Prevotella maculosa sp. nov., isolated from the human oral cavity

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Three strains of anaerobic Gram-negative bacilli isolated from human oral sites were subjected to a comprehensive range of phenotypic and genotypic tests and were found to comprise a homogeneous group. 16S rRNA gene sequence analysis revealed the strains to constitute a novel group within the genus *Prevotella*, most closely related to *Prevotella oris* and *Prevotella salivae*. A novel species, *Prevotella maculosa* sp. nov., is proposed to accommodate these strains.

Prevotella maculosa is saccharolytic and produces acetic and succinic acids as end products of fermentation. The G+C content of the DNA of the type strain is 48 mol%. The type strain of *Prevotella maculosa* is W1609^T (=DSM 19339^T=CCUG 54766^T).

Prevotella species are part of the normal human oral microbiota and are also frequently isolated from oral infections such as periodontitis, dental caries and abscesses (Dymock *et al.*, 1996; Paster *et al.*, 2001; Nadkarni *et al.*, 2004). However, many isolates encountered in clinical samples cannot be assigned to species with validly published names. In this study, a group of three such strains were characterized by a variety of phenotypic and genotypic methods.

Strain W1609^T was isolated from the exudate from pericoronitis associated with a third molar, W2051 from an infection associated with a dental implant and W6974 from subgingival plaque in a periodontally healthy subject. *Prevotella oris* ATCC 33573^T was obtained from the ATCC and *Prevotella salivae* DSM 15606^T from the DSMZ. *Prevotella oris* W1864 and *Prevotella salivae* W1640 were isolated from exudates from pericoronitis in third molars.

Strains were grown at 37 °C on fastidious anaerobe agar (FAA; LabM) supplemented with 5% horse blood under anaerobic conditions (80% N₂, 10% H₂, 10% CO₂) in an anaerobic workstation (Don Whitley Scientific). Colonial morphologies were determined after 3 days incubation using a dissecting microscope. Cellular morphology was recorded after Gram-staining of smears prepared from 2-day FAA cultures. Hanging-drop preparations of 18 h

cultures of peptone/yeast extract/glucose (PYG) broth were examined under phase-contrast microscopy for cellular motility.

Biochemical tests were performed using standard methods (Holdeman *et al.*, 1977; Jousimies-Somer *et al.*, 2002). Fermentation tests were performed using prereduced, anaerobically sterilized (PRAS) sugars prepared in an anaerobic workstation (Holdeman *et al.*, 1977). Susceptibility to special-potency antibiotic discs separately containing vancomycin (5 µg), kanamycin (1 mg) and colistin (10 µg) was determined on FAA (Jousimies-Somer *et al.*, 2002). Bacterial strains were grown in peptone/yeast extract (PY) broth with and without glucose and short-chain volatile and non-volatile fatty acids were extracted by standard methods and analysed by gas chromatography (Holdeman *et al.*, 1977). Enzyme profiles were generated in duplicate with the Rapid ID 32A anaerobe identification kit (bioMérieux), according to the manufacturer's instructions, using bacteria harvested from Columbia agar plates (LabM) supplemented with 5% horse blood.

Cellular fatty acid composition was determined as described previously (Sutcliffe, 2000; Downes *et al.*, 2005). Fatty acid methyl esters (FAMES) were identified by comparison with authentic FAME standards (Sigma). The G+C content of the DNA of strains W1609^T and W6974 was determined by an HPLC method as described previously (Wade *et al.*, 1999). A thermal renaturation method (Huß *et al.*, 1983) was used to estimate the DNA–DNA relatedness between strain W1609^T and the type strains of *P. oris* and *P. salivae*.

The 16S rRNA genes of the strains were sequenced as described previously (Downes *et al.*, 2005). Sequences were

Abbreviation: FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession numbers for the two 16S rRNA gene sequences obtained from *Prevotella maculosa* W1609^T are EF534314 and EF534315.

An extended phylogenetic tree, a summary of the characteristics of the novel species and a comparison of FAME profiles are available as supplementary material with the online version of this paper.

connected using the BioEdit programme (Hall, 2004) and provisionally identified by BLAST interrogation of the GenBank database (Altschul *et al.*, 1990). Phylogenetic analysis was performed using MEGA version 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were constructed by the neighbour-joining method from distance matrices prepared using the Jukes–Cantor correction.

Individual PCR-amplified 16S rRNA genes from strain W1609^T were isolated by cloning using the TOPO TA cloning kit and TOP10 chemically competent cells (Invitrogen). Ten cloned genes were partially sequenced with primer 519R.

The results of the phenotypic tests are tabulated in Supplementary Table S1, available in IJSEM Online. The strains were obligately anaerobic, non-motile, non-pigmenting, Gram-negative bacilli, 0.7 µm wide by 1–3 µm long, with occasional cells up to 6 µm long. After 3 days incubation on FAA medium, colonies were 1.4–1.8 mm in diameter, circular, entire, convex, grey, shiny and opaque with a slightly mottled internal appearance when viewed under a plate microscope. Strains were resistant to special-potency discs containing vancomycin, colistin and kanamycin. Growth of all strains in PY broth produced a moderately turbid suspension (2 to 3+ on a scale of 0 to 4+). Growth was enhanced by the addition of 1% fermentable carbohydrates (3 to 4+). Strains were saccharolytic (see species description for sugar reactions) and major amounts of acetic and succinic acids were produced as end products of glucose metabolism. Aesculin was hydrolysed but other biochemical tests were negative (see species description). The G + C content of the DNA of strains W1609^T and W6974 was 48 mol%.

The strains gave positive reactions in the Rapid ID 32A panel for α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, α-arabinosidase, N-acetyl-β-glucosaminidase, mannose fermentation, raffinose fermentation, alkaline phosphatase, leucyl glycine arylamidase and alanine arylamidase, while β-glucuronidase production was weak and variable. Negative reactions were obtained for the remaining 17 tests, resulting in a profile of 45³/7 4402 00. The enzyme profiles for the two strains of *P. oris* were 4537 4402 20 and 4537 4402 22 for the two strains of *P. salivae*. *P. salivae* and *P. oris* strains gave a positive reaction for α-fucosidase while the strains of the novel group were negative; for glutamyl glutamic acid arylamidase, *P. salivae* was positive and *P. oris* and the novel group were negative; β-glucuronidase was variable for the novel group but negative for *P. oris* and *P. salivae*.

Assembly of the 16S rRNA gene sequences for strain W1609^T revealed a number of ambiguous bases in helix 6 in variable region 1. Sequencing of individual amplified genes isolated by cloning revealed two versions of the stem section of helix 6, which differed by 12 bases (Fig. 1). The two versions were designated A and B and version A was used for the subsequent sequence identity comparisons. Heterogeneity within multiple rRNA operons within

Fig. 1. Sequence variation in helix 6 between variants A and B of the 16S rRNA gene of strain W1609^T.

genomes is well-known. Among 83 bacterial genomes with multiple rRNA operons, 62% were found to exhibit 16S rRNA gene intragenomic heterogeneity (Case *et al.*, 2007). However, this heterogeneity is rarely noted in species descriptions; it was so marked for strain W1609^T that the two versions of the gene have been submitted to GenBank.

Phylogenetic analysis of the two 16S rRNA gene sequences for W1609^T revealed this organism to belong to the genus *Prevotella* (Fig. 2; see Supplementary Fig. S1 for a fuller phylogenetic tree). The 16S rRNA gene was also sequenced from strains W2051 and W6974 and showed 98.3% sequence identity or greater to strain W1609^T sequence variant A (W1609A) over 1454 unambiguously aligned bases. The most closely related sequences in GenBank were from oral clones *Prevotella* AA020 (GenBank accession no. AY005057) and *Prevotella* AA016 (AY005058), with sequence identities to W1609A of 98.8 and 98.6% respectively. The most closely related species to W1609A with validly published names were *P. oris* (94.0% sequence identity to the type strain) and *P. salivae* (94.1%).

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence comparisons over 1411 aligned bases showing the relationship between *Prevotella maculosa* sp. nov. W1609^T and related species. The tree was constructed using the neighbour-joining method following distance analysis of aligned sequences. Numbers represent bootstrap values for each branch based on data for 100 trees. Accession numbers for 16S rRNA gene sequences are given for each strain. Bar, 0.02 substitutions per nucleotide site.

Table 1. Phenotypic characteristics that differentiate strains W1609^T, W2051 and W6974 (*Prevotella maculosa* sp. nov.) from other *Prevotella* species

Species: 1, *P. buccae*; 2, *P. dentalis*; 3, *P. oris*; 4, *P. salivae*; 5, *P. maculosa* sp. nov.; 6, *P. bergensis*; 7, *P. oralis*; 8, *P. baroniae*; 9, *P. buccalis*; 10, *P. loeschii*; 11, *P. veroralis*; 12, *P. multiformis*; 13, *P. denticola*; 14, *P. melaninogenica*; 15, *P. shahii*; 16, *P. oulorum*; 17, *P. enoeca*; 18, *P. bivia*; 19, *P. tanneriae*; 20, *P. intermedia*; 21, *P. nigrescens*; 22, *P. corporis*; 23, *P. marshii*; 24, *P. pallens*; 25, *P. disiens*. w, Weak; v, variable; NA, no data available. Data for reference species (with the exception of α -fucosidase and glutamyl glutamic acid arylamidase activities, which were determined in this study from the reference strains mentioned in the text) were taken from the original descriptions.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Pigment*	–	–	–	–	–	–	–	–	–	+	–	–	+	+	+	–	–	–	+	+	+	+	–	+	–
Fermentation of:																									
Arabinose	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	v	–	–	–	–	–	–
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	v	+	v	v	+	+	v	–	–
Raffinose	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	v	v	+	–	–	+	–
Salicin	+	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sucrose	+	w	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	v	+	+	–	–	+	–
Indole production	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	+	–
Aesculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	–	+	v	–	+	v	–	–	–	–	–	–	–	–
Gelatin hydrolysis	+	–	v	–	–	–	v	w	–	+	v	+	+	+	+	–	+	+	+	+	+	+	+	+	+
α -Fucosidase	NA	NA	+	+	–	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Glutamyl glutamic acid arylamidase	NA	NA	–	+	–	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

*Pigmentation on blood agar may take up to 14 days and varies from tan through brown to black depending on the species.

Cellular FAME results for the three strains are shown in Supplementary Table S2. The FAME profiles were consistent with previous analyses of *Prevotella* species (Supplementary Table S2), with a relatively high proportion of palmitic acid noted. These data further confirm the need for a comprehensive, standardized survey of fatty acid composition in *Prevotella* and closely related genera.

DNA–DNA relatedness between strain W1609^T and the type strains *P. oris* ATCC 33573^T and *P. salivae* DSM 15606^T was determined to be 6 and 12 %, respectively.

The strains studied here constitute a homogeneous group and are clearly distinct from any species with validly published names. We therefore propose the name *Prevotella maculosa* sp. nov. for this group. Phenotypic characteristics that distinguish *P. maculosa* from the other *Prevotella* species are shown in Table 1. Conventional biochemical tests, commonly used to differentiate members of the genus *Prevotella*, were unable to distinguish *P. maculosa* from *P. oris* and *P. salivae*. However, tests for α -fucosidase and glutamyl glutamic acid arylamidase, included in the Rapid ID 32A kit, could differentiate the species, as described above. However, when additional strains become available, these should be tested to confirm the reliability of species differentiation based on these tests.

Description of *Prevotella maculosa* sp. nov.

Prevotella maculosa (ma.cu.lo'sa. L. fem. adj. *maculosa* speckled, referring to the internal appearance of colonies of the organism growing on agar media).

The description is based on three strains isolated from the human mouth. Cells are obligately anaerobic, non-motile, non-pigmenting, Gram-negative bacilli, 0.7 μ m wide by 1–3 μ m long, with occasional cells up to 6 μ m long. After 3 days incubation on FAA medium, colonies are 1.4–1.8 mm in diameter, circular, entire, convex, opaque, shiny and grey, with a slightly mottled internal appearance. Growth in broth media produces a moderate turbidity that is enhanced by the addition of fermentable carbohydrates. Cells are saccharolytic and ferment arabinose, cellobiose, fructose, glucose, lactose, maltose, mannose, melibiose, raffinose, rhamnose, salicin, sucrose and xylose; mannitol, melezitose and sorbitol are not fermented. Major amounts of acetic and succinic acids are produced as end products of metabolism in PYG broth. The non-hydroxylated fatty acid profile is predominantly anteiso-C_{15:0} and C_{16:0}, with lesser amounts of iso-C_{15:0} and iso-C_{17:0}. Aesculin is hydrolysed; arginine, gelatin and urea are not hydrolysed. Indole and catalase are not produced and nitrate is not reduced. There is no growth in 20 % bile. The G+C content of the DNA of the type strain is 48 mol%.

The type strain, W1609^T (=DSM 19339^T =CCUG 54766^T), and strains W2051 and W6974 were isolated from the human oral cavity.

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