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# Tetracycline Resistance Heterogeneity in *Enterococcus faecium*

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**The tetracycline (Tet) determinants, which encode resistance either to tetracyclines without minocycline ($Tc^r$) or to tetracyclines including minocycline ($Tc^r$-$Mn^r$), of 30 wild-type clinical isolates of *Enterococcus faecium* were identified and localized. The Tet determinants were transferred by conjugation into a plasmid-free *Enterococcus faecalis* recipient at frequencies of $10^{-6}$ to $10^{-9}$ transconjugants per donor, as follows: $Tc^r$, 6 strains; $Tc^r$-$Mn^r$, 14 strains; both $Tc^r$ and $Tc^r$-$Mn^r$, 6 strains; no detectable transfer, 4 strains. Classes L ($Tc^r$ phenotype) and M and O ($Tc^r$-$Mn^r$ phenotype) of the Tet determinants were identified by DNA-DNA hybridization experiments. The Tet L determinant was plasmid-borne in 18 strains and was chromosomal in 2 strains. Tet M was chromosomal in 27 strains and plasmid-borne (pIP1534) in 1 strain; pIP1534 also carried Tet L. Tet M was located on Tn*916*-like elements in 22 strains and on a Tn*916*-modified element in 1 strain. Tet O was detected in only one strain in which it was plasmid-borne. Both Tet L and Tet M determinants were carried by 19 strains. One strain carried, in addition to chromosomal nonconjugative Tet L and Tet M determinants, a conjugative $Tc^r$-$Mn^r$ marker which did not correspond to any Tet determinant tested in this study. These results attest to the genetic complexity of tetracycline resistance in *E. faecium* strains.**

Enterococci are common inhabitants of the human and animal gut. *Enterococcus faecalis* and *Enterococcus faecium* are the most prevalent species isolated from human enterococcal infections, such as urinary tract, genital, and biliary infections and subacute endocarditis. Even though tetracyclines are no longer used to treat enterococcal infections, resistance to these antibiotics occurs in about 70 and 50% of *E. faecalis* and *E. faecium* isolates, respectively (20). The continued use of tetracyclines as antibiotics of choice against certain microbial infections apparently results in an in vivo selection of tetracycline-resistant strains (26). As reported previously for other bacteria (26), most *E. faecalis* and almost all *E. faecium* strains which emerge under tetracycline usage are resistant not only to tetracyclines but also to many other unrelated antibiotics (20).

Tetracycline resistance (Tet) determinants in enterococci and streptococci belong to classes L, M, and O (27). While the Tet M and Tet O determinants encode resistance to all tetracyclines, including minocycline ($Tc^r$-$Mn^r$), Tet L encodes resistance to all tetracyclines except minocycline ($Tc^r$). We recently demonstrated that the previously reported Tet N determinant (4) does not exist (18). Another class of Tet determinants is Tet P, which was first identified on a conjugative plasmid in *Clostridium perfringens* (1).

Tet L is located either on small, nonconjugative plasmids isolated from *Streptococcus agalactiae* (4) or on large conjugative plasmids harbored by *E. faecalis* (30).

Tet M is chromosomally located in group A, B, C, and G streptococci (4, 24), *Streptococcus sanguis* (13), *Streptococcus anginosus* (*S. milleri*) (8), enterococci (7, 9, 14, 15, 17, 29, 30), and *Aerococcus viridans* (5). In most cases, Tet M is carried by a conjugative transposon similar in structure to Tn*916* and is located, as in Tn*916*, on a 4.8-kb *Hin*cII fragment (10). More rarely, Tet M is carried by large conjugative plasmids; these have been found in both *E. faecalis* (4, 6, 11, 30) and *E. faecium* (14). Tet M is also widespread among other bacterial genera (27).

Tet O was initially identified on R plasmids harbored by *Campylobacter coli* (33) and *Campylobacter jejuni* (34); Tet O is occasionally present on the chromosome of *Streptococcus mutans* (22), *Streptococcus pyogenes*, *Streptococcus agalactiae* (24), *S. anginosus* (8), and *A. viridans* (5).

In a previous report (25), we suggested that at least two tetracycline resistance determinants are present in *E. faecium*, one located on plasmids and the other located on the chromosome. The aims of the present study were to determine which classes of Tet determinants are present in *E. faecium* and to localize them. Thirty wild-type *E. faecium* clinical isolates were examined for the conjugative transfer of $Tc^r$ and $Tc^r$-$Mn^r$ markers and for the presence of plasmids. To identify and localize these determinants, DNA-DNA hybridization experiments were performed with probes containing Tet determinants of classes L, M, O, and P.

## MATERIALS AND METHODS

**Bacterial strains.** The 30 wild-type clinical isolates used in this study, listed in Table 1, were isolated in different French hospitals between 1956 and 1987 from patients with urinary tract, biliary, genital, pleural, and peritoneal infections and subacute endocarditis. All the strains were identified in our laboratory as *E. faecium* (20), and all strains except strain 28 were resistant to tetracyclines, including minocycline; strain 28 was resistant to tetracyclines but not to minocycline. Except for strains 7 and 27, which were resistant to only tetracycline-minocycline, the strains were also resistant to one or several of the following antibiotics: chloramphenicol, erythromycin (macrolide-lincosamide-streptogramin B phenotype), kanamycin, penicillin, spectinomycin, and streptomycin. *E. faecalis* JH2-2 (21), which was resistant to fusidic acid and rifampin, was used as the recipient in mating experiments.

**Media and antibiotics.** Media and growth conditions for enterococcal and streptococcal strains have been described previously (19). *Escherichia coli* strains were grown in LB medium (12). Ampicillin (100 μg/ml) and tetracycline (4 μg/ml) were used to maintain *E. coli* plasmids.

**Determination of resistance to antibiotics.** Antibiotic resis-

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tance was tested by the disk diffusion method and, for tetracycline, by MIC determination on brain heart infusion agar supplemented with 5% horse blood. The MIC was determined by plating $10^5$ bacteria per spot on agar containing serially diluted antibiotic.

**Mating conditions.** Matings were done on membrane filters (pore diameter, 0.45 μm; filter diameter, 0.47 mm; type HAEP; Millipore Corp., Bedford, Mass.) by mixing 0.1 ml each of 18-h broth cultures of donor and recipient strains plus 0.1 ml of broth. Selection was done with 4 μg of tetracycline per ml or 3 μg of minocycline per ml. Counterselection was done as described previously (19).

**DNA isolation.** Plasmids were isolated from *E. faecalis* transconjugants and *E. faecium* wild-type strains by ultracentrifugation in dye-buoyant density gradients as described previously (23). Plasmids were isolated from *E. coli* by the procedure of Birnboim and Doly (3). Chromosomal DNA was isolated from enterococcal strains as described previously (2, 23).

**DNA analysis.** The restriction endonucleases used were *Eco*RI, *Hin*cII, *Hin*dIII, *Hha*I, and *Sph*I (Amersham International, Little Chalfont, England). Electrophoresis of digested DNA was done on 0.7% agarose gels. Bacteriophage λ DNA, which was double-digested with *Eco*RI and *Hin*dIII, and a 1-kb DNA ladder (Bethesda Research Laboratories, Inc., Cockeysville, Md.) were used as molecular size markers. Plasmid DNA restriction fragments were isolated from 0.7 or 1% low-melting-point agarose gels by the technique described in the Multiprime DNA-labeling kit (Amersham International).

**DNA-DNA hybridization.** *Hin*cII-digested plasmid and chromosomal DNAs were transferred from agarose gels to nitrocellulose filters by the bidirectional method described by Smith and Summers (32). The probes were labeled with [α-$^{32}$P]dCTP (3000 Ci/mmol; Amersham International) with the Amersham Multiprime DNA-labeling system. Hybridization was carried out under stringent conditions (65°C), as reported previously (23). The radioactivity of the probe (specific activity, about $10^9$ cpm/μg) corresponded to approximately $10^7$ cpm per filter.

**Hybridization probes.** The probes used were (i) the 1.1-kb *Hha*I fragment of pMV158 (Tet L probe) (4); (ii) pAT101, which carries the 0.85-kb *Hin*dIII-*Cla*I fragment of Tn*1545* (Tet M probe) (28); (iii) pAM170, which carries the *Eco*RI-D::Tn*916* fragment of pAD1 (Tn*916* probe) (16); (iv) pAM170LT, which is pAM170 without Tn*916* (16) and which was used as a negative control for pAM170; (v) pUOA4, which carries the 1.8-kb *Hin*cII fragment of pUA466 (Tet O probe) (34); and (vi) the 0.8-kb *Eco*RI-*Sph*I fragment of pJIR39 (Tet P probe) (1).

## RESULTS

**Mating experiments and plasmid isolation.** Each of the 30 wild-type *E. faecium* strains was mated with *E. faecalis* JH2-2. As presented in Table 1, the wild-type strains were divided into four categories according to the resistance to tetracyclines of the corresponding transconjugants. Transconjugants obtained from strains of categories I and II each carried a single Tet determinant, Tc$^r$ or Tc$^r$-Mn$^r$, respectively, whereas both of these types of transconjugants were obtained from each strain of category III. No conjugative transfer of Tc$^r$ or Tc$^r$-Mn$^r$ markers was detected from strains of category IV.

The Tc$^r$ marker of the strains of categories I and III transferred into strain JH2-2 at frequencies which varied from $1.5 \times 10^{-6}$ to $1 \times 10^{-9}$ transconjugants per donor cell. In each case, the other antibiotic resistance markers carried by the wild-type strains, except penicillin resistance, were linked to Tc$^r$. For strains of category I, no conjugative transfer was detected (frequency, $< 5 \times 10^{-10}$ per donor) when selection was done with minocycline. The Tc$^r$-Mn$^r$ marker of the strains of categories II and III transferred into JH2-2 at frequencies ranging from $10^{-7}$ to $10^{-9}$ per donor, whether selection was done with tetracycline or minocycline.

The transconjugants carrying the Tc$^r$ marker harbored plasmids ranging in size from 27.8 to 69.9 kb, but those derived from strains 3, 21, and 23 were plasmid-free. All the transconjugants resistant to tetracycline-minocycline, except that derived from strain 17, were plasmid-free. Plasmids were found in three of the four wild-type strains of category IV.

**Hybridization with Tet L.** Sequences homologous to the Tet L probe were detected in 20 wild-type strains (Table 1) on 1.1-kb *Hin*cII fragments. Tet L was located in 18 strains on plasmids, including pIP1534 (strain 27), which also carried the Tc$^r$-Mn$^r$ determinant. In strains 17 and 21 and in the transconjugants derived from strains 3, 21, and 23, Tet L was found on the chromosome.

**Hybridization with Tet M.** Sequences homologous to the Tet M probe were detected in 28 strains (Table 1). In 23 of these strains, Tet M was associated with genetic elements related to Tn*916* (see next section). Tet M was located on the chromosome in all 28 strains except strain 27, in which it was plasmid-borne (pIP1534). In strains 1, 12, and 17, Tet M was located on two fragments, suggesting either the presence of two copies of Tet M or, perhaps, the presence of a *Hin*cII site in this gene.

Strain 17 carried chromosomal sequences homologous to both Tet M and Tet L determinants. Apparently, neither of these markers transferred by conjugation, although transconjugants resistant to tetracycline-minocycline were obtained. No sequence homology to any of the probes used here, including Tet P, was detected either on the cellular DNA of the derived transconjugant or on pIP1173. To verify this result, six additional transconjugants were tested; none of them displayed homology with any of the probes. Therefore, strain 17 carries, in addition to nonconjugative Tet L and Tet M determinants, another plasmid-borne or chromosomal conjugative Tc$^r$-Mn$^r$ marker that has not yet been identified.

**Hybridization with Tn*916*.** Sequences homologous to the Tn*916* probe were detected on the chromosomes of 22 strains and on pIP1534 (strain 27) (Table 1). All strains except strain 21 were considered to carry a Tn*916*-like element (Fig. 1), since DNA homology with Tn*916* was revealed on three or four *Hin*cII fragments equivalent in size to the internal *Hin*cII fragments of Tn*916* (5.5, 4.8, 1.6, 1.1, and 0.4 kb) (10). In the chromosomes of strains 4 and 30, there was no 5.5-kb *Hin*cII fragment that hybridized to Tn*916* (Fig. 1, lane 7). In most of the strains, sequences homologous to Tn*916* were also detected on one or two additional hybridizing fragments which might represent junctions between the chromosome and a Tn*916*-like element (Fig. 1). No hybridization with pAM170LT was detected on the chromosomal or plasmid DNAs of strains that hybridized with Tn*916*.

Strain 21 (as well as its corresponding transconjugant) was considered to carry a Tn*916*-modified element, since only one (1.6 kb) of the four *Hin*cII fragments (7.0, 2.6, 2.4, 1.6

TABLE 1. Bacterial strains and DNA-DNA hybridization results

| No. (designation) of bacterial strain | Tet class[a] (*Hin*cII fragment [kb]) or Tn*916*-like element | | | |
|---|---|---|---|---|
| | Wild-type strain | | Transconjugant | |
| | Plasmid (designation) | Chromosome | Plasmid (designation) | Chromosome |
| Category I | | | | |
| 1 (D229) | Tet L[b] | Tet M (8.6, 2.2) | Tet L | NT[c] |
| 2 (D360) | Tet L | Tn*916* | Tet L | NT |
| 3 (D453) | Tet L | Tn*916* | No plasmid | Tet L[b] |
| 4 (D456) | Tet L | Tn*916* | Tet L | NT |
| 5 (D457) | Tet L | Tn*916* | Tet L | NT |
| 6 (D459) | Tet L | Tn*916* | Tet L | NT |
| Category II | | | | |
| 7 (D146) | No plasmid | Tet M (2.2) | No plasmid | Tet M (2.2) |
| 8 (D319) | Tet L | Tet M (6.7) | No plasmid | Tet M (6.7) |
| 9 (D331) | +[d] | NT | No plasmid | Tn*916* |
| 10 (D344) | + | NT | No plasmid | Tn*916* |
| 11 (D357) | + | Tn*916* | No plasmid | Tet M (4.6) |
| 12 (D370) | Tet L | Tet M (8.2, 4.5) | No plasmid | Tet M (8.2, 4.5) |
| 13 (D372) | Tet L | NT | No plasmid | Tn*916* |
| 14 (D446) | No plasmid | NT | No plasmid | Tn*916* |
| 15 (D447) | Tet L | Tn*916* | No plasmid | Tet M (5.0) |
| 16 (D448) | + | NT | No plasmid | Tn*916* |
| 17 (D449) | + (pIP1173) | Tet L, Tet M (12.5, 4.0) | + (pIP1173) | —[e] |
| 18 (D452) | + | Tn*916* | No plasmid | Tet M (5.0) |
| 19 (D454) | No plasmid | NT | No plasmid | Tn*916* |
| 20 (D461) | Tet O (1.8) (pIP1543) | Tet O (1.8) | No plasmid | Tet O (1.8) |
| Category III[f] | | | | |
| 21 (D297) | No plasmid | Tet L, Tn*916*[g] | No plasmid | Tet L |
| | | | No plasmid | Tn*916*[g] |
| 22 (D382) | Tet L | NT | Tet L | NT |
| | | | No plasmid | Tn*916* |
| 23 (D445) | Tet L | NT | No plasmid | Tet L |
| | | | No plasmid | Tn*916* |
| 24 (D450) | Tet L | NT | Tet L | NT |
| | | | No plasmid | Tn*916* |
| 25 (D451) | Tet L | NT | Tet L | NT |
| | | | No plasmid | Tn*916* |
| 26 (D455) | Tet L | NT | Tet L | NT |
| | | | No plasmid | Tn*916* |
| Category IV | | | | |
| 27 (D301) | Tet L, Tn*916* (pIP1534) | NT | NA[h] | NA |
| 28 (D320) | Tet L | NT | NA | NA |
| 29 (D458) | Tet L | Tn*916* | NA | NA |
| 30 (D451) | No plasmid | Tn*916* | NA | NA |

[a] The Tet L determinant encodes resistance for tetracyclines except minocycline; the Tet M and Tet O determinants encode resistance to tetracyclines, including minocycline.
[b] In each case, Tet L was located on a 1.1-kb *Hin*cII fragment.
[c] NT, not tested.
[d] +, presence of one or several plasmids, none of which detectably hybridized with the Tet L, Tet M, or Tet O probes.
[e] —, no homology detected on the cellular DNA of this transconjugant or on pIP1173 with any of the probes tested here (including Tet P).
[f] Two types of transconjugants were obtained from each strain of category III, one carrying Tet L and the other carrying Tet M.
[g] Presence of a Tn*916*-modified structure (see text).
[h] NA, not applicable (no conjugative transfer; frequency $<5 \times 10^{-10}$).

kb) that hybridized with Tn*916* corresponded in size to that of an internal fragment of Tn*916* (data not shown).

Chromosomal and plasmid DNAs with homology with Tn*916* were probed with the Tet M determinant. As in Tn*916* (10), Tet M was situated, in each case, on a 4.8-kb *Hin*cII fragment (Fig. 1), but in pIP1534 and strain 21, Tet M was situated on a 4.3-kb (Fig. 1, lane 8) and a 7.0-kb *Hin*cII fragment, respectively.

**Hybridization with Tet O.** In all strains carrying the Tet M determinant, weak hybridization with Tet O was detected on the same fragments on which Tet M was found, probably because of the 76% identity in the nucleotide sequences of these two genes (33). Sequences homologous to the Tet O probe, but not to the Tet M probe, were detected in strain 20 on a 1.8-kb *Hin*cII fragment of pIP1543 and in the derived transconjugant on a chromosomal fragment of the same size (Table 1). The chromosomal and plasmid DNAs were also digested with *Hin*dIII; in both cases, homology to Tet O was revealed on a 1.6-kb *Hin*dIII fragment (data not shown). We considered that only the strains in which the DNA hybrid-

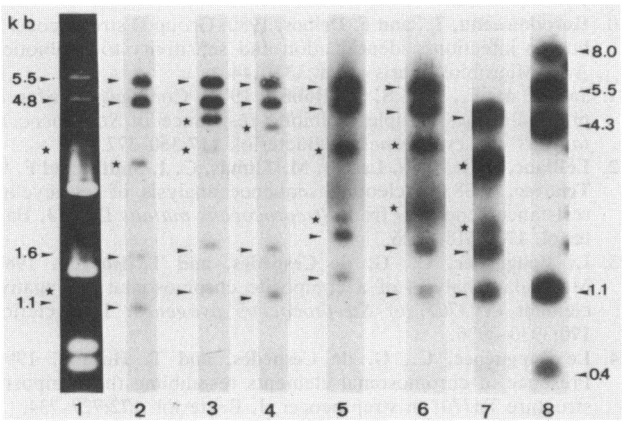


FIG. 1. Hybridization of *Hin*cII-digested chromosomal and plasmid DNA with the Tn*916* probe (autoradiogram). Nitrocellulose filters were probed with $^{32}$P-labeled pAM170. Lane 1, agarose gel electrophoretic pattern of pAM170; lanes 2 to 6, transconjugants derived from strains 13, 25, 23, 22, 26, respectively; lane 7, strain 30; lane 8, pIP1534 (strain 27). Molecular sizes (in kilobases) of internal Tn*916* fragments are shown on the left. Molecular sizes (in kilobases) of hybridizing fragments of pIP1534 are shown on the right. Arrows indicate the fragments equivalent in size to those of the internal fragments of Tn*916* (DNAs in lanes 3 to 8 were from gels different from those used for lanes 1 and 2). Stars indicate junction fragments. Tet M was localized on a 4.8-kb fragment in all cases except pIP1534, in which it was on a 4.3-kb fragment.

ized to the Tet O probe, but not to the Tet M probe, actually carried the Tet O determinant. By these criteria, Tet O was also identified in an *A. viridans* strain (5) and in other streptococci (8, 24).

**Correlation between tetracycline MICs and the presence of tetracycline resistance classes in wild-type *E. faecium* strains.** The MICs of tetracycline for the strains studied here ranged from 8 to 256 μg/ml, and the MIC was 0.25 μg/ml for the tetracycline-susceptible *E. faecalis* JH2-2 control strain. In 10 of the 11 strains in which only one Tet class was identified (strains 7, 9 to 11, 14, 16, 18, 20, 28, and 30), the MICs of tetracycline ranged from 8 to 64 μg/ml; exceptionally, the MIC for strain 19 was 256 μg/ml. In 15 of the 19 strains in which two Tet classes were detected, the MICs of tetracycline ranged from 128 to 256 μg/ml. Thus, the MIC of tetracycline was generally higher for strains that carried two classes of Tet determinants than it was for strains with only one Tet determinant. We previously obtained similar results for *E. faecalis* strains (30).

## DISCUSSION

The three classes of Tet resistance determinants described so far in various streptococci, *A. viridans*, and *E. faecalis* (4, 5, 8, 22, 24, 27, 30) were also found in the *E. faecium* strains studied here: Tet L, Tet M, and Tet O. The results of this study also suggest the existence of a new class of Tet determinant.

In the 30 strains studied here, classes L and M were predominant, while class O was found in only 1 strain; both Tet L and Tet M were carried by 19 of the strains. In most cases Tet L was plasmid-borne and Tet M was chromosomal.

The Tet L determinant was located on a plasmid in wild-type strains 3 and 23 but on the chromosome in the transconjugants derived from these strains; this suggests that Tet L may be associated with an element which transposed from the plasmids of these wild-type strains onto the transconjugant chromosome. Alternatively, the plasmid carrying the Tet L determinant may have transferred by conjugation to the *E. faecalis* recipient, but was perhaps restricted or prevented from replicating in this host, and the Tet L determinant, or a genetic element carrying it, may have transposed to the host chromosome. A similar situation was observed for the Tet O determinant carried by strain 20.

The Tet M determinant was associated with sequences homologous to the conjugative transposon Tn*916* (10) in 23 strains. In 22 of these strains, we detected genetic elements which had a structure resembling that of Tn*916* (Tn*916*-like elements), while we found a Tn*916*-modified element on the chromosome of strain 21.

The Tn*916*-like elements of 11 strains did not transfer by conjugation (Table 1). The Tn*916*-like nonconjugative elements carried by strains 4 and 30 lacked a *Hin*cII fragment of 5.5 kb that hybridized with Tn*916*. The transfer functions of Tn*916* have been localized, by Tn*5* insertion mutagenesis, to a region of the transposon that includes the entire 5.5-kb *Hin*cII fragment (31). Therefore, strains 4 and 30 may carry genetic elements derived from Tn*916* by deletions that render them nonconjugative. However, in the other nine strains carrying Tn*916*-like elements that failed to transfer by conjugation, four *Hin*cII fragments, including a 5.5-kb fragment, hybridized to Tn*916*. Therefore, we could not correlate the capacity to transfer by conjugation with detectable alterations in the structure of the elements. Possibly, a point mutation is responsible for the loss of transfer functions in these cases. Alternatively, DNA sequences in the donor chromosome may contribute to the capacity of Tn*916*-like elements to transfer by conjugation; perhaps some of the *E. faecium* strains do not possess such sequences.

Strains 11, 15, and 18 appeared to carry, in addition to a nonconjugative Tn*916*-like element, a second genetic element encoding the Tet M determinant which transferred by conjugation. The same type of conjugative elements may also have been present in strains 7, 8, and 12, in which homology with Tn*916* was limited to Tet M, suggesting the presence of a DNA segment including, but larger than, Tet M.

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