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Potential Role of *Veillonella* spp. as a Reservoir of Transferable Tetracycline Resistance in the Oral Cavity

D. Ready,[1]* J. Pratten,[2] A. P. Roberts,[2] R. Bedi,[3] P. Mullany,[2] and M. Wilson[2]

Eastman Dental Hospital, UCLH NHS Foundation Trust, 256 Gray's Inn Road, London WC1X 8LD,[1] Division of Microbial Diseases, UCL Eastman Dental Institute, 256 Gray's Inn Road, London WC1X 8LD,[2] and King's College London, Strand, London WC2R 2LS,[3] United Kingdom

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Twelve out of 96 *Veillonella* spp. isolated from oral samples harbored tetracycline resistance genes. The most common resistance gene was *tet*(M). A *tet*(M)-positive *Veillonella dispar* strain was shown to transfer a Tn*916*-like element to four *Streptococcus* spp. by conjugation at a frequency of 5.2×10^{-6} to 4.5×10^{-5} per recipient.

The tetracyclines are broad-spectrum, bacteriostatic antibiotics used for the treatment of skin, respiratory, and oral infections (18); however, bacterial resistance is limiting their usefulness. The oral cavity harbors a diverse community of microorganisms and has been shown to be a reservoir for antibiotic-resistant bacteria (5, 9, 14). The most widespread tetracycline resistance (Tc^r) gene identified from oral bacteria is *tet*(M) (9, 17, 19). It has been identified in 42 genera (16) and is usually found on conjugative transposons of the Tn*916*/Tn*1545* family (4). Transfer of Tn*916*-like elements between oral streptococci has been observed (7, 8, 15). However, the role of oral bacteria other than streptococci in the transfer of Tc^r genes has not been thoroughly investigated. Previous work has shown that the most prevalent group of tetracycline-resistant oral bacteria, other than the oral streptococci, are *Veillonella* spp. (9). *Veillonella* spp. are anaerobic, gram-negative cocci and are residents of the oral cavity, gastrointestinal tract, and vagina (2). The aim of this study was to determine if oral *Veillonella* spp. harbored transferable Tc^r.

Ninety-six *Veillonella* isolates were tested for Tc^r. The isolates were recovered from the dental plaque of 52 healthy subjects who had not received antibiotics in the previous three months (ethical approval 98/56 [Local Health Authority Research and Ethics Committee]). Of these isolates, 12.5% were shown to be tetracycline resistant by agar dilution (12) (MIC $\geq$ 16 μg/ml), and five different Tc^r genes were detected (Table 1) by PCR and sequencing using previously published primers (13, 19). The most common Tc^r gene was *tet*(M), followed by *tet*(S) (Table 1). *Veillonella dispar* (34.2A) containing *tet*(M) was used as a donor of Tc^r. The genetic support for *tet*(M) was demonstrated by PCR. Reactions were carried out to amplify the region from the start of *tet*(M) to the *int* gene using previously published primer pairs RT1 and RT2, RT3 and RT4 (15), orf6 (5′ TGGATATTTGTGTCCTGTATGTG) and xis (5′ CGTAGCTTGTTTTCGCCAAT), and intxis1 and intxis2 (15). The results of the PCR and sequencing demonstrated that the *tet*(M) gene was likely to be contained within a Tn*916*-like conjugative transposon.

Determination of conjugative transfer of the Tn*916*-like element from *V. dispar* to four oral streptococci (Table 2) was achieved by filter-mating and transformation experiments in the presence and absence of DNase I. The donor *V. dispar* 34.2A (MIC, 32 μg/ml) was grown anaerobically overnight on tetracycline containing anaerobic agar (8 μg/ml; Oxoid Ltd., Basingstoke, United Kingdom) supplemented with 5% defibrinated horse blood (E&O Laboratories, Bonnybridge, United Kingdom). The four tetracycline-susceptible recipients (MIC $\leq$ 0.25 μg/ml; Table 2) were grown overnight in air with 5% CO_2 on antibiotic-free Columbia blood agar (Oxoid) supplemented with 5% defibrinated horse blood. The cells from these plates were suspended in 20 ml of antibiotic-free brain heart infusion (BHI) broth (Oxoid) and grown overnight at 37°C anaerobically. Ten milliliters of antibiotic-free BHI broth was inoculated with 100 μl of overnight culture and incubated at 37°C until mid-exponential phase (optical density at 600 nm, 0.4 to 0.6). The cultures were centrifuged (1,910 × *g*) for 10 min, and the supernatant was discarded. The cells were resuspended in 1 ml of BHI broth with or without 50 mg/liter DNase I (3), and 100 μl aliquots from the donor and one of the recipients were spread onto 0.45 μm nitrocellulose filters (Fisher Scientific, London, United Kingdom) on antibiotic-free anaerobic agar and incubated anaerobically overnight at 37°C. To control for transformation (as all the recipients are naturally competent), the pelleted recipient cells were mixed with 371 ng of pAM120 DNA and spread onto filters as described above. The plasmid pAM120 is pGL101 containing Tn*916* and flanking DNA on an EcoRI fragment. This plasmid has previously been shown to transform gram-positive cells to have Tc^r (6). As with the filter-mating experiments, these transformation experiments were carried out both in the presence and in the absence of DNase I. The filters were removed from the agar plates and placed in sterile universals with 1 ml of prewarmed BHI broth and vortexed (20 s). Aliquots of 100 μl were then spread onto Columbia blood agar plates containing tetracycline (8 μg/ml). These plates were incubated at 37°C for 48 h in air supplemented with 5% CO_2, providing selection against the obligate anaerobic donor. Colonies were counted

* Corresponding author. Mailing address: Microbiology, Eastman Dental Hospital, UCLH NHS Foundation Trust, 256 Gray's Inn Road, London WC1X 8LD, United Kingdom. Phone: 44 20 7915 1050. Fax: 44 20 7915 1127. E-mail: d.ready@eastman.ucl.ac.uk.

TABLE 1. Distribution of tetracycline resistance genes among *Veillonella* spp.

Identity of bacterium	No. tested	No. (%) resistant to tetracycline	Tetracycline MIC_{50} (mg/liter) (range)	No. of isolates from which the indicated Tc^r gene was isolated					No. of isolates with more than one Tc^r gene
				tet(M)	*tet*(S)	*tet*(O)	*tet*(A)	*tet*(L)	
Veillonella parvula	52	6 (11.5)	32 (16–64)	3	3	0	1	1	2[a]
Veillonella dispar	37	5 (13.5)	16 (16–32)	4	2	1	0	0	2[b]
Veillonella atypica	3	0 (0)		0	0	0	0	0	0
Unspeciated *Veillonella*	4	1 (25.0)	16 (16–32)	1	0	0	0	0	0
Total	96	12 (12.5)	16 (16–64)	8	5	1	1	1	4

[a] One with *tet*(M) plus *tet*(L); one with *tet*(S) plus *tet*(A).
[b] One with *tet*(M) plus *tet*(S); one with *tet*(M) plus *tet*(O).

and subcultured onto tetracycline-containing agar to ensure resistance and purity. Putative transconjugants were identified by partial 16S rRNA gene sequencing (10, 20). Sequences were analyzed using relevant databases (1, 11). The presence of the Tn*916*-like element within the streptococcal transconjugants was confirmed by PCR amplification and sequencing of the *tet*(M) gene and *int* and *xis* (15).

Results of the filter-mating experiments are shown in Table 2. None of the recipients became spontaneously resistant to tetracycline, and tetracycline-resistant transconjugants (MIC, 32 μg/ml) arose from each of the mating experiments. PCR and sequencing demonstrated that the donor and transconjugants contained identical *tet*(M), *int*, and *xis* genes. DNase I was added to the matings to ensure that transfer occurred by conjugation. Mating experiments in which DNase I was not added were also carried out. The transformation experiments with pAM120 in the absence of DNase I showed that the four recipients were all naturally transformable while incubating on the filters. The same experiment in the presence of DNase I showed a dramatic reduction in the number of transformants (Table 2), confirming the observation that most of the extracellular DNA present during these experiments was being degraded. Therefore, the majority of the transfer observed during these filter-mating experiments was likely to be due to conjugation.

Other studies have shown transfer of Tc^r between oral streptococci (7, 8, 15). However, the potential of *Veillonella* spp. to harbor and transfer Tc^r has not previously been investigated. In this study, 12.5% of *Veillonella* spp. were shown to be tetracycline resistant, and *tet*(M) was found to be the most common resistance gene. Furthermore, the transfer of *tet*(M) from *V. dispar* to four streptococcal species was demonstrated.

Tetracycline-resistant *Veillonella* spp. have the opportunity to come into close contact with, and consequently transfer resistance elements to, other oral bacteria and bacteria which pass through the oral cavity. Additionally, oral bacteria have the opportunity to transfer from person to person, and this transfer could further allow the spread of a resistant bacterium to a new host and subsequent dissemination of the mobile Tc^r genes to susceptible bacteria.

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TABLE 2. Frequency of transfer detected in filter-mating experiments

Filter-mating expt parameters	No. and type of Tc^r isolates	Transfer frequency
Streptococcus mitis NCTC 12261	No spontaneous mutants	
S. mitis + *V. dispar*	1.1×10^3 colonies	1.6×10^{-6}/recipient
S. mitis + *V. dispar* + DNase	2.9×10^3 transconjugants	4.1×10^{-6}/recipient
S. mitis + pAM120	29 transformants	7.8×10^{-2}/ng DNA
S. mitis + pAM120 + DNase	No transformants	
S. oralis NCTC 11427	No spontaneous mutants	
Streptococcus oralis + *V. dispar*	3.3×10^3 colonies	5.6×10^{-5}/recipient
S. oralis + *V. dispar* + DNase	6.7×10^4 transconjugants	5.7×10^{-5}/recipient
S. oralis + pAM120	3,120 transformants	8.4/ng DNA
S. oralis + pAM120 + DNase	6 transformants	1.6×10^{-2}/ng DNA
Streptococcus parasanguinis NCTC 55898	No spontaneous mutants	
S. parasanguinis + *V. dispar*	1.8×10^3 colonies	4.6×10^{-6}/recipient
S. parasanguinis + *V. dispar* + DNase	8.0×10^3 transconjugants	5.2×10^{-6}/recipient
S. parasanguinis + pAM120	512 transformants	1.4/ng DNA
S. parasanguinis + pAM120 + DNase	5 transformants	1.3×10^{-2}/ng DNA
S. salivarius NCTC 8618	No spontaneous mutants	
S. salivarius + *V. dispar*	2.6×10^3 colonies	8.7×10^{-5}/recipient
S. salivarius + *V. dispar* + DNase	4.6×10^3 transconjugants	4.5×10^{-5}/recipient
S. salivarius + pAM120	22 transformants	5.9×10^{-2}/ng DNA
S. salivarius + pAM120 + DNase	No transformants	

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