroides* species (27).

Proteins encoded by members of the same class of tetracycline resistance determinants possess a high degree of identity: 92 to 97% amino acid identity for M-class members and as high as 98% for Tet(O) proteins. As expected, the homology between proteins of different classes is lower, i.e., 75% identity between members of the M and O classes and 52 and 53% homology to Tet(Q) for the Tet(M) and Tet(O) proteins, respectively.

The genus *Bacteroides* consists of a large, heterogeneous group of non-spore-forming, nonmotile, obligatorily anaerobic, gram-negative rods which can colonize the human gastrointestinal tract, as well as the vaginal tract and oral cavity (15, 28, 32). Resistance to tetracycline is widespread among *Bacteroides* clinical isolates. In many colonic *Bacteroides* isolates, a nonplasmid conjugation system that mediates transfer of tetracycline resistance is induced by low levels of tetracycline (27, 28). We report herein the sequence of a second *tet*(Q) gene isolated from a member of this genus and the comparison of this gene to that first reported by Nikolich et al. (27).

(A preliminary report of this research has been presented previously [19].)

Origin of the *tetA*(Q)2 gene. The *tetA*(Q)2 gene originated from *Bacteroides fragilis* 1126, a clinical strain isolated between 1978 and 1981 at University Hospital, San Diego, Calif., and identified by standard culture and biochemical methods (11). The *tetA*(Q)2 gene has been localized on a large transfer element and is linked to a clindamycin resistance locus whose self-transfer is regulated by exposure to tetracycline or clindamycin (13), as has also been reported for *tetA*(Q)1 (27). *B. fragilis* tetracycline resistance gene *tetA*(Q)2 was first identified on a 13-kb DNA fragment, subsequently mapped to a 2.8-kb region, and subcloned into the *Sma*I-*Cla*I sites of plasmid Bluescript II SK$^+$, producing plasmid pBSK1.2-5 (13), as kindly provided to us by D. G. Guiney. The strain of origin, *B. fragilis* 1126, as well as transconjugants obtained from the mating between Tc^s *B. fragilis* 638 and 1126 exhibited resistance to 40 μg of tetracycline per ml (13).

Transformation. Recombinant plasmid DNA was transformed into *E. coli* DH5α [F$^-$ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* (r_k^- m_k^+) *supE44* λ$^-$ *thi-1 gyrA96 relA1*] (Bethesda Research Laboratories, Gaithersburg, Md.) in accordance with the $CaCl_2$ method described by Sambrook et al. (34). Selection was accomplished on Luria-Bertani (LB) agar plates (Bacto Tryptone [Difco Laboratories, Detroit, Mich.] at 10 g/liter, NaCl at 10 g/liter, Bacto Yeast Extract [Difco] at 5 g/liter, agar at 15 g/liter) supplemented with 50 μg of ampicillin per ml, 0.5 mM isopropyl-β-D-thiogalactopyranoside (Promega Corp., Madison, Wis.), and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Promega Corp.) per ml at 37°C overnight.

Preparation of DNA. Recombinant plasmid DNA was prepared by the alkaline lysis method in accordance with the modification of Ish-Horowicz and Burke of the procedure of Birnboim and Doly (34). The covalently closed circular DNA was purified by equilibrium centrifugation in a continuous

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CsCl-ethidium bromide gradient (34). DNA destined for sequence analysis was additionally precipitated with polyethylene glycol (1).

Subcloning. All enzymes were purchased from Promega Corp., except *Nru*II, which was acquired from Stratagene, La Jolla, Calif. The desired DNA fragments obtained following digestion were recovered from agarose gels (0.7% agarose in Tris-borate-EDTA buffer [34]) by a freeze-thaw procedure (36). DNA fragments used in subcloning were inserted into plasmid pUC19 (Bethesda Research Laboratories), creating subclones pLL1 (*Hin*cII-*Pvu*II 689-bp fragment of pBSK1.2-5), pLL2 (*Eco*RI-*Sma*I 378-bp fragment of pBSK1.2-5), pLL3 (*Pvu*II-*Nru*I 611-bp fragment of pBSK1.2-5), and pLL4 (*Nru*I-*Eco*RI 926-bp fragment of pBSK1.2-5), or into vector Bluescript II SK$^+$ (Stratagene), giving pLL5 (*Nru*I-*Bam*HI 1,308-bp fragment of pBSK1.2-5). These five subclones were used for sequencing. The ligations were performed overnight at 16°C with T4 DNA ligase.

Sequence. Double-stranded DNA sequencing was performed by the University of Florida Interdisciplinary Center for Biotechnology Research DNA Sequencing Core Laboratory. Sequencing was accomplished by employing the *Taq* Dye Primer and *Taq* Dye Terminator cycle sequencing protocols (Applied Biosystems, Inc., Foster City, Calif.) with fluorescent primers and dideoxynucleotides, respectively. The labeled extension products were analyzed on an ABI 373a DNA Sequencer (Applied Biosystems, Inc.). The entire sequence was obtained for both strands of DNA by using the appropriate subclones or synthetic oligonucleotides synthesized by the University of Florida DNA Synthesis Core Facility. The sequencing strategy was designed to sequence overlapping sites used in DNA subcloning. The complete sequence was analyzed with Genetics Computer Group sequence analysis software (6).

MICs. The level of expression of the tetracycline resistance gene in *E. coli* DH5α was assayed by testing its susceptibility to tetracycline by the broth macrodilution method (26). Briefly, *E. coli* DH5α, *E. coli* DH5α bearing plasmid Bluescript II SK$^+$, and *E. coli* DH5α bearing recombinant plasmid pBSK1.2-5 were grown overnight in Mueller-Hinton broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 50 μg of ampicillin per ml. On the next day, the cell density was adjusted with Mueller-Hinton broth to a 0.5 McFarland standard ($\sim 10^8$ CFU/ml). One milliliter of a 1:100 dilution of this bacterial suspension was added to 1-ml tubes of Mueller-Hinton broth containing doubling dilutions of 0.25 to 64 μg of tetracycline per ml. The final inoculum was 5×10^5 CFU/ml. The tubes were then incubated for 24 h without shaking at 37°C before growth was evaluated. Each MIC experiment was repeated in triplicate to ensure the validity of the results. MIC determinations were also done with an inoculum 100 times more concentrated or with LB broth instead of Mueller-Hinton broth. To determine whether the *tetA*(Q)2 gene can be induced in *E. coli* DH5α, an additional MIC experiment was done with cells grown in the presence of a subinhibitory concentration of tetracycline (0.25 μg/ml) in Mueller-Hinton broth.

Transcription-translation assay. The proteins encoded by recombinant plasmid pBSK1.2-5 were analyzed with the *E. coli* S30 Extract System for Circular DNA Templates (Promega Corp.). The assay was performed in accordance with the instructions provided by the manufacturer, with [^{35}S]methionine (>800 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). The labeled proteins were separated on a sodium dodecyl sulfate-polyacrylamide gel (5% stacking gel and 15% separating gel) as described by Laemmli (20). The sizes of the proteins were calculated by comparing their migration with that of ^{14}C-labeled, methylated protein midrange molecular weight markers (Amersham Corp.).

The nucleic acid sequence of the gene isolated from *B. fragilis* 1126 which encodes tetracycline resistance and is defined as *tetA*(Q)2 is shown in Fig. 1. Two potential translational start sites are evident, a conventional ATG sequence at position 362 and a GTG sequence at position 314 of the DNA fragment. The potential ATG start site is preceded by putative −10 (TATATT; position 342 of the DNA) and −35 (TCGACA; position 321 of the DNA) sequences. These two sequences differ by only one nucleotide from the *E. coli* consensus promoter sequences TATAAT and TTGACA, respectively. However, no match to the consensus Shine-Dalgarno sequence was found 5′ to the ATG. An open reading frame beginning at this ATG site would encode a 642-amino-acid protein with a deduced molecular mass of 72.5 kDa possessing an isoelectric point of 5.34. The second potential but unconventional GTG site for initiation of translation is also preceded by putative −10 (ATTAAT; position 258 of the DNA) and −35 (TTGTAA; position 225 of the DNA) sequences. Such promoter sequences are distinct from the *E. coli* consensus sequences in two positions and are located farther upstream than what has commonly been reported for *E. coli*. In contrast to the ATG start site, a ribosome-binding site (AGGAGG; position 299 of the DNA) matching the *E. coli* consensus sequence perfectly is located 16 nucleotides upstream of the potential GTG initiation sequence. A 658-amino-acid protein with a deduced molecular mass of 74.4 kDa and a pI of 5.34 would be encoded by this longer open reading frame. It is not possible, given only the sequence data, to identify which of these start codons, GTG or ATG, is used to translate the mRNA. By using *tetA*(Q)1, Nikolich et al. (27) tried to determine the start codon with exonuclease III. By constructing deletions extending 26 bp into the beginning of the large open reading frame of the *tetA*(Q)1 gene, thereby removing the putative −35 sequence, Nikolich and coworkers were able to reduce the level of resistance in *E. coli* without eliminating it entirely, suggesting that the ATG start site is the one used in *E. coli* (27). However, the same deletion completely eliminated the resistance when the modified gene was transferred into Tc^s *B. thetaiotaomicron* BT4001, a Rif^r derivative of type strain 5482 (27), indicating that the GTG start site is probably used in *Bacteroides* spp. We hypothesize that both GTG and ATG may be utilized in *Bacteroides* species under different conditions, depending upon the amount of Tet(Q) protein needed; GTG is under the control of a stronger promoter than ATG.

Comparison of the tetracycline genes isolated from *B. thetaiotaomicron* [*tetA*(Q)1] (27) and *B. fragilis* [*tetA*(Q)2] revealed 97.3% identity at the nucleic acid level and 97.0% identity at the amino acid level with ATG as the initiation site for translation. The same comparison done with GTG as the start site produced 97.5 and 97.9% identities based on the nucleic acid and amino acid sequences, respectively. The molecular G+C content for the small open reading frame of *tetA*(Q)2 is 39.5%, compared with 40.1% for the *tetA*(Q)1 gene. Very similar results were obtained for the G+C content of the reading frame beginning with GTG, i.e., 39.4% for *tetA*(Q)2 and 39.9% for *tetA*(Q)1. The values obtained for either reading frame are comparable to the 42% G+C of the chromosomes of *B. thetaiotaomicron* and *B. fragilis* (16). The high degree of identity reported between

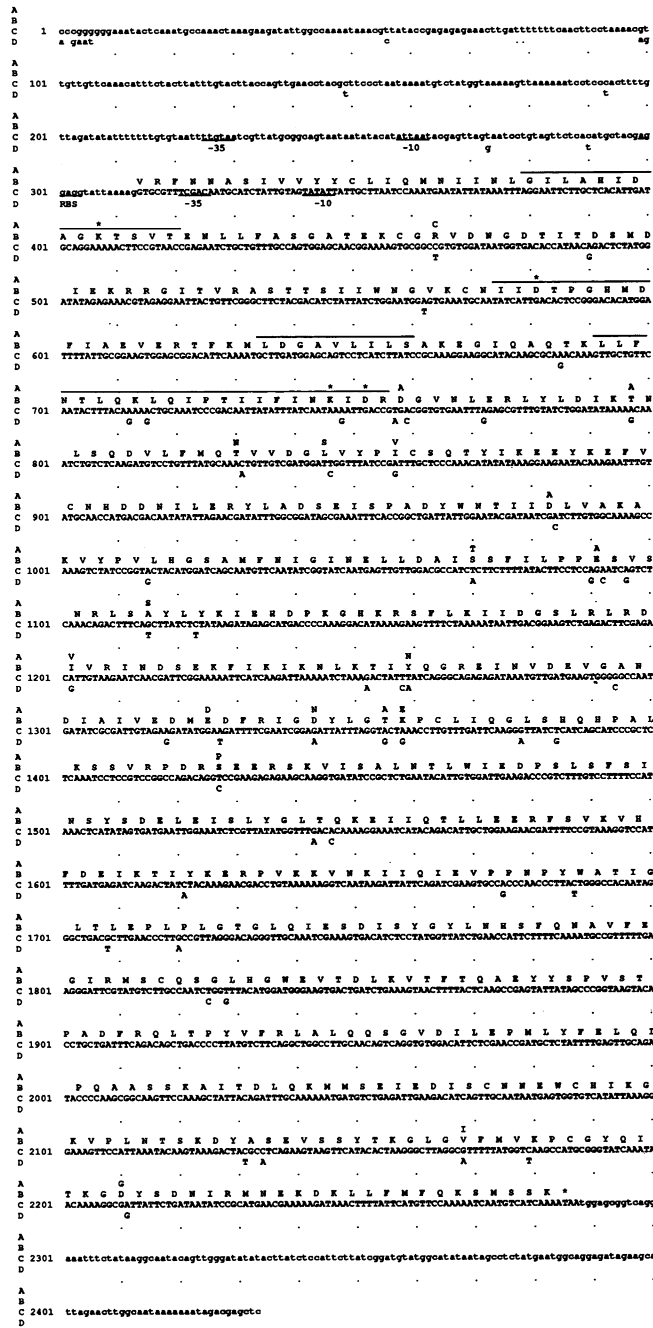

FIG. 1. Nucleotide and deduced amino acid sequences of the *tetA*(Q)2 gene compared with those of the *tetA*(Q)1 gene. Lines: A, differences in the amino acid sequences of the TetA(Q)2 and TetA(Q)1 proteins; B, deduced amino acid sequence of the TetA(Q)2 protein for the large open reading frame; C, sequence of the *tetA*(Q)2 gene; D, differences between the *tetA*(Q)2 and *tetA*(Q)1 genes. Two putative sets of −10 and −35 regions are indicated, one pair for each of the possible start codons, GTG (position 314) and ATG (position 362). A potential ribosome-binding site (RBS) preceding the GTG codon is indicated at position 299. The nucleotide part of the open reading frame is shown in uppercase letters. The four principal regions of sequence similarity found in GDP-GTP-binding proteins are indicated by bars over the sequence. Within these regions, the invariant amino acids making direct contact with the GDP ligand are labeled with an asterisk (14, 24).

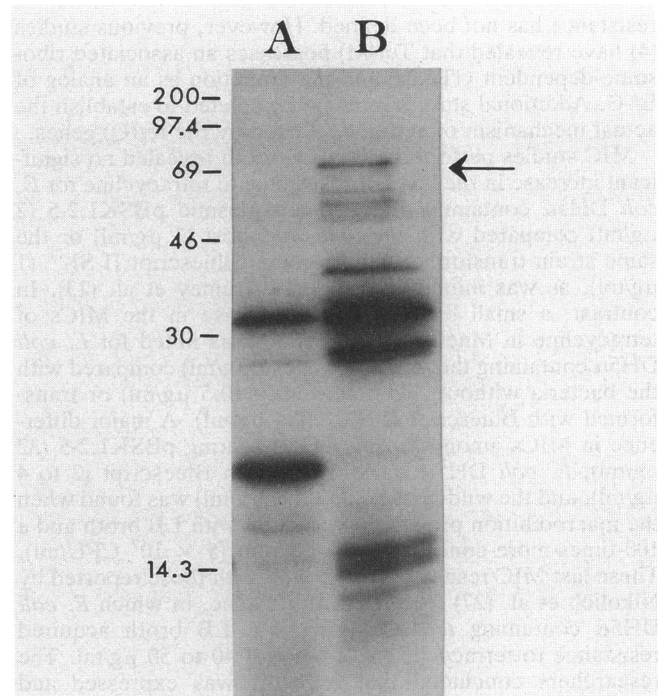

FIG. 2. Protein expressed by pBSK1.2-5 in an in vitro transcription-translation assay. Lanes: A, products of Bluescript II SK$^+$; B, products of pBSK1.2-5. The arrow indicates the 70- to 72-kDa TetA(Q)2 protein. Protein molecular size standards are in kilodaltons.

the *tetA*(Q)1 and *tetA*(Q)2 genes may at first glance appear surprising as their hosts of origin, i.e., *B. thetaiotaomicron* and *B. fragilis*, respectively, share an average of only 20% DNA-DNA homology (16), suggesting recent horizontal transmission of the Tet Q determinant in the *Bacteroides* group. Furthermore, DNA homologous to the Tet Q determinant has been identified by Southern hybridization in many other colonic, as well as oral, *Bacteroides* isolates resistant to tetracycline, even though these isolates may have less than 7% DNA homology (12, 16).

The upstream regions of *tetA*(Q)1 and *tetA*(Q)2 have a very high degree of homology. However, these upstream regions are different from the DNA sequences upstream of the *tet*(O) and *tet*(M) open reading frames (38). It is unknown whether the upstream region plays a role in the full expression or regulation of the *tetA*(Q) genes in *E. coli* or *Bacteroides* spp., as has been postulated for *tet*(O) and *tet*(M).

The results obtained from transcription-translation assays are presented in Fig. 2. They corroborate the nucleic acid sequence analysis, revealing the production of a 70- to 72-kDa protein in the in vitro *E. coli* system. Comparison of the deduced TetA(Q)1 and TetA(Q)2 proteins revealed that only 19 amino acids were different. As previously noticed for Tet(M) (35), Tet(O) (24), and TetA(Q)1 (27), comparison of TetA(Q)2 and GDP-GTP-binding proteins, such as Ef-Tu and Ef-G, revealed four short conserved regions where none of the 19 different amino acids are present. These conserved regions have been shown by X-ray crystallographic studies to be implicated in GDP-GTP binding for Ef-Tu (17). In addition, one of these regions has been found to be essential for the tetracycline resistance conferred by Tet(O) (9). The biochemical mechanism by which these proteins confer

resistance has not been defined. However, previous studies (4) have revealed that Tet(M) possesses an associated ribosome-dependent GTPase and may function as an analog of Ef-G. Additional studies must be completed to establish the actual mechanism of action conferred by the *tet*(Q) genes.

MIC studies performed with LB broth revealed no significant increase in the level of resistance to tetracycline for *E. coli* DH5α containing recombinant plasmid pBSK1.2-5 (2 μg/ml) compared with the wild-type host (1 μg/ml) or the same strain transformed with plasmid Bluescript II SK$^+$ (1 μg/ml), as was initially reported by Guiney et al. (13). In contrast, a small but significant increase in the MICs of tetracycline in Mueller-Hinton broth was noted for *E. coli* DH5α containing the *tetA*(Q)2 gene (2 μg/ml) compared with the bacteria without this determinant (0.5 μg/ml) or transformed with Bluescript II SK$^+$ (0.5 μg/ml). A major difference in MICs among *E. coli* DH5α bearing pBSK1.2-5 (32 μg/ml), *E. coli* DH5α transformed with Bluescript (2 to 4 μg/ml), and the wild-type host (2 to 4 μg/ml) was found when the macrodilution procedure was done with LB broth and a 100-times-more-concentrated inoculum (5×10^7 CFU/ml). These last MIC results correlate well with those reported by Nikolich et al. (27) for the *tetA*(Q)1 gene, in which *E. coli* DH5α containing *tetA*(Q)1 grown in LB broth acquired resistance to tetracycline at a level of 40 to 50 μg/ml. The researchers concluded that *tetA*(Q)1 was expressed and conferred tetracycline resistance in *E. coli*, contrary to other antibiotic resistance determinants cloned from *Bacteroides* species (10, 33, 37). However, the noticeable variations in drug sensitivity conferred upon *E. coli* by the Tet Q determinants in these MIC studies can be partly explained by differences in the procedures used, such as the type of broth, incubation with or without agitation, or what seems to be the most important variable, the concentration of the final inoculum utilized. Thus, caution should be used when comparing MIC results from different sources, and MIC studies using standardized techniques (26) should be performed on *E. coli* DH5α transformed with *tetA*(Q)1 to obtain a definite answer as to its ability to change host susceptibility to the drug. However, we do not exclude the possibility of a real difference in the expression of *tet*(Q) genes, even though comparison of the TetA(Q)1 and TetA(Q)2 protein sequences revealed no major differences which would explain some discrepancies in their abilities to confer tetracycline resistance. Indeed, all of the amino acid residues possibly involved in GTP binding or GTPase activity (24) are exactly identical, but slight changes in the overall protein sequence can still be observed. Because not all of the protein regions implicated in the ribosome protection mechanism are known, modification of an amino acid in one important area would greatly affect the ability of the protein to protect the *E. coli* ribosome against tetracycline, affecting host susceptibility to the drug. Thus, even though TetA(Q)1 and TetA(Q)2 are very similar, they may act differently when present in an *E. coli* host.

When *E. coli* DH5α cells containing pBSK1.2-5 grown with a subinhibitory concentration of tetracycline were used as the inoculum in MIC studies, the MIC of tetracycline for these cells was the same (2 μg/ml) as when cells grown without tetracycline were used for the inoculum. These results indicate that *tetA*(Q)2 gene expression is not induced by tetracycline.

We reported the sequence of a second member of the Q class of tetracycline resistance determinants which confer resistance by protection of ribosomes. Our data indicate that *tet*(Q) genes possess an impressive degree of identity, as is the case for genes of the other two ribosome protection groups, Tet M and Tet O.

Nucleotide sequence accession number. The sequence presented in this report has been deposited in the EMBL data library under accession no. Z21523.

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