Prevotella dentasini sp. nov., a black-pigmented species isolated from the oral cavity of donkeys

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Four strains (NUM 1903^T, NUM 1904, NUM 1912 and NUM 1925) that were obligately anaerobic, pigmented, Gram-negative-staining rods were isolated from the oral cavity of donkeys. These strains were analysed using the Rapid ID 32A, API 20A and API ZYM systems, by DNA–DNA hybridization with other related species and by 16S rRNA gene sequencing. 16S rRNA gene sequence analysis showed that each of the new isolates was a member of the genus *Prevotella* and related to *Prevotella multiformis* PPA21^T, showing about 93 % sequence similarity. Based on phylogenetic and phenotypic evidence, it is proposed that the four strains are representatives of a novel species, for which the name *Prevotella dentasini* sp. nov. is proposed. The type strain is NUM 1903^T (=JCM 15908^T=DSM 22229^T).

Black-pigmented, Gram-negative, anaerobic rods are frequently found in the oral cavity of periodontally healthy and diseased individuals (Slots *et al.*, 1986; Hirasawa & Takada, 1994; Könönen *et al.*, 1994). During our studies on the oral microbial flora of donkeys, we isolated a number of black-pigmented, obligately anaerobic, Gram-negative-staining rods on *Brucella* agar (Becton Dickinson) supplemented with kanamycin (0.5 mg ml⁻¹) and 5 % (v/v) horse blood (BBA).

Four black-pigmented strains, NUM 1903^T, NUM 1904, NUM 1912 and NUM 1925, were randomly selected for this study from among 25 black-pigmented isolates obtained from the oral cavity of five donkeys from a zoo. The strains formed black, small and smooth colonies on BBA after incubation at 37 °C for 2 days in an anaerobic chamber. Biochemical analysis was conducted using the Rapid ID 32A, API 20A and API ZYM systems (bioMérieux) according to the manufacturer's instructions. The colony and biochemical characteristics of the novel strains resembled those of the *Prevotella* group. The phenotypic characteristics that differentiate strain NUM 1903^T (designated as the type strain from the four isolates) from the type strains of recognized species of the genus *Prevotella* are shown in Table 1.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NUM 1903^T is AB477014.

A maximum-parsimony phylogenetic tree constructed using 16S rRNA gene sequences and a table of DNA–DNA hybridization values are available as supplementary material with the online version of this paper.

The four isolates were subjected to further taxonomic study. DNA was extracted from bacterial cultures using the

Table 1. Phenotypic characteristics and enzyme production of strain NUM 1903^T and related strains of the genus *Prevotella*

Strains: 1, NUM 1903^T; 2, *P. multiformis* JCM 12541^T; 3, *P. denticola* ATCC 35308^T; 4, *P. oris* ATCC 33573^T; 5, *P. histicola* T05-04^T; 6, *P. veroralis* ATCC 33779^T; 7, *P. melaninogenica* ATCC 25845^T. Data were taken from this study, Sakamoto *et al.* (2005) and Downes *et al.* (2008). All strains were positive for fermentation of sucrose and raffinose and negative for production of indole. +, Positive; v, variable; –, negative reaction; ND, no data available.

Characteristic	1	2	3	4	5	6	7
Pigmentation	+	–	+	–	v	–	+
Gelatin digestion	+	+	+	+	+	–	+
Aesculin hydrolysis	–	–	+	+	–	+	–
Rapid ID 32A							
L-Arabinose	–	–	–	+	–	–	–
Cellobiose	–	+	–	+	–	+	–
Glycerol	+	+	+	–	ND	–	–
Lactose	–	+	+	+	+	+	+
D-Mannose	–	+	+	+	+	+	+
D-Rhamnose	–	–	–	+	–	–	–
Salicin	–	–	–	+	–	–	–
D-Xylose	–	–	–	+	–	–	–
DNA G+C content (mol%)	50.2	51.1	50.5	42	43	42.1	40–43

Wizard Genome DNA purification kit (Promega) according to the manufacturer's instructions. The G + C content of the DNA was determined by HPLC employing a method described previously (Hirasawa & Takada, 1994). The G + C content of strains NUM 1903^T, NUM 1904, NUM 1912 and NUM 1925 ranged from 49 to 51 mol%. DNA–DNA hybridization was performed according to the microtitration plate method (Ezaki *et al.*, 1989) with minor modifications (Takada & Hirasawa, 2007) using labelled DNA from strain NUM 1903^T (see Supplementary Table S1, available in IJSEM Online). DNA from strains NUM 1904, NUM 1912 and NUM 1925 showed high levels of DNA–DNA relatedness to strain NUM 1903^T (96.8, 95.9 and 97.4 %, respectively) and DNA from *Prevotella denticola* ATCC 35308^T, *Prevotella multiformis* JCM 12541^T, *Prevotella melaninogenica* ATCC 25845^T and *Prevotella nigrescens* ATCC 33563^T showed low levels of

DNA–DNA relatedness to strain NUM 1903^T (43.1, 33.0, 40.3 and 35.8 %, respectively).

The almost-entire 16S rRNA gene of each of the four clinical isolates was sequenced and subjected to a comparative analysis. The 16S rRNA genes of the isolates were amplified by PCR and directly sequenced using a Big Dye Terminator v.1.1 Cycle Sequencing kit and an automatic DNA sequencer (ABI Prism model 373A; Applied Biosystems). Database searches of sequences available in DDBJ, EMBL and GenBank identified the closest known relatives of the new isolates. Sequence data were aligned using CLUSTAL W software (Thompson *et al.*, 1994) and corrected by manual inspection. Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura, 1980) after gaps and unknown bases had been eliminated. A phylogenetic tree was constructed by employing the

Fig. 1. Neighbour-joining phylogenetic tree derived from 16S rRNA gene sequences showing the position of strain NUM 1903^T in the genus *Prevotella*, based on a comparison of approximately 1350 nucleotides. Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position.

neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Tamura *et al.*, 2007) methods. Bootstrap resampling analysis (Felsenstein, 1985) was performed to estimate the confidence of the tree topologies. The neighbour-joining and maximum-parsimony trees depicting the phylogenetic affinity of strain NUM 1903^T within the genus *Prevotella* are presented in Fig. 1 and Supplementary Fig. S1, respectively. The closest phylogenetic neighbours of strain NUM 1903^T were *P. multiformis* PPA21^T (93.4% 16S rRNA gene sequence similarity) and *P. denticola* ATCC 35308^T.

Serotypes were examined with an agar-gel immunodiffusion test using rabbit antisera raised against reference strains, prepared as described previously (Hirasawa *et al.*, 1980; Takada *et al.*, 1984). Antigens were prepared from cells cultured in Gifu anaerobic medium (GAM; Nissui, Japan) overnight and extracted by sonication (50 W, 15 min) with cooling. Immunodiffusion experiments with strain NUM 1903^T, showed that no reactive precipitin bands were formed with typing sera prepared against oral black-pigmented *Prevotella* strains, *P. denticola* ATCC 35308^T, *P. melaninogenica* ATCC 25845^T, *P. intermedia* ATCC 25611^T, *P. nigrescens* ATCC 33563^T and *P. loescheii* ATCC 15930^T. These results suggest that strain NUM 1903^T has a hitherto-unknown surface antigen.

Therefore, on the basis of the chemotaxonomic and phylogenetic evidence that show similarities among the four strains and the phenotypic characteristics of strain NUM 1903^T that differentiate it from type strains of recognized species of the genus *Prevotella*, we believe that the four isolates should be classified in a novel species of the genus *Prevotella*, for which the name *Prevotella dentasini* sp. nov. is proposed.

Description of *Prevotella dentasini* sp. nov.

Prevotella dentasini (den.ta.si'ni. L. gen. n. *dens* -entis a tooth; L. gen. n. *asini* of a donkey; N.L. gen. n. *dentasini* of the tooth of a donkey).

Cells are obligately anaerobic, non-spore-forming, non-motile, Gram-negative-staining rods, 0.6–0.8 × 1.5–2.5 µm. Colonies on blood agar after 2 days at 37 °C are small and black, 1–2 mm in diameter, circular, entire, smooth and non-haemolytic. There is no growth in 20% bile. Produces acid from D-glucose, glycerol, maltose, sucrose and raffinose, but not from arabinose, cellobiose, lactose, D-mannitol, D-mannose, rhamnose, salicin, sorbitol, trehalose or xylose. Digests gelatin. Does not hydrolyse aesculin or produce indole. Alanine arylamidase, acid phosphatase, alkaline phosphatase, leucine glycine arylamidase, glutamyl glutamic acid arylamidase and naphthol-AS-BI-phosphohydrolase are produced, but catalase and urease are not produced. Susceptibility to kanamycin is negative.

The type strain is NUM 1903^T (=JCM 15908^T=DSM 22229^T), isolated from the oral cavity of a donkey. The DNA G+C content of the type strain is 50.2 mol%.

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References

- Downes, J., Hooper, S. J., Wilson, M. J. & Wade, W. G. (2008). *Prevotella histicola* sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol* **58**, 1788–1791.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Hirasawa, M. & Takada, K. (1994). *Porphyromonas gingivicanis* sp. nov. and *Porphyromonas crevioricanis* sp. nov., isolated from beagles. *Int J Syst Bacteriol* **44**, 637–640.
- Hirasawa, M., Kiyono, H., Shiota, T., Michalek, S. M. & McGhee, J. (1980). Virulence of *Prevotella mutans*: immunochemical characterization of a serotype g-defective mutant (C307). *Infect Immun* **27**, 697–699.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Könönen, E., Jousimies-Somer, H. & Asikainen, S. (1994). The most frequently isolated gram-negative anaerobes in saliva and subgingival samples taken from young women. *Oral Microbiol Immunol* **9**, 126–128.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sakamoto, M., Huang, Y., Umeda, M., Ishikawa, I. & Benno, Y. (2005). *Prevotella multiformis* sp. nov., isolated from human subgingival plaque. *Int J Syst Evol Microbiol* **55**, 815–819.
- Slots, J., Bragd, L., Wikström, M. & Dahlén, G. (1986). The occurrence of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* in destructive periodontal disease in adults. *J Clin Periodontol* **13**, 570–577.
- Takada, K. & Hirasawa, M. (2007). *Streptococcus orisui* sp. nov., isolated from the pig oral cavity. *Int J Syst Evol Microbiol* **57**, 1272–1275.
- Takada, K., Wyszomirska, J. & Shiota, T. (1984). Serological characterization of *Streptococcus mutans* serotype polysaccharide g and its different molecular weight forms. *Infect Immun* **45**, 464–469.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.