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New Tetracycline Resistance Determinants Coding for Ribosomal Protection in Streptococci and Nucleotide Sequence of *tet*(T) Isolated from *Streptococcus pyogenes* A498

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An approach based on PCR has been developed to identify new members of the *tet* gene family in streptococci resistant to tetracycline and minocycline. Degenerate primers, corresponding to portions of the conserved domains of the proteins Tet(M), Tet(O), TetB(P), Tet(Q), and Tet(S), all specifying the tetracycline-minocycline resistance phenotype, were used to selectively amplify DNA fragments within the coding sequences. Nine streptococcal strains which do not carry the genes *tet*(M), *tet*(O), *tetB*(P), *tet*(Q), or *tet*(S) were investigated. Four of them gave no detectable PCR products. The five remaining strains each yielded a PCR product of 1.1 kbp. DNA hybridization experiments showed that these putative Tet determinants fell into four new hybridization classes, of which one, Tet T, was further analyzed. The gene *tet*(T) was isolated from *Streptococcus pyogenes* A498, and the nucleotide sequence that was necessary and sufficient for the expression of tetracycline resistance in *Escherichia coli* was determined. The deduced Tet(T) protein consists of 651 amino acids. The protein most closely related to Tet(T) was Tet(Q), which has 49% identical amino acid residues. A phylogenetic analysis revealed that Tet T represents a novel branching order among the Tet determinants so far described.

The tetracycline group of antibiotics exhibits a broad spectrum of activity and little toxicity. Such properties led to the widespread use of tetracyclines and related drugs for human and animal therapy. Tetracyclines inhibit protein synthesis by blocking the binding of aminoacyl-tRNA to the A site of the ribosomes (45). Three known mechanisms of resistance to tetracyclines have been reported: drug inactivation, efflux by proton antiporters, and protection of the ribosomes (7). The last mechanism, although not yet completely elucidated, appears to be the most frequent in nature (5, 23, 45, 47); it mediates resistance to the narrow- and expanded-spectrum tetracyclines (22, 35).

To date, six classes of Tet determinants encoding ribosomal protection toward tetracycline and minocycline, i.e., Tet M, Tet O, Tet P, Tet Q, Tet S, and Tet U, have been identified by DNA-DNA hybridization in several bacterial genera of clinical importance, including *Bacteroides*, *Campylobacter*, *Clostridium*, *Enterococcus*, *Gardnerella*, *Haemophilus*, *Listeria*, *Mycoplasma*, *Neisseria*, *Staphylococcus*, *Streptococcus*, and *Ureaplasma* (6, 22, 34, 35). Numerous genes, prototypes, and variants representative of the Tet M-Tet S classes have been cloned and sequenced (4, 6, 14, 19, 21, 25, 28–30, 40, 42, 43, 46). The similarities of the deduced polypeptides range from 60 to 99%. Structure-function relationships have been highlighted by comparison with other Tet proteins, including the Otr(A) protein, found in *Mycobacterium* and *Streptomyces* species (11, 12, 32). The regions of greatest homology are centered around five highly conserved GTP-binding motifs, which were shown to be functional in Tet(M) and Tet(O) (5, 23, 45, 47). In contrast, Tet(U) has no GTP-binding motif and thus appears quite distinct in terms of size and similarity (<20% amino acid identity with each of the members belonging to this family of proteins).

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In our previous studies focusing on the distribution of the *tet* genes in streptococci, we stated that among 158 plasmid-free strains resistant to tetracycline-minocycline, the genes *tet*(M) and *tet*(O) were present in 88 and 6.4% of the strains, respectively (3, 8, 9, 18). No hybridization with *tet*(M), *tet*(O), *tetB*(P), *tet*(Q), or *tet*(S) was detected for the nine remaining strains (5.6% of the total). The aim of the present study was to investigate the molecular basis of the chromosomal tetracycline-minocycline resistance of these nine strains. By using an approach based on PCR, similar to that described previously for the genes *erm* (2), *cat* (49), and *vat* (1), we amplified *tet*-specific DNA fragments from five of the nine streptococcal strains investigated. These DNA fragments could be grouped into four new hybridization classes. The gene *tet*(T), located on the chromosome of *Streptococcus pyogenes* A498, was cloned and sequenced.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in the study are described in Table 1. Most of the streptococcal strains investigated were obtained between 1979 and 1988 in various French hospitals from human patients with diverse infections; those of group C, however, originated from animals. The medium used for streptococcal strains was brain heart infusion broth or agar (Difco Laboratories, Detroit, Mich.), and that used for *Escherichia coli* strains was Luria-Bertani broth or agar (1.5%, wt/vol) containing 10 g of tryptone per liter, 5 g of yeast extract per liter, and 10 g of sodium chloride per liter. Antibiotics were used at the following concentrations: 100 μg of ampicillin per ml for selection of *E. coli* transformants and 10 μg of tetracycline or minocycline per ml for analysis of the transformants by replica plating.

Enzymes and chemical reagents. Restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase, and Klenow polymerase (Amersham International, Little Chalfont, England, or Pharmacia Biotech, Uppsala, Sweden) were used according to the manufacturers' instructions. Acrylamide-bisacrylamide solution was purchased from Appligène (Illkirch, France), and agarose of analytical grade was purchased from FMC BioProducts (Rockland, Maine). The blue dye 5-bromo-4-chloro-3-indolyl-β-D-galactoside and the inducer isopropyl-α-D-thiogalactoside were both supplied by Appligène. The oligodeoxynucleotides were synthesized and purified by Eurogentec (Seraing, Belgium). Three of them, designed for PCR experiments, were degenerate; they are described below in terms of the conventional one-letter code (31): oligodeoxynucleotide DI (4,096-fold degeneracies; forward primer [Fig. 1]), 5′-GAYACICCIGGICAYRTIGAYTT; oligodeoxynucleotide DII (4,096-fold degeneracies; reverse primer [Fig. 1]), 5′-G

TABLE 1. Bacterial strains and plasmids used in the study

Strains[a] (species, group) and plasmids	Relevant characteristics of the strains and plasmids[b]	Tet determinant (PCR product)	Reference or source
Strains			
A498 (*Streptococcus pyogenes*, A)	Tc^r-Mn^r, Tra$^-$, human origin	Tet T (+)	This study
B130 (*Streptococcus agalactiae*, B)	Cm^r Em^r Tc^r-Mn^r, Tra$^-$, human origin	ND[c] (+)	This study
C94 (*Streptococcus equisimilis*, C)	Sm^r Tc^r-Mn^r, Tra$^-$, animal origin	ND (−)	This study
C95 (*Streptococcus equisimilis*, C)	Sm^r Tc^r-Mn^r, Tra$^-$, animal origin	ND (−)	This study
C96 (*Streptococcus equisimilis*, C)	Tc^r-Mn^r, Tra$^-$, animal origin	ND (−)	This study
D135 (*Streptococcus bovis*, D)	Em^r Tc^r-Mn^r, Tra$^-$, human origin	ND (+)	9
D295 (*Streptococcus bovis*, D)	Em^r Tc^r-Mn^r, Tra$^-$, human origin	ND (+)	9
G59 (*Streptococcus* sp., G)	Em^r Km^r-Nm^r Sm^r Tc^r-Mn^r, Tra$^-$, human origin	ND (−)	This study
MG32 (*Streptococcus anginosus*)	Tc^r-Mn^r, Tra$^-$, human origin	ND (+)	8
MG16 (*Streptococcus anginosus*)[d]	Em^r Km^r-Nm^r Sm^r Tc^r-Mn^r, Tra$^-$, human origin	Tet M (+)	8
MG23 (*Streptococcus anginosus*)[d]	Em^r Tc^r-Mn^r, Tra$^-$, human origin	Tet O (+)	8
TG1 (*Escherichia coli*)[e]	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	None	Amersham
Plasmids			
pUC18[f]	Ap^r *lacZ*α	None	Appligène
pAT101[g]	pUC18Ω0.85-kb *Hin*dIII-*Cla*I fragment of Tn*1545*	Tet M (NT[h])	25
pUOA4[g]	pUC18Ω1.8-kb *Hin*cII fragment of pUA466	Tet O (NT)	46
pJIR939[d,g]	pWSK29Ω3-kb *Xba*I-*Cla*I fragment of pCW3	Tet P (+)	42
pBSK1.2-5[d,g]	pBluescript II SKΩ2.8-kb *Sma*I-*Cla*I fragment of *Bacteroides fragilis* 1126	Tet Q (+)	15
pAT451[d]	pUC18Ω4.5-kb *Cla*I fragment of pIP811	Tet S (+)	6
pAT456[g]	pUC19Ω0.9-kb *Eco*RI-*Bgl*II fragment of pAT451	Tet S (NT)	6
pIP1695	pUC18Ω1.1-kbp amplified DNA from A498	Tet T (+)	This study
pIP1696	pUC18Ω7.3-kbp chromosomal DNA from A498	Tet T (+)	This study
pIP1697	pUC18Ω2.8-kbp *Hin*cII-*Kpn*I fragment of pIP1696	Tet T (+)	This study

[a] All the strains are plasmid-free.
[b] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance (macrolide-lincosamide-streptogramin B phenotype); Km^r, high-level kanamycin resistance; Mn^r, minocycline resistance; Nm^r, high-level neomycin resistance; Sm^r, high-level streptomycin resistance; Tc^r, tetracycline resistance; Tra, conjugative transfer.
[c] ND, not determined.
[d] Strains or plasmids used as positive controls in PCR assays.
[e] Host strain used for bacterial transformation.
[f] Plasmid used as a cloning vector.
[g] Plasmid used as a probe.
[h] NT, not tested.

CCCARWAIGGRTTIGGIGGIACYTC; and oligodeoxynucleotide DIII (262,144-fold degeneracies; reverse primer [Fig. 1]), 5′-CKRAARTCIGCIGG IGTISWIRCIGG.

DNA manipulations. Cellular DNA of the streptococcal strains was prepared as reported earlier (20). Plasmid DNA was recovered from *E. coli* cells by using a Nucleobond kit (Macherey-Nagel, Düren, Germany). DNA analysis, cloning, and sequencing were carried out by standard methods (38). DNA fragments were extracted from agarose gels and purified with the GeneClean II kit (Bio 101, Inc., La Jolla, Calif.). Hanahan's procedure (16) served to transform *E. coli* TG1. Hybond-N$^+$ membranes (Amersham International) were used for Southern and colony blotting. Probes were labelled with [α-^{32}P]dCTP by using the Megaprime DNA labelling system (Amersham International). Prehybridization and hybridization experiments were carried out under stringent conditions, as described previously (20). The recombinant plasmids used as probes are listed in Table 1. Sequencing was performed on alkali-denatured plasmid DNA with Sequenase, version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio), according to the dideoxy-chain termination method (41).

PCR experiments. Amplification was done in a 0.5-ml microcentrifuge tube containing 10 ng of purified cellular DNA or 1 ng of linearized plasmid DNA, 0.6 μM (each) primer, 200 μM (each) deoxynucleoside triphosphate, 2.5 U of *Taq* polymerase (Amersham International), and 1× supplied buffer. The final 100-μl reaction volume was overlaid with 50 μl of heavy white mineral oil (Sigma Chemical Co., St. Louis, Mo.). The reaction was performed in a Crocodile II incubator (Appligène) programmed with the following times and temperatures: 5 min at 95°C, 2 min at 45°C, then 35 cycles of 2 min at 72°C, 40 s at 92°C, and 40 s at 45°C, and, finally, 10 min at 72°C. Ten percent of each PCR-amplified sample was analyzed by electrophoresis through a 1% (wt/vol) agarose gel in Tris-borate buffer. The amplified DNA fragments were stained with ethidium bromide and then visualized with UV light. The sizes of the bands were evaluated in comparison with those of the 1-kb DNA ladder (Bethesda Research Laboratories, Inc., Bethesda, Md.).

Computer analysis of sequence data. The programs contained within the Sequence Analysis Software Package, licensed from the Genetics Computer Group, Inc., University of Wisconsin, were used for all DNA sequence analyses (10). Amino acid homology searches were run through the GenBank-EMBL Data Library by using the TFastA method (33). Multiple alignments and evaluation of relatedness among homologous proteins were determined by appropriate algorithms delivered in the Clustal V package (17) and in the PHYLIP 3.5 package (13), respectively.

Nucleotide sequence accession number. The 2.8-kbp DNA sequence reported in this paper was deposited in the GenBank-EMBL Data Library under accession number L42544.

RESULTS AND DISCUSSION

Development of PCR assays for the amplification of DNA fragments within the genes encoding tetracycline-minocycline resistance. The alignment of the amino acid sequences of the Tet proteins implicated in ribosomal protection against tetracyclines has revealed highly conserved stretches located at the amino and carboxyl ends of the proteins (Fig. 1). The reverse translation of portions of domains I, II, and III served to design the degenerate oligodeoxynucleotides DI, DII, and DIII (see Materials and Methods), which were paired according to their convergent polarities. Two oligodeoxynucleotide pairs, DI-DII and DI-DIII, were used as primers in preliminary PCR assays performed with genomic or plasmid DNA carrying the *tet*(M), *tet*(O), *tetB*(P), *tet*(Q), or *tet*(S) gene (Table 1). The use of the oligodeoxynucleotide pair DI-DIII enabled us to amplify a DNA fragment of 1.5 kbp for each of the *tet* genes tested. However, multiple amplicons were regularly obtained in each

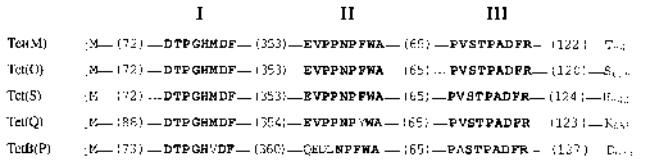

FIG. 1. Three conserved domains of the Tet(M), Tet(O), TetB(P), Tet(Q), and Tet(S) proteins. The amino acid sequences were translated from the nucleotide sequences registered in the GenBank-EMBL databases under the indicated accession numbers: *tet*(M) from *Enterococcus faecalis* (X56353) (4), *tet*(O) from *Campylobacter jejuni* (M18896) (24), *tet*(S) from *Listeria monocytogenes* (L09756) (6), *tet*(Q) from *Bacteroides fragilis* (Z21523) (21), and *tetB*(P) from *Clostridium perfringens* (L20800) (42). Alignment was performed with the Clustal V package. Identical residues are indicated in boldface type. Numbers in parentheses indicate the length of each peptide fragment.

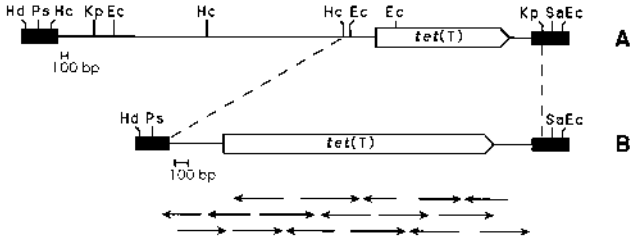

FIG. 2. Restriction maps of pIP1696 (A) and pIP1697 (B). The location and orientation of *tet*(T) are indicated. Arrows represent the direction and extent of the dideoxy-sequencing reactions performed with pIP1697. Thick line, pUC18 polylinker; thin line, cloned DNA. Only relevant restriction sites are shown. Abbreviations: Ec, *Eco*RI; Hc, *Hin*cII; Hd, *Hin*dIII; Kp, *Kpn*I; Ps, *Pst*I; Sa, *Sac*I.

PCR assay carried out with this combination of primers, perhaps because the oligodeoxynucleotide DIII is highly degenerate. By using DI-DII, a DNA fragment of 1.1 kbp was amplified for all the *tet* genes except *tetB*(P). In all positive cases, the DNA fragment of the predicted size was the predominant, if not the unique, product of amplification.

Identification of four new classes of Tet determinants in streptococci resistant to tetracycline-minocycline. The cellular DNAs of the nine streptococcal isolates resistant to tetracycline-minocycline, which had not hybridized under stringent conditions with *tet*(M), *tet*(O), *tetB*(P), *tet*(Q), or *tet*(S) (18), were investigated by the PCR assay described above. Amplified DNA fragments of 1.1 kbp were obtained with the oligodeoxynucleotide pair DI-DII for five of the nine strains (Table 1). In contrast, no DNA was amplified with the four remaining streptococcal strains, with three of them belonging to group C (animal origin) and one belonging to group G (human origin). These negative results were confirmed by using the oligodeoxynucleotide pair DI-DIII. That four strains gave negative PCR results suggested the existence of other *tet* genes, as yet uncharacterized, unrelated to those conferring ribosomal protection. This hypothesis is more likely than that of the occurrence of chromosomal mutations which would render the 30S ribosomal subunit insensitive to tetracyclines. Indeed, to our knowledge, such isolates have not yet been selected either in vivo or in vitro, perhaps because several components of the translational process would have to be simultaneously altered by reciprocal mutations.

Each of the 1.1-kbp DNA fragments, obtained from the five strains which gave positive results by PCR amplification, was purified and used as a probe in DNA-DNA hybridization experiments. No hybridization was found with the *tet*(M), *tet*(O), *tetB*(P), *tet*(Q), or *tet*(S) gene. The PCR products of strains A498, B130, and MG32 hybridized only with the respective chromosomal DNA of each strain. In contrast, the amplified fragments and chromosomal DNA of *Streptococcus bovis* D135 and D295 cross-hybridized. Thus, there were four new classes of Tet determinants in the five streptococcal strains which were positive by PCR (Table 1).

Genetic characterization of the Tet T determinant. The 1.1-kbp DNA fragment amplified from A498 was treated with Klenow polymerase and T4 polynucleotide kinase and was cloned into *Hin*cII-linearized pUC18. The insert of the resulting recombinant plasmid, pIP1695, was used as a probe to screen 4,000 *E. coli* transformants obtained by cloning the whole-cell DNA of A498 partially digested with *Sau*3A (average size, 5 to 10 kbp) into pUC18 cut with *Bam*HI. Of the seven hybridizing clones, only three were able to confer resistance to tetracycline and minocycline in *E. coli*. The recombinant plasmid isolated from one of them, pIP1696, contained a 7.3-kbp insert which was mapped (Fig. 2A). Subcloning of the 2.8-kbp *Hin*cII-*Kpn*I fragment of pIP1696 into pUC18 gave the recombinant plasmid pIP1697 (Fig. 2B). This plasmid also conferred resistance to tetracycline and minocycline in *E. coli.*

The sequences of both strands of the insert of pIP1697 were determined (Fig. 3). The gene, designated *tet*(T), was located between the ATG codon at coordinate 478 and the TGA codon at coordinate 2430. It was predicted to encode a polypeptide of 651 amino acid residues with a calculated molecular mass of 73.5 kDa. The presumptive start codon of the *tet*(T) gene is preceded seven nucleotides upstream by a ribosome-binding site which is thought to bind tightly with the 16S

FIG. 3. Nucleotide and predicted amino acid sequences of the 2.8-kbp DNA insert of pIP1697 carrying *tet*(T). Putative −35 and −10 promoter elements as well as the ribosome-binding site (RBS) sequence are underlined. Convergent arrows indicate imperfect palindromic structures. Amino acids in boldface type are presumed to be involved in the formation of the GTP-binding site (39).

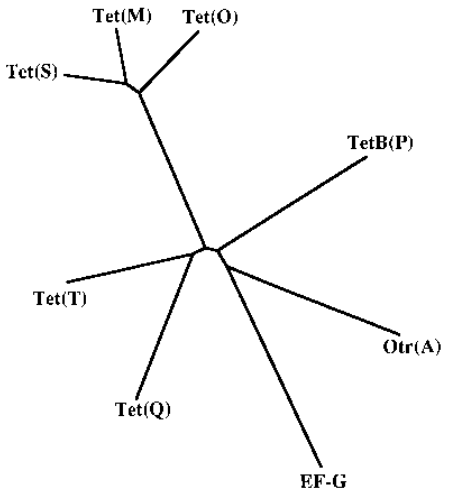

FIG. 4. Unrooted tree showing the evolutionary relationships among the Tet(T)-related proteins. Analysis included eight proteins present in the GenBank-EMBL databases under the indicated accession numbers: Tet(M) from *Enterococcus faecalis* Tn*916* (X56353) (4), Tet(O) from *Streptococcus mutans* (M20925) (19), TetB(P) from *Clostridium perfringens* (L20800) (42), Tet(Q) from *Bacteroides fragilis* (Z21523) (21), Tet(S) from *Listeria monocytogenes* (L09756) (6), Tet(T) from *S. pyogenes* (L42544) (this study), Otr(A) from *Streptomyces rimosus* (X53401) (12), and EF-G from *Thermus thermophilus* (X16278) (51). The length of the branch is proportional to the presumed degree of evolutionary divergence.

rRNA of gram-positive bacteria (26, 27), according to the ΔG value calculated for the sequence GGAGG (−14.4 kCal/mol) (48). The 32.2% G+C content of the coding sequence is slightly lower than that of the *S. pyogenes* chromosome, which ranges from 34.5 to 38.5% (36). Sequences almost identical to the canonical sequences found at the −35 and −10 regions of bacterial promoters were identified at coordinates 145 to 150 and 122 to 127, respectively. Three potential secondary structures that might form in the corresponding mRNA were also detected in the vicinity of the *tet*(T) gene (Fig. 3). These structures might play a role in the regulation of gene expression, as has previously been suggested for analogous structures adjacent to *tet*(M), *tet*(O), and *tet*(S) (6, 24, 44, 50). There is no evidence of any leader peptide in the case of *tet*(T).

Phylogeny of the Tet(T)-related proteins. The amino acid sequence of the Tet(T) protein was compared to those contained in the GenBank-EMBL databases. The best matches were observed with all the Tet proteins conferring tetracycline-minocycline resistance by ribosomal protection except Tet(U). Amino acid identity was scored as 49% with Tet(Q) and about 44% with Tet(M), Tet(O), TetB(P), and Tet(S). The other sequences homologous to Tet(T) included other Tet proteins, such as Otr(A) of *Streptomyces rimosus* (12) and that of *Streptomyces lividans* (11), or translational elongation factors, such as EF-Tu and EF-G, originating from various bacteria. For example, 31% of the residues of Tet(T) and EF-G of *Thermus thermophilus* were identical (51). This percentage of identity lends support to the hypothesis that the Tet proteins encoding ribosomal modification may have emerged as a result of mutations of resident chromosomal genes coding for the translational elongation factors.

The evolutionary distances among the Tet(T)-related proteins were analyzed by the neighbor-joining method (37). The topology of the resulting tree (Fig. 4) indicates that the proteins Tet(M), Tet(O), and Tet(S) are clearly clustered, as stated previously (45), whereas TetB(P), Tet(Q), Tet(T), Otr(A), and EF-G belong to separate branching orders. Our data suggest that the divergence might have occurred a long time ago and that the common ancestor of these proteins, if it still exists, remains to be discovered. Alternatively, the relationships of TetB(P), Tet(Q), and Tet(T) with Tet(M), Tet(O), and Tet(S) might reflect structural homologies rather than evolutionary links. The determination of the nucleotide sequences of other *tet* genes, including those carried by B130, by MG32, and by both D135 and D295, will help us to elucidate this issue.

Concluding remarks. The present study reveals that, in addition to the genes *tet*(M) and *tet*(O), at least four other *tet* genes can confer tetracycline-minocycline resistance in streptococci. The gene *tet*(M) is largely distributed among the tetracycline-minocycline-resistant streptococci (3, 8, 9, 20). An explanation of this predominance could be that in most cases *tet*(M) is carried either by conjugative transposons, such as Tn*916* (4), or by composite structures, such as Tn*3701* (20), which can translocate from chromosome to chromosome. Evidence of similar carriers for *tet*(O), *tet*(T), or the three other *tet* genes identified here has not been found so far.

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