Veillonella denticariosi sp. nov., isolated from human carious dentine

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Selective culture of human carious dentine for *Veillonella* strains resulted in the isolation of two strains of a Gram-negative, coccus-shaped bacterium that has not been described previously. Comparative 16S rRNA and *dnaK* gene sequence analysis indicated that the two strains were homogeneous and comprised a distinct lineage within the genus *Veillonella*, phylogenetically most closely related to *Veillonella rodentium*. This was supported by DNA–DNA hybridization, which showed clearly that the two strains were similar and distinct from other *Veillonella* species, and the production of major cellular fatty acids (C_{13:0} and C_{17:1ω8}), which is consistent with other members of the genus *Veillonella*. Based on these observations, strains RBV81 and RBV106^T represent a novel species, for which the name *Veillonella denticariosi* sp. nov. is proposed, with the type strain RBV106^T (=CIP 109448^T =CCUG 54362^T =DSM 19009^T).

The genus *Veillonella* comprises small, Gram-negative, non-fermentative, anaerobic cocci that are commonly isolated from the oral cavities and gastrointestinal tracts of humans and animals (Rogosa, 1964). Presently, the genus *Veillonella* belongs to the family ‘*Acidaminococcaceae*’ of the phylum *Firmicutes*, and is represented by eight species, *Veillonella parvula*, *V. dispar*, *V. atypica*, *V. caviae*, *V. rodentium*, *V. ratti*, *V. criceti* (Mays *et al.*, 1982; Rogosa, 1965), *V. montpellierensis* (Jumas-Bilak *et al.*, 2004) and the recently identified ‘*V. rogosa*’ (Arif *et al.*, 2008). Of these, only *V. parvula*, *V. dispar*, *V. atypica*, *V. montpellierensis* and ‘*V. rogosa*’ have been isolated from humans, and they are seldom associated with disease.

With the exception of *V. criceti*, *Veillonella* species are unable to ferment carbohydrates or amino acids and rely on the fermentation of lactate, pyruvate, malate, fumarate and/or oxaloacetate as a source of carbon and energy (Foubert & Douglas, 1948; Rogosa, 1984). *Veillonella* species are frequently isolated from the oral cavity and,

in the polymicrobial community of dental plaque, are commonly associated with bacteria that are capable of fermenting carbohydrates to lactic acid, such as strains of *Actinomyces*, *Streptococcus* and *Lactobacillus*. It is believed that veillonellae play an important role in modulating the acid flux (Hoshino *et al.*, 1981), where the metabolic conversion of lactate into the weaker acetic and propionic acids (Ng & Hamilton, 1971) is considered to be anti-cariogenic, as the weaker acids are less capable of decalcifying enamel (Mikx *et al.*, 1972; van der Hoeven *et al.*, 1978). Likewise, in the anaerobic environment of deep dentinal caries, the conversion of a lactate-dominant to a less acidic environment would favour the growth and proliferation of various anaerobic bacterial species that are commonly found in the deeper regions of carious dentine (Chhour *et al.*, 2005; Edwardsson, 1974; Hoshino, 1985). In this study, we describe the isolation and characterization of two strains of a novel species of *Veillonella* isolated from human carious dentine.

The bacterial isolates RBV81 and RBV106^T were selectively isolated from carious lesions of two independently extracted teeth of patients serviced at the Westmead Centre for Oral Health (Sydney, Australia) in 2005. Carious dentine was extracted as described previously (Martin *et al.*, 2002) and plated on *Veillonella* agar (Rogosa, 1956; Rogosa *et al.*, 1958) supplemented with vancomycin (final concentration 7.5 µg ml⁻¹). Plates were

The GenBank/EMBL/DDJB accession numbers for the 16S rRNA and *dnaK* gene sequences of strains RBV106^T and RBV81 are EF185167 and EF185168 (16S rRNA genes) and EF219278 and EF219279 (*dnaK*), respectively.

A matrix of DNA–DNA hybridization results and 16S rRNA gene sequence similarities is available as supplementary material with the online version of this paper.

incubated at 37 °C in an anaerobic atmosphere (90 % N₂, 5 % H₂, 5 % CO₂ by vol.) for 48 h in an anaerobic workstation (Don Whitley Scientific). Isolates were presumptively identified by PCR-RFLP analysis of 16S rRNA genes (Sato *et al.*, 1997). Strains RBV81 and RBV106^T were identified as having an identical restriction profile to that of *V. rodentium* ATCC 17743^T.

Colonies of strains RBV106^T and RBV81 cultured on *Veillonella* agar in an anaerobic atmosphere for 48 h at 37 °C were circular, smooth, beige coloured, opaque, convex and approximately 2 mm in diameter. Both strains had Gram-negative, coccus-shaped cells arranged singularly or in pairs. Cells were non-spore-forming and non-motile.

For negative staining, bacteria were grown in *Veillonella* broth at 37 °C in an anaerobic atmosphere for 48 h. A drop of the bacterial suspension was held on a 200-mesh copper grid with a carbon-coated pioloform/Formvar support film for 1 min and removed. The grids were then negatively stained with phosphotungstic acid for 30 s and removed. After allowing the grids to dry for 30 min, they were observed under a Philips CM10 electron microscope. The cells were spherical in shape with a convoluted surface and varied in size from 0.3 to 0.5 µm, with a mean size of 0.4 µm. This is consistent with previous observations of other members of the genus *Veillonella* (Bladen & Mergenhagen, 1964; Jumas-Bilak *et al.*, 2004).

For electron microscopy of ultrathin sections, cells were fixed in Karnovsky's fixative for 1 h. The bacteria were then washed in phosphate buffer and dehydrated using graded ethanol and embedded in fresh Spurr's resin. Ultrathin sections were cut using a Reichert Ultracut E microtome and picked up on 400-mesh copper grids. The sections were stained with uranyl acetate and lead citrate and observed under a Philips CM10 electron microscope at high magnification to distinguish cellular structures (Fig. 1). The ultrathin sections allowed visualization of structural components (outer membrane, thin peptidoglycan layer and cytoplasmic membrane) that are characteristic of a Gram-negative cell wall and consistent with previous reports for other members of the genus *Veillonella* (Bladen & Mergenhagen, 1964; Jumas-Bilak *et al.*, 2004).

Conventional biochemical analysis for anaerobic organisms and subsequent determination of metabolic end products were performed as described previously (Carlier, 1985; Holdeman *et al.*, 1977). For the Rapid ID 32A system (bioMérieux), cells were grown for 24 h on *Veillonella* agar at 37 °C in an anaerobic atmosphere and then collected and resuspended in sterile distilled water and analysis was performed according to the manufacturer's instructions. Cellular fatty acid composition was analysed by GC according to Veys *et al.* (1989). Briefly, strains were grown anaerobically in 10 ml trypticase-glucose-yeast extract (TGY) medium at 37 °C for 48 h in an anaerobic jar containing 5 % H₂, 5 % CO₂ and 90 % N₂ (by vol.). Methyl esters were chromatographed on a fused-silica capillary

Fig. 1. Electron micrograph of an ultrathin section of a cell of strain RBV106^T. Bar, 100 nm.

column (25 m × 0.25 mm i.d.) coated with 5 % methyl phenyl silicone (film thickness 0.25 µm).

Strains RBV81 and RBV106^T were strictly anaerobic, catalase-negative, indole-negative and reduced nitrate to nitrite. The strains were resistant to vancomycin (5 µg) but susceptible to kanamycin (1 mg), colistin (10 µg), metronidazole (4 µg) and bile identification discs, which is characteristic of other *Veillonella* species, except *V. montpellierensis* and *V. ratti* (Jumas-Bilak *et al.*, 2004). Interestingly, gas was not produced in TGY deep agar, which is atypical for the genus *Veillonella*. Biochemical analysis showed that neither strain was able to ferment carbohydrates (glucose, fructose, lactose, maltose, mannose and sucrose) but could metabolize lactate to acetate and propionate (5.5 and 5.4 mM, respectively, for strain RBV106^T; 5.8 and 6.4 mM, respectively, for strain RBV81). This was supported by the use of the Rapid ID 32A system, which showed that both strains were only positive for the reduction of nitrates (NIT) and for the hydrolysis of arginine (ARG). Cellular fatty acid profiles for RBV81 and RBV106^T were similar to each other and consistent with those of other *Veillonella* species (Table 1), with the dominant fatty acids C_{13:0} and C_{17:1ω8} being synthesized (Jumas-Bilak *et al.*, 2004). Minor fatty acids included C_{12:0}, iso-C_{14:0}, C_{14:0}, anteiso-C_{15:0}, C_{15:0}, C_{16:1ω9c}, C_{16:0}, C_{18:1ω9c} and C_{18:0}. Fatty acids C_{11:0}, iso-C_{16:0}, C_{17:0Δ}, C_{17:0}, C_{16:0} 2-OH, C_{18:1ω9t} and C_{20:0} were found in trace amounts. Strains RBV81 and RBV106^T were found to differ from other *Veillonella* species in the production of significant amounts of iso-C_{14:0} as well as three unknown compounds.

Table 1. Relative content of cellular fatty acids of strains RBV106^T and RBV81 and other *Veillonella* species

Reference strains (data from Jumas-Bilak *et al.*, 2004): 1, *V. atypica* ATCC 17744^T; 2, *V. caviae* DSM 20738^T; 3, *V. criceti* DSM 20734^T; 4, *V. dispar* ATCC 17748^T; 5, *V. ratti* ATCC 17746^T; 6, *V. rodentium* ATCC 17743^T; 7, *V. parvula* ATCC 17745; 8, *V. montpellierensis* CIP 107992^T. Data are percentages of total cellular fatty acids. For unsaturated fatty acids, the position of the double bond can be located by counting from the methyl (ω) end of the carbon chain; *cis* and *trans* isomers are respectively indicated by the suffixes *c* and *t*. Cyclopropane acid are identified by Δ . On the basis of their retention times, unknown compounds 2 and 3 may correspond to anteiso-C_{16:0} and C_{16:1} ω 7*c*, respectively. —, Not detected; NA, no data available (these components were not reported by Jumas-Bilak *et al.*, 2004).

Fatty acid	RBV106 ^T	RBV81	1	2	3	4	5	6	7	8
C _{11:0}	0.5	0.7	1.1	1.4	2	0.8	2.1	1.6	1.8	2.3
C _{12:0}	3.0	4.5	3.8	4	1.4	3.8	4.5	5	4.9	7
C _{13:0}	12.9	19.2	20.4	7.3	16.3	23.5	20	25.3	24	6.3
iso-C _{14:0}	5.1	5.8	0.6	1.2	—	1.5	0	—	1.9	—
C _{14:0}	3.9	5.2	5.7	3.1	1.2	4.9	2	9.2	6.5	5
anteiso-C _{15:0}	2.4	4.1	—	—	—	—	—	—	—	—
C _{15:0}	4.4	6.3	17.7	7	12.7	7	10.3	11.6	12	6.3
C _{14:0} 2-OH	—	—	0.3	—	0.3	—	1	—	—	—
iso-C _{16:0}	3.2	0.8	—	—	—	—	—	—	—	—
Unknown 1	4.2	3.6	—	—	—	—	—	—	—	—
Unknown 2	5.1	5.4	—	—	—	—	—	—	—	—
C _{16:1} ω 9 <i>c</i>	4.5	5.5	5.2	10.6	4.2	7.7	4.8	5.5	5.2	8.4
Unknown 3	4.2	1.3	—	—	—	—	—	—	—	—
C _{16:0}	8.6	6.0	7.5	10.3	3.7	8.4	4.6	6.7	7	12.9
C _{17:1} ω 8	10.1	13.1	25.7	29.9	48.9	25.6	36.9	17	22.3	20.7
C _{17:0} Δ	3.7	1.4	—	—	—	—	—	—	—	—
C _{17:0}	3.2	2.3	2.4	2.5	2.6	2.5	2.4	4.2	4.1	2
C _{16:0} 2-OH	2.3	0.7	—	—	—	—	—	—	—	—
C _{18:1} ω 9 <i>c</i>	5.7	6.8	4.3	14.3	2.8	8	6.1	6.7	6.2	17.4
C _{18:1} ω 9 <i>t</i>	3.5	2.8	—	1.9	1.2	—	—	—	—	2
C _{18:0}	6.6	3.5	2.7	6.2	2.1	5.7	5.8	6.7	3.9	11.1
C _{19:0} Δ	—	0.5	NA	NA	NA	NA	NA	NA	NA	NA
C _{20:0}	2.6	1.6	NA	NA	NA	NA	NA	NA	NA	NA

DNA was extracted from cells collected from *Veillonella* agar plates resuspended in 50 mM Tris/HCl (pH 8.0) using the QIAamp DNA Mini kit (Qiagen) as described by the manufacturer. PCR and sequencing of the 16S rRNA and *dnaK* genes was performed using primers described previously (Marchandin *et al.*, 2003; Paster *et al.*, 2001). Sequences were aligned using the program CLUSTAL_X (version 1.83) and phylogenetic analysis was performed using MEGA (version 3.1). Distance matrices were constructed using Kimura's two-parameter method and phylogenetic trees were constructed by the neighbour-joining method (Figs 2 and 3). Bootstrap analysis was based on 1000 replicates and support is represented as percentages.

A contiguous sequence of 1497 bp of the 16S rRNA gene was obtained from strains RBV106^T and RBV81, with two and three ambiguous positions, respectively, observed in the chromatograms, which is consistent with the observation of intrachromosomal variation between 16S rRNA gene copies in some *Veillonella* strains (Marchandin *et al.*,

2003). The 16S rRNA gene sequences of strains RBV106^T and RBV81 differed at only a single site (99.9%) and were phylogenetically most closely related to *V. rodentium* ATCC 17743^T, with 99.1 and 99.0% sequence identity, respectively (Supplementary Table S1, available in IJSEM Online).

Because of the high level of sequence conservation in the 16S rRNA gene sequence between several *Veillonella* species and the relatively high level of intrachromosomal heterogeneity in some *Veillonella* isolates (Jumas-Bilak *et al.*, 2004; Marchandin *et al.*, 2003), the 70 kDa heat-shock protein gene (*dnaK*) was also partially sequenced as an alternate genetic marker. Analysis of partial *dnaK* sequences (640 bp) of strains RBV106^T and RBV81 showed that they share 99.5% identity. Consistent with observations in the 16S rRNA gene, both strains were phylogenetically most closely related to *V. rodentium* ATCC 17743^T (Fig. 3), with 95.2 and 95.7% sequence identity, respectively. This is comparable to the level of sequence identity observed between the *dnaK* sequences of the type

Fig. 2. Neighbour-joining phylogenetic tree based on the 16S rRNA sequences (1497 bp) of type strains of *Veillonella* species. Bootstrap values at nodes are represented as percentages of 1000 replicates. Bar, genetic distance of 1 %.

strains of the closely related species *V. parvula* and *V. dispar* (Jumas-Bilak *et al.*, 2004), supporting the claim that strains RBV106^T and RBV81 represent a novel species. The phylogenetic tree for *dnaK* is congruent with that of the 16S rRNA gene, which shows that strains RBV106^T and RBV81 are distinct from other *Veillonella* species and most closely related to *V. rodentium*.

DNA–DNA hybridization to determine genetic relatedness was performed in NucleoLink micro-well strips (Nalge Nunc International) as described previously (Christensen *et al.*, 2000), except that hybridization was performed at 42 °C for 18 h with 50 ng photobiotin acetate-labelled DNA per well in 100 µl 2 × SSC buffer, 5 × Denhardt's solution, 3 % (w/v) dextran sulphate, 50 % (v/v) formamide and 50 µg salmon sperm DNA ml⁻¹ (Sigma). The control wells contained purified genomic DNA from *Escherichia coli* XL1-Blue. Strains RBV81 and RBV106^T showed a high level of DNA relatedness (87 %; Supplementary Table S1) and are most closely related to *V. rodentium* ATCC 17743^T (49 and 48 %, respectively).

Fig. 3. Neighbour-joining phylogenetic tree based on partial *dnaK* sequences (640 bp) of type strains of *Veillonella* species. Bootstrap values at nodes are represented as percentages of 1000 replicates. Bar, genetic distance of 5 %.

Description of *Veillonella denticariosi* sp. nov.

Veillonella denticariosi (den.ti.ca.ri.o'si. L. n. *dens*, *dentis* tooth; L. adj. *cariosus* rotten, decayed; N.L. gen. n. *denticariosi* of a decayed tooth).

Cells are non-motile, non-spore-forming cocci, arranged singly or in pairs, with a mean diameter of 0.4 µm. Irregular masses of cells are also observed in TGY broth. Colonies on *Veillonella* agar are circular, smooth, beige coloured, opaque and convex and are approximately 2 mm in diameter after 48 h at 37 °C in an anaerobic atmosphere. Cells are strict anaerobes, Gram-negative, with a convoluted surface. Cells are catalase-negative, reduction of nitrates is positive, arginine dihydrolase is present and gas is not produced. Lactate is fermented, with the major metabolic end products being acetate and propionate. Major cellular fatty acids produced are C_{13:0} and C_{17:1}ω8, consistent with other *Veillonella* species, with unique fatty acids iso-C_{14:0} and three unknown compounds being produced. Can also be differentiated from other *Veillonella* species by 16S rRNA gene and *dnaK* sequencing.

The type strain RBV106^T (=CIP 109448^T =CCUG 54362^T =DSM 19009^T) and a reference strain RBV81 (=CIP 109449 =CCUG 54361 =DSM 19010) were isolated from human carious dentine.

Acknowledgements

We are grateful to Mary Simonian for providing assistance with electron microscopy and Maria Manich for technical assistance. This work was supported by the NIDCR grant R01 DE015272-07.

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