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Transferable Tet M in *Fusobacterium nucleatum*

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Two tetracycline-resistant *Fusobacterium nucleatum* strains were able to transfer, by conjugation, tetracycline resistance and the Tet M determinant to recipient *F. nucleatum*, *Peptostreptococcus anaerobius*, and *Enterococcus faecalis* strains at a frequency of 10^{-1} to 10^{-8}.

We recently showed that the tetracycline-resistant (Tet^r) anaerobic gram-negative rod *Fusobacterium nucleatum* owes its resistance to the presence of the Tet M determinant located on the chromosome (14, 20). These strains also hybridized with pJI2.14, a plasmid which carries the right side of a Tet M transposon from *Streptococcus agalactiae* B109 but does not carry the structural Tet M gene or confer tetracycline resistance (2, 13). On the basis of this information, we hypothesized that the *F. nucleatum* strains would carry functional conjugative transposons and be able to transfer tetracycline resistance to other members of the species (13).

To test this hypothesis, we selected for further study two Tet^r *F. nucleatum* strains, one isolated from the urogenital tract (2014.15) and the other isolated from a periodontal pocket (6601). Strain 2014.15 was Tet^r and susceptible to streptomycin (Str^s), rifampin (Rif^s), erythromycin (Ery^s), and fusidic acid (Fus^s), while strain 6601 was Tet^r, Str^r, Fus^r, Ery^s, and Rif^s. Two clinical recipients, 152.16 and 2016.9, isolated from the urogenital tract were Tet^s, Str^r, Fus^r, and Ery^r and were selected for Rif^r by plating of a turbid suspension of each strain on Columbia agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% sheep blood and 10 μg of rifampin per ml under anaerobic conditions at 37°C. Colonies which grew retained the other chromosomal markers and were now able to grow on blood agar supplemented with 60 μg of rifampin per ml. Donors and recipients were grown anaerobically on four Columbia blood agar plates at 37°C for 2 to 3 days and then removed, and turbid suspensions were made in 1 ml of GCK broth (12, 18). Equal volumes of the donor and the recipient were mixed, and 0.2 to 0.3 ml was plated onto four blood agar plates and incubated anaerobically at 37°C (12). After incubation, the cells were removed and placed in 1 ml of GCK broth. Serial 10-fold dilutions were prepared, and 0.1 ml was plated on blood agar supplemented with 10 μg of tetracycline and 10 μg of rifampin per ml for selection of transconjugants or plated on blood agar supplemented with 10 μg of rifampin or 10 μg of tetracycline per ml to determine the number of recipients and donors (12, 18). Putative transconjugants were screened for erythromycin, streptomycin, and fusidic acid resistance markers, and their Gram stain reactions were determined. The frequency of transconjugants per recipient was then determined (12, 18).

Since no data exist for mating experiments with *F. nucleatum*, we examined what effect changing the incubation time of the mating mixture had on the transfer of the Tet M determinant. Duplicate matings were prepared; one set was incubated overnight (18 h), and the other set was incubated for 60 h. Differences in transfer frequency were seen between the 18- and 60-h incubation periods for matings with the donors *F. nucleatum* 6601 and *Enterococcus faecalis* DS160 (Table 1). In contrast, little difference between the two incubation times was observed with the donor 2014.15 (Table 1). We also found that the recipient 2016.9 had consistently higher transfer frequencies than did the recipient 152.16 with the three donors used.

Transconjugants from the various mating pairs were selected, and DNA was extracted and cut with *Hin*dIII and *Eco*RI (13). Southern blots were prepared and probed with the 1.8-kilobase (kb) Tet M internal probe from plasmid pUW-JKB1 (1) to verify the presence of the Tet M determinant. All transconjugants tested hybridized with the probe (data not shown). *Hin*dIII cuts the Tet M transposon once and has been found in all Tet M-containing strains tested (1, 2, 12, 13). Therefore, when *Hin*dIII-restricted DNAs are hybridized with the 5-kb *Hin*cII fragment from pJI3, which includes the whole structural gene and about 2 kb of the transposon not involved in tetracycline resistance (2), the number of inserted Tet M determinants can be determined on the basis of the number of hybridizing bands (3, 4, 17). In Fig. 1, lanes D and F, the three hybridizing bands suggested that more than one copy of Tet M was inserted into the transconjugants produced from the *E. faecalis-F. nucleatum* mating; the two bands in lanes A and E and the one band in lanes B and C suggested that just one copy of Tet M was present (3, 4, 17). Three or more bands appear to be a feature of Tn*916*, since multiple inserts were previously demonstrated with other recipient species when Tn*916* was introduced by either conjugation or transformation (4, 7, 8, 17).

We have shown that oral and urogenital *Peptostreptococcus* spp. carry the Tet M, Tet K, Tet L, and Tet O determinants (14, 20). Since *Peptostreptococcus* spp. are found in association with *F. nucleatum* in both the oral and urogenital tracts, we were interested in determining if Tet^r *F. nucleatum* could transfer the Tet M determinant to a *Peptostreptococcus* recipient. A Tet^s, Str^r *Peptostreptococcus anaerobius* strain (192.19) which was found negative by use of a DNA probe for Tet K, Tet L, Tet M, Tet O, and Tet P (14) was chosen as a recipient, and strain 2014.15 was chosen as the donor. Mating mixtures were incubated anaerobically, and transconjugants were selected on blood agar plates supplemented with 10 μg of tetracycline and 250 μg of streptomycin per ml. Tet^r *P. anaerobius* was obtained (Table 1), and selected transconjugants were Gram stained to ensure that they were gram-positive cocci. DNA was extracted and restricted, and Southern blots were prepared and probed with the 1.8-kb probe to verify the presence of the Tet M determinant (data not shown).

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TABLE 1. Transfer of Tet M among *F. nucleatum* strains

Donor	Recipient	Frequency[a] of Tet M transfer at:	
		18 h	60 h
F. nucleatum	*F. nucleatum*		
6601	2016.9	1.8×10^{-3}	4.9×10^{-1}
6601	152.16	2.0×10^{-8}	2.0×10^{-4}
2014.15	2016.9	1.5×10^{-1}	1.0×10^{-2}
2014.15	152.16	5.5×10^{-7}	1.6×10^{-7}
E. faecalis	*F. nucleatum*		
DS160	2016.9	4.1×10^{-5}	1.2×10^{-1}
DS160	152.16	1.5×10^{-7}	3.8×10^{-4}
F. nucleatum 2014.15	*E. faecalis* JH2-2	1.0×10^{-7}	1.0×10^{-7}
F. nucleatum 2014.15	*P. anaerobius* 192.19	2.1×10^{-6}	8.0×10^{-5}

[a] Frequencies represent the average of two or more experiments and were calculated as the number of transconjugants divided by the number of recipients after mating mixtures were incubated for 18 or 60 h.

The *F. nucleatum* donors used in this study could transfer the Tet M determinant to a variety of recipients representing both gram-positive and gram-negative species. We have since tested two other Tet^r *F. nucleatum* strains and found that both were able to transfer the Tet M determinant with frequencies similar to those reported in Table 1. This result suggests that many, if not all, of the Tet^r *F. nucleatum* strains found in nature are able to transfer tetracycline resistance. We previously showed that Tet^r strains represented 30% of a randomly selected group of *F. nucleatum* strains (14); therefore, it is possible that Tet^r *F. nucleatum* may act as a reservoir for the Tet M determinant in the urogenital tract. Tet^r *F. nucleatum* strains are much less frequent in the oral cavity and thus are less likely to contribute to the spread of the Tet M determinant in this ecosystem (20).

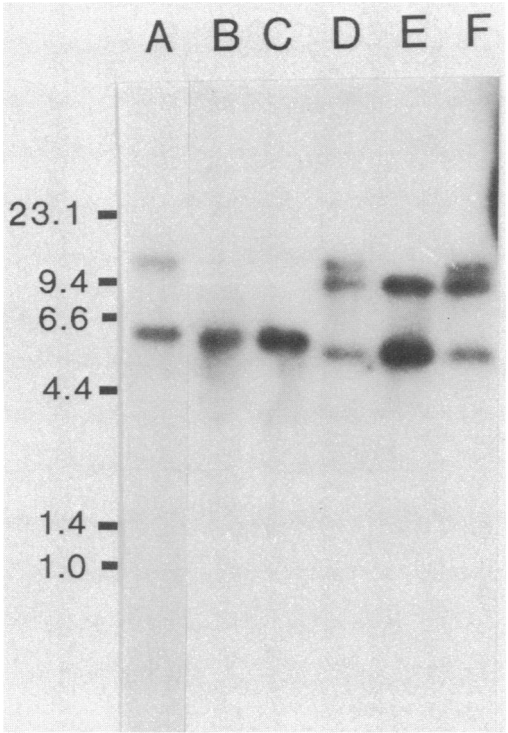

FIG. 1. *F. nucleatum* transconjugant DNAs cut with *Eco*RI and *Hin*dIII and hybridized with the radiolabeled 5-kb *Hin*cII fragment from pJI3. Lanes: A and B, transconjugants from an *F. nucleatum* 6601-*F. nucleatum* 2016.9 mating; C, transconjugants from an *F. nucleatum* 2014.15-*F. nucleatum* 2016.9 mating; D and F, transconjugants from an *E. faecalis* DS160-*F. nucleatum* 2016.9 mating. Numbers at left are in kilobases.

Transferable tetracycline resistance in *Clostridium difficile* (23, 24), due to the presence of the Tet M determinant (5), and transferable tetracycline resistance in *Bacteroides* spp. (6, 11, 21, 22), due to genetically distinct Tet determinants (21), have both been described. The work presented here describes the third anaerobic genus to show conjugation of a chromosomal tetracycline resistance determinant and is the first work to demonstrate the presence of a functional conjugative chromosomal Tet M determinant in a gram-negative rod. These data also support the hypothesis that once the Tet M determinant enters one strain or a few strains of *F. nucleatum*, it can then easily be transferred to other strains within the species. Thus, conjugation among different strains of *F. nucleatum* may account for the dissemination of the Tet M determinant within this species.

We recently showed that *Peptostreptococcus* spp. and *Veillonella parvula* also have DNA sequences which are homologous throughout the length of Tn*916* (13), suggesting that they also carry functional conjugative Tet M transposons. Preliminary experiments indicated that both *Peptostreptococcus* and *V. parvula* donor strains can transfer the Tet M determinant to recipient strains (unpublished observations).

In the last few years, the number of identified genera which owe their resistance to the presence of the Tet M determinant has increased dramatically (5, 9, 10, 12, 14–16, 19, 20). Therefore, it seems reasonable to assume that the number of species and genera shown to carry the Tet M determinant will continue to increase over time. These species and genera will need to be examined to determine if they carry the DNA sequence for a complete transposon and whether these transposons are functional.

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