Oral Microbiology Immunology 2008: 23: 344–347
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY
AND IMMUNOLOGY

Short communication

The predominant cultivable *Veillonella* spp. of the tongue of healthy adults identified using *rpoB* sequencing

D. Beighton[1,2], D. Clark[1], B. Hanakuka[1], S. Gilbert[1], T. Do[1]

[1]Infection Research Group, King's College London, Dental Institute, London, UK, [2]Biomedical Research Centre, Guy's and St Thomas Hospital Foundation Trust, London, UK

Beighton D, Clark D, Hanakuka B, Gilbert S, Do T. The predominant cultivable Veillonella *spp. of the tongue of healthy adults identified using* rpoB *sequencing. Oral Microbiol Immunol 2008: 23: 344–347.* © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard.

The predominant *Veillonella* spp. were isolated from the dorsum surface of the tongues of 11 healthy adults and identified to species level using *rpoB* sequencing because 16S ribosomal RNA sequence analysis does not reliably differentiate between all members of this genus. In all, 253 isolates were identified and the mean proportion (± SE) of *Veillonella* spp. per sample was 16.2 (± 3.6) with a range of 3.0% to 36.3% of the total anaerobic colony count. The predominant species were *Veillonella atypica* (10/11), *Veillonella dispar* (9/11) and *Veillonella rogosae* (8/11) because they were isolated from the majority of subjects. *Veillonella parvula* was isolated from only one subject while *Veillonella dentocariosi* and *Veillonella montpelleriensis* were not isolated from any subject.

Key words: normal flora; tongue; *Veillonella*

David Beighton, Department of Microbiology, King's College London, Dental Institute, Floor 17, Guys Tower, London Bridge, SE1 9RT, UK
Tel.: + 44 (0)207 188 7465;
fax: + 44 (0)207 188 7466;
e-mail: david.beighton@kcl.ac.uk
Accepted for publication October 16, 2007

The genus *Veillonella* has for many years been restricted to the species *Veillonella parvula*, *Veillonella atypica*, *Veillonella dispar*, *Veillonella criceti*, *Veillonella ratti*, *Veillonella rodentium*, and *Veillonella caviae* (14, 20, 22). However, in recent years three new species, *Veillonella montpellierensis*, *Veillonella denticariosi*, and *Veillonella rogosae* (2, 3, 13), have been validly published. Veillonellae are small, non-fermentative, anaerobic, gram-negative cocci isolated from the oral cavity and intestinal tract of humans and animals that gain energy from the utilization of short-chain organic acids (5, 24). The human species are *V. parvula*, *V. atypica*, *V. dispar*, *V. montpelliierensis*, *V. denticariosa*, and *V. rogosae*, the other species being isolated primarily from rodents. The human species may be associated with mono-microbial infections (17) and a strain identified as a member of the *V. ratti–V. criceti* group has been found in a semen sample from a patient attending for infertility at a urology unit (18).

Veillonella spp. are routinely isolated from the oral cavity and are found on all the mucosal and tooth surfaces but the identification of members of this genus is not reliable using either phenotypic characteristics or by comparison of 16S ribosomal RNA (rRNA) nucleotide sequences (19). To overcome these problems sequence analyses of *rpoB*, *dnaK*, or *gyrB* have been used to define *Veillonella* species (3, 13). This approach has enabled clear discrimination between species that are, on the basis of DNA–DNA homology determination, well defined but that are poorly defined on the basis of 16S rRNA sequencing. This difficulty applies, in particular, to the identification of *V. dispar*, *V. parvula*, *V. denticariosi*, and *V. rogosae.* The predominant *Veillonella* spp. on healthy individuals have been identified using 16S rRNA sequencing as '*Veillonella* spp.', *V. parvula*, *V. dispar*, '*V. parvula/V. dispar*', or *V. atypica* (1, 4, 10, 15) while, using sodium dodecyl sulfate–polyacrylamide gel electrophoresis protein profile analysis, *V. dispar* and *V. atypica* were reported as the predominant *Veillonella* spp. but *V. parvula* was not isolated (12). The *Veillonella* spp. from the dorsum of the tongue of patients with halitosis have been identified, on the basis of 16S rRNA sequencing, as '*Veillonella* spp.', *V. parvula*, *V. dispar*, or *V. atypica* (7, 10, 25, 26) and those exhibiting nitrate reduction activity have been identified as *V. atypica* or *V. dispar* on the basis of 16S rRNA sequencing (6,8) while checkerboard

DNA–DNA hybridization studies identified *V. parvula* as one of the predominant tongue organisms (8, 16). The identification of *V. parvula* and *V. dispar* on the basis of 16S rRNA sequencing is not reliable (19) and since the above studies were reported three new species have been identified (2, 3, 13) so we have examined the composition of the *Veillonella* populations on the dorsum of the tongue of healthy adult subjects and identified these using DNA-dependent RNA polymerase subunit B (*rpoB*) sequence analysis.

Materials and methods

Sample collection and processing

Sterile cotton wool swabs were used to take a sample of the microbial biofilm adherent to the dorsum of the tongue of 11 healthy adult subjects. The swabs were placed in 1 ml Fastidious Anaerobe Broth (FAB, LabM, Bury, UK), vortexed to disperse the biofilm and decimally diluted in FAB. Aliquots (100 µl) were plated onto Fastidious Anaerobe Agar (LabM) supplemented with 5% (volume/volume) defibrinated horse blood and *Veillonella* agar (21). The *Veillonella* agar contained 5 g BactoTryptone (Difco Media, BD, Oxford, UK), 5 g Bacto Yeast Extract (Difco), 0.75 g sodium thioglycollate (Sigma, Poole, Dorset, UK), 0.002 g Bacto Basic Fuchsin (Difco), 21 ml 60% sodium lactate (Sigma) and 15 g Bacto Agar (Difco), which were added to 1 l of deionized water. The pH was adjusted to 7.5 before autoclaving and the mixture was supplemented with 7.5 µg/ml vancomycin (Sigma) after autoclaving. All media were incubated in an atmosphere consisting initially of 90% (volume/volume) nitrogen and 5% (volume/volume) each of hydrogen and carbon dioxide at 37°C for 4 days for the *Veillonella* Agar and 7 days for the FAA.

The total number of bacteria in the samples was determined by counting the total number of colonies on the FAA while the number of *Veillonella* was determined by counting the total number of typical colonies on the selective isolation medium. The numbers of each colony type on the *Veillonella* agar were counted and each colony type was examined by Gram staining. Typical *Veillonella* colonies were 2–4 mm in diameter, regular and slightly domed in shape with an entire edge and were composed of small, gram-negative coccal cells, mainly as single cells but with some short chains visible. The detection limit was dependent upon the numbers of bacteria in the sample but it was <0.1% of the total colony count.

Identification of *Veillonella* spp.

To confirm the identity of the presumptive *Veillonella* isolates representative colonies composed of gram-negative cocci were subcultured on FAA and incubated anaerobically for 2 days. DNA was extracted from individual colonies from each isolate by the 'tooth pick' method and a portion of the *rpoB* gene was amplified using the following primers: Veill-rpoBF – GTAACAAAGGTGTCGTTTCTCG and Veill-rpoBR – GCACCRTCAAATACAGGTGTAGC (2, 3). The sequences of these isolates and those of type strains [*V. rogosae* DSM18960 (EF211831), *V. parvula* ATCC10790 (EF185158), *V. atypica* ATCC17744 (EF185159), *V. dispar* ATCC17748 (EF185161), *V. denticariosi* CIP109448 (EF185162), *V. caviae* DSM20738 (EF185163), *V. criceti* ATCC17747 (EF185164), *V. ratti* ATCC17746 (EF185165), *V. rodentium* ATCC17743 (EF185166) and *V. montpellierensis* CIP107992 (EF411194)] and of the reference strain *V. parvula* ATCC17745 (EF185160) were aligned using BioEdit (9) and the neighbor-joining method of Saitou and Nei (23) was used to construct a phylogenetic tree. The isolates in this study were assigned to a species if they were associated in clusters containing one of the valid *Veillonella* species and also had ≥ 98% sequence identity with the species with which they were clustered. From these data the total number of *Veillonella* spp. and the total number of each individual species in each sample were determined and expressed as percentages of the total anaerobic count.

Results and discussion

The tongue swabs yielded high numbers of bacteria [mean ± SE colony-forming units (CFU) per swab as $\log_{10}$(CFU per swab) was 7.29 ± 0.15, median 7.17]. *Veillonella* spp. were isolated from the dorsum of the tongue of each subject (Table 1); the mean proportion (± SE) of *Veillonella* spp. per sample was 16.2 (± 3.6) with a range of 3.0% to 36.3%. In all, 253 isolates were identified using *rpoB* sequencing and all isolates were identified as either *V. dispar*, *V. rogosae*, *V. atypical*, or *V. parvula*. While 16S rRNA sequencing has revealed the phylogenetic relationships between organisms in the genus *Veillonella* in considerable detail, it has proven to be an unreliable tool for identifying all species (2, 3, 13, 19). This is unusual but within the streptococci the species *Streptococcus pneumoniae*, *Streptococcus mitis* and *Streptococcus oralis* are also not reliably identified using 16S rRNA sequencing and sequence comparison of the *sodA* gene has been recommended for their identification (11). Alternative genes have recently been used to establish the identity of organisms and *rpoB* was reported to be the most discriminatory for *Veillonella* (3); it also has the advantage that organisms have only one copy of the gene unlike the multiple copies of the 16S rRNA gene (18), which are not necessarily all identical and may cause difficulties in obtaining unambiguous sequence data. Fig. 1 is a representative dendrogram of all the *Veillonella* isolates from one subject, who harboured all the different *Veillonella* spp. identified in this study, showing the clustering of individual isolates with valid *Veillonella* species. None of the isolates were identified as *V. montpellierensis*, previously isolated from clinical specimens (13), and none was identified as belonging to the species previously isolated from various rodents, although a clinical isolate had been identified previously as belonging to the *V. ratti–V. criceti* group (18). Furthermore, none of the 253 isolates were identified as *V. dentocariosi*, which has

Table 1. Total anaerobic colony counts [as $\log_{10}$(CFU)], proportions of *Veillonella* spp. and individual species as percentage of the total anaerobic colony count and the number of isolates from each sample (n = 11) identified using partial *rpoB* sequencing

Sample	Total count [$\log_{10}$(CFU)]	Total % *Veillonella* spp.	*V. rogosae* (%)	*V. dispar* (%)	*V. atypica* (%)	*V. parvula* (%)	Number of isolates
1	7.53	3.0	1.6	ND	1.4	ND	24
2	7.61	6.8	0.3	3.2	3.2	ND	21
3	7.59	36.3	17.3	6.3	12.6	ND	23
4	7.40	15.5	13.0	2.5	ND	ND	25
5	7.23	30.0	4.0	16.6	9.3	ND	45
6	8.33	12.5	ND	4.3	8.1	ND	23
7	6.92	5.9	0.3	1.1	3.7	0.7	20
8	6.77	32.2	ND	3.6	28.6	ND	18
9	6.88	18.9	13.5	2.7	2.7	ND	21
10	7.11	3.8	ND	ND	3.8	ND	19
11	7.00	13.1	1.9	0.9	10.3	ND	14

CFU, colony-forming unit; ND, not detected; detection limit <0.1% of the total count.

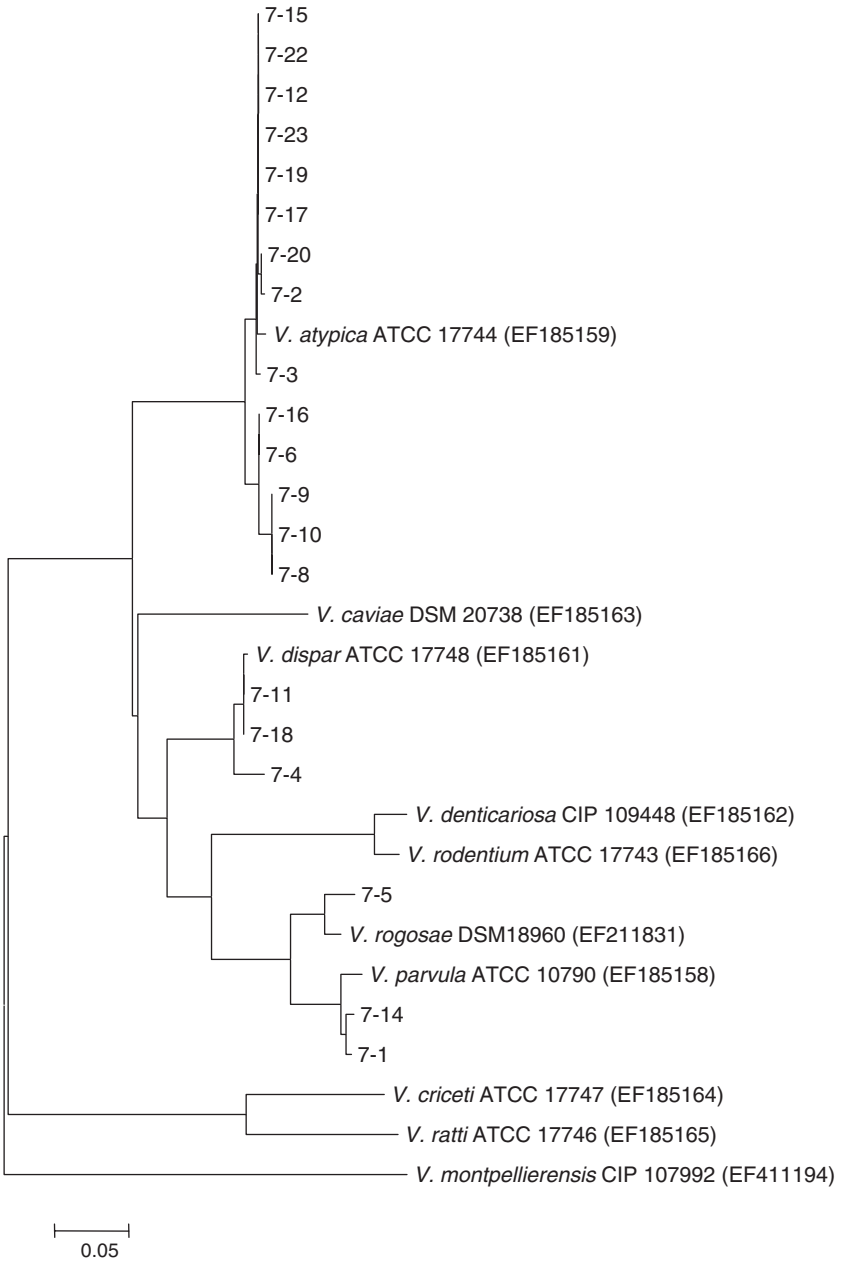

Fig. 1. Dendrogram showing relationships between *Veillonella* spp. isolated from the tongue sample of subject 7 and their clustering with all accepted *Veillonella* type strains listed as species name, strain number (GenBank sequence number).

recently been described and isolated from carious lesions (3; Arif et al., submitted for publication) but not from dental plaque of caries-free children (Arif et al., submitted for publication).

V. atypica was the most frequently isolated species, being recovered from the tongues of 91% of subjects (10/11), and in subject 10 it was the only species isolated. This was the only subject from whom only a single *Veillonella* species was isolated, suggesting that *V. atypica* may have the ability to occupy all niches on the tongue or that presence of some strains of this species may preclude the colonization of other members of the genus. *V. dispar* was recovered from 82% (9/11) of the subjects and *V. rogosae* was isolated from the tongues of 73% (8/11) of the subjects. Previously Hughes et al. (12) used sodium dodecyl sulfate–polyacrylamide gel electrophoresis protein profiles to identify isolates and reported that *V. parvula* was not present on the tongue, while others have suggested that *V. parvula* was present widely on the dorsum of the tongue (8, 10, 16, 17). These data support the previous report (12) because *V. parvula* was isolated from only one subject and formed only 0.74% of the total cultivable flora in that subject. In previous studies, *V. dentocariosi* was isolated from infected human dentine (3; Arif et al. submitted for publication) while *V. parvula* is routinely isolated from the oral biofilm on the dentition. However, *V. rogosae* was not recovered from carious lesions in children but only from the oral biofilm of the teeth of caries-free children (Arif et al. submitted for publication). Therefore there are at least two factors that may influence the distribution of *Veillonella* spp. in the mouth; the surface colonized (tongue or enamel) and the caries status of the enamel surface colonized (decayed or intact).

The intraoral distribution of *Veillonella* spp. is more complex than previously reported and while phenotypic testing is not useful for identifying members of this genus (14) the present data support the use of *rpoB* sequencing rather then 16S rRNA sequencing to identify the members of this genus.

References

1. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. J Clin Microbiol 2005: **43**: 5721–5732.
2. Arif N, Do T, Byun R et al. *Veillonella rogosae* sp. nov., a novel anaerobic Gram-negative coccus isolated from the dental plaque of caries-free children. Int J Syst Evol Microbiol (in press).
3. Byun R, Carlier JP, Jacques NA, Marchandin H, Hunter N. *Veillonella denticariosi* sp. nov., isolated from human carious dentine. Int J Syst Evol Microbiol 2007: **57**: 2844–2848.
4. da Silva CM, Colombo AV, do Souto RM, Colombo AP. In vivo evaluation of the effect of essential oil-containing oral strips on salivary bacteria using the checkerboard method. J Clin Dent 2005: **16**: 38–43.
5. Delwiche EA, Pestka JJ, Tortorello ML. The Veillonellae: gram-negative cocci with a unique physiology. Annu Rev Microbiol 1985: **39**: 175–193.
6. Doel JJ, Benjamin N, Hector MP, Rogers M, Allaker RP. Evaluation of bacterial nitrate reduction in the human oral cavity. Eur J Oral Sci 2005: **113**: 14–19.
7. Donaldson A, McKenzie D, Riggio M et al. Microbiological culture analysis of the tongue anaerobic microflora in subjects with and without halitosis. Oral Dis 2005: **11**: S61–S63.
8. Faveri M, Feres M, Shibli JA, Hayacibara RF, Hayacibara MM, de Figueiredo LC. Microbiota of the dorsum of the tongue after plaque accumulation: an experimental study in humans. J Periodontol 2006: **77**: 1539–1546.

9. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 1999: **41**: 95–98.
10. Haraszthy VI, Zambon JJ, Sreenivasan PK et al. Identification of oral bacterial species associated with halitosis. J Am Dent Assoc 2007: **138**: 1113–1120.
11. Hoshino T, Fujiwara T, Kilian M. Use of phylogenetic and phenotypic analyses to identify nonhemolytic streptococci isolated from bacteremic patients. J Clin Microbiol 2005: **43**: 6073–6085.
12. Hughes CV, Kolenbrander PE, Andersen RN, Moore LV. Coaggregation properties of human oral *Veillonella* spp.: relationship to colonization site and oral ecology. Appl Environ Microbiol 1988: **54**: 1957–1963.
13. Jumas-Bilak E, Carlier JP, Jean-Pierre H et al. *Veillonella montpellierensis* sp. nov., a novel, anaerobic, Gram-negative coccus isolated from human clinical samples. Int J Syst Evol Microbiol 2004: **54**: 1311–1316.
14. Kolenbrander PE, Moore LVH The genus *Veillonella*. In: Balows HG, Trüper M, Dworkin W, Harder W, Schleifer K-H eds. The Prokaryotes, 2nd edn. New York: Springer, 1992: 2034–2047.
15. Kononen E, Asikainen S, Jousimies-Somer H. The early colonization of gram-negative anaerobic bacteria in edentulous infants. Oral Microbiol Immunol 1992: **7**: 28–31.
16. Mager DL, Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Distribution of selected bacterial species on intraoral surfaces. J Clin Periodontol 2003: **30**: 644–654.
17. Marchandin H, Jean-Pierre H, Carrière C, Canovas F, Darbas H, Jumas-Bilak E. Prosthetic joint infection due to *Veillonella dispar*. Eur J Clin Microbiol Infect Dis 2001: **20**: 340–342.
18. Marchandin H, Teyssier C, Jumas-Bilak E, Robert M, Artigues AC, Jean-Pierre H. Molecular identification of the first human isolate belonging to the *Veillonella ratti–Veillonella criceti* group based on 16S rRNA and dnaK gene sequencing. Res Microbiol 2005: **156**: 603–607.
19. Marchandin H, Teyssier C, Simeon De Buochberg M et al. Intra-chromosomal heterogeneity between the four 16S rRNA gene copies in the genus *Veillonella*: implications for phylogeny and taxonomy. Microbiology 2003: **149**: 1493–1501.
20. Mays TD, Holdeman LV, Moore WEC, Rogosa M, Johnson JL. Taxonomy of the genus *Veillonella* Prévot. Int J Syst Bacteriol 1982: **32**: 28–36.
21. Rogosa M. A selective medium for the isolation and enumeration of the veillonella from the oral cavity. J Bacteriol 1956: **72**: 533–536.
22. Rogosa M Anaerobic Gram-negative cocci. In: Krieg NR, Holt JG eds. Bergey's Manual of Systematic Bacteriology. Baltimore: Williams & Wilkins, 1984: 680–685.
23. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987: **4**: 406–425.
24. Sutter VL. Anaerobes as normal oral flora. Rev Infect Dis 1984: **6**: S62–S66.
25. Tyrrell KL, Citron DM, Warren YA, Nachnani S, Goldstein EJ. Anaerobic bacteria cultured from the tongue dorsum of subjects with oral malodor. Anaerobe 2003: **9**: 243–246.
26. Washio J, Sato T, Koseki T, Takahashi N. Hydrogen sulfide-producing bacteria in tongue biofilm and their relationship with oral malodour. J Med Microbiol 2005: **54**: 889–895.