Prevotella multiformis sp. nov., isolated from human subgingival plaque

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Four bacterial strains isolated from the human oral cavity, PPPA19, PPPA21^T, PPPA28 and PPPA30, were characterized by determining phenotypic and biochemical features, cellular fatty acid profiles and the phylogenetic position based on 16S rRNA gene sequence analysis. 16S rRNA gene sequence analysis showed that each of the isolates was a member of the genus *Prevotella*. These strains were related to *Prevotella denticola* with about 95 % similarity. The strains were obligately anaerobic, non-pigmented, non-spore-forming, non-motile, Gram-negative rods. However, the cells of these strains were often cocci (coccobacilli), depending on the cultivation time. Colonies of different sizes were detected on Eggerth Gagnon agar plates for these strains. The cells forming large colonies were cocci, whereas those forming small colonies were cocci and rods. However, 16S rRNA gene sequence comparison of colonies of different sizes revealed that only a single organism was present. Although these strains had phenotypic characteristics that were similar to those of *P. denticola* JCM 8528, they could be differentiated from *P. denticola* JCM 8528 by aesculin hydrolysis and D-cellobiose fermentation in API 20A tests. DNA–DNA hybridization experiments revealed the genomic distinction of these four strains with respect to *P. denticola* JCM 8528. On the basis of these data, a novel *Prevotella* species, *Prevotella multiformis* sp. nov., is proposed, with PPPA21^T (=JCM 12541^T=DSM 16608^T) as the type strain.

During a study of novel oral phylotypes associated with periodontitis (Sakamoto *et al.*, 2002a), we isolated, from the human oral cavity, a number of strains that were obligately anaerobic, non-pigmented, non-spore-forming, non-motile, Gram-negative rods. These isolates were phenotypically related to the genus *Prevotella*. *Prevotella* species are mainly isolated from the oral cavity, and some species are associated with periodontitis. 16S rRNA gene sequence analysis showed that each of the isolates was a member of the genus *Prevotella*. These strains were

divided into four groups: group 1 (one strain) was related to *Prevotella loescheii* with about 95 % similarity; group 2 (one strain) was related to *Prevotella oris* with about 94 % similarity; group 3 (four strains) was related to *Prevotella denticola* with 95 % similarity; and group 4 (six strains) was related to *Prevotella dentalis* with 89 % similarity. We have already proposed that groups 1 and 2 should be classified as two novel species of the genus *Prevotella*, *Prevotella shahii* and *Prevotella salivae*, respectively (Sakamoto *et al.*, 2004). The present study was designed to determine the taxonomic status of group 3. On the basis of the results presented here, we propose that these strains should be classified as a novel species of the genus *Prevotella*, *Prevotella multiformis* sp. nov.

The strains used in the present study were maintained on Eggerth Gagnon (EG) agar (Merck) supplemented with 5 % (v/v) horse blood for 2 days at 37 °C in an atmosphere containing 100 % CO₂. Strains PPPA19, PPPA21^T, PPPA28 and PPPA30 were isolated, on EG agar, from subgingival plaque from a patient with chronic periodontitis. Growth of the isolates on Bacteroides bile aesculin agar (Shah, 1992) was tested.

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Abbreviations: G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *P. multiformis* strains PPPA21^T, PPPA19, PPPA28 and PPPA30 are AB182483–AB182486.

Photomicrographs of *P. multiformis* cells from EG agar, an extended phylogenetic tree and tables of phenotypic and biochemical characteristics and cellular fatty acid compositions are available as supplementary material in IJSEM Online.

Physiological reactions were determined with an API 20A anaerobic test kit in duplicate as recommended by the manufacturer (bioMérieux). The metabolic end-products were prepared as described previously (Holdeman *et al.*, 1977) and were analysed by GLC (GC-7A; Shimadzu) using a 2.1 m glass column (2.8 mm, FAL-M 25 %, Chromosorb W, 80/100 mesh, AW-DMCS H₃PO₄; Shimadzu). Fatty acid methyl esters were obtained from about 40 mg wet cells by saponification, methylation and extraction using minor modifications (Kuykendall *et al.*, 1988) of the method of Miller (1982). Cellular fatty acid profiles were determined by using the MIDI microbial identification system (Microbial ID). Isoprenoid quinones were extracted as described previously by Komagata & Suzuki (1987) and were analysed by HPLC with a Cosmosil 5C₁₈ column (4.6 × 150 mm; Nacalai Tesque). Glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), malate dehydrogenase and glutamate dehydrogenase activities were determined spectrophotometrically as described previously (Gharbia & Shah, 1991; Bailey & Love, 1995). API ZYM and API An-Ident enzymic substrate tests were performed in duplicate as recommended by the manufacturer (bioMérieux). Chromosomal DNA was isolated by using previously described methods (Marmur, 1961; Saito & Miura, 1963), with some modifications. The DNA G + C content was determined by using the HPLC method of Tamaoka & Komagata (1984), with some modifications. DNA–DNA hybridization experiments were carried out in microplate wells, as described by Ezaki *et al.* (1989). Hybridization was performed at 45 °C for 16 h. The 16S rRNA gene was analysed as described previously (Sakamoto *et al.*, 2002b). The previously determined 16S rRNA gene sequences used for comparisons in this study were retrieved from the DDBJ, EMBL and GenBank nucleotide sequence databases. Sequence data were aligned using the CLUSTAL W program (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. The phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). Bootstrap resampling analysis (Felsenstein, 1985) was performed to estimate the confidence of tree topologies.

Strains PPPA19, PPPA21^T, PPPA28 and PPPA30 were obligately anaerobic, non-spore-forming, non-motile, Gram-negative rods or cocci (coccobacilli). Cells on EG agar were 0.5–0.8 × 1.6–6.6 µm or 0.8 × 0.9–1.0 µm and occurred singly (see Supplementary Fig. A available in IJSEM Online). Colonies were 1–2 mm in diameter, grey to light brown, circular, entire, slightly convex and smooth on EG agar plates. Colonies of different sizes were detected on EG agar plates for all four strains. The cells forming large colonies were cocci, whereas those forming small colonies were cocci and rods. These four strains did not hydrolyse aesculin on Bacteroides bile aesculin agar, whereas *P. loescheii* JCM 8530^T, *Prevotella oralis* JCM 6330, *P. oris* JCM 8540^T, *P. salivae* JCM 12084^T and *Prevotella veroralis*

JCM 6290^T hydrolysed aesculin on the same agar plates. Growth of the isolates and other *Prevotella* species was inhibited on Bacteroides bile aesculin agar. The results of phenotypic tests are given in the species description below, while the phenotypic characteristics of the other *Prevotella* species are available in Supplementary Table A. The phenotypic characteristics of strains PPPA19, PPPA21^T, PPPA28 and PPPA30 were similar to those of *P. denticola* JCM 8528. These strains could be differentiated from *P. denticola* JCM 8528 by aesculin hydrolysis and D-cellobiose fermentation in API 20A tests.

The cellular fatty acid composition of *Bacteroides* species has been determined (Mayberry *et al.*, 1982; Miyagawa *et al.*, 1979; Shah & Collins, 1980) and was reviewed for the classification of the genus *Bacteroides* (Shah & Collins, 1983). In this study, the cellular fatty acid compositions of strains PPPA19, PPPA21^T, PPPA28 and PPPA30 were found to be very similar to that of *P. denticola* JCM 8528. These four strains and *P. denticola* JCM 8528 contain greater relative amounts of anteiso-15:0 (≥ 30 %) than do other *Prevotella* species. The findings are summarized in the species description. In addition, the cellular fatty acid compositions of other *Prevotella* species are available in Supplementary Table B.

The major menaquinones of the clinical isolates and the other *Prevotella* species were MK-10 and MK-11. This result supports findings reported previously (Shah & Collins, 1980, 1983). In our study, only the clinical isolates possessed MK-13 (Table 1).

The dehydrogenase enzyme pattern is an important criterion that differentiates the genus *Bacteroides sensu stricto* from other Gram-negative, anaerobic, non-spore-forming rods (Shah & Collins, 1989; Shah, 1992). *Bacteroides fragilis* and related species possess enzymes of the hexose monophosphate shunt/pentose phosphate pathway, such as G6PDH, 6PGDH, malate dehydrogenase and glutamate dehydrogenase. Shah & Collins (1988, 1990) proposed the new genera *Porphyromonas* and *Prevotella* because of the lack of particular enzymes (G6PDH and 6PGDH). In the present study, G6PDH and 6PGDH activities were not detected in extracts of the clinical isolates or the other *Prevotella* species. On the other hand, both malate dehydrogenase and glutamate dehydrogenase activities were detected in extracts of all strains tested.

The API ZYM and API An-Ident systems have been reported to be useful in the identification of oral and non-oral Gram-negative bacteria (Laughon *et al.*, 1982; Slots, 1981; Tanner *et al.*, 1985). In addition, the RAPID-ANA system (Innovative Diagnostics Systems) has been reported to be helpful in the identification of some phenotypically similar bile-inhibited *Bacteroides* species (Dellinger & Moore, 1986). The biochemical characteristics of the clinical isolates and the other *Prevotella* species are available in Supplementary Table C. All strains were tested with API ZYM and API An-Ident. The biochemical

Table 1. Menaquinone compositions of strains of *P. multiformis* sp. nov. and related species

Values are percentages of total menaquinones.

Strain	MK-8	MK-9	MK-10	MK-11	MK-12	MK-13
<i>Prevotella multiformis</i> PPA19		1	9	55	30	2
<i>Prevotella multiformis</i> PPPA21 ^T		1	9	54	31	3
<i>Prevotella multiformis</i> PPA28	1	1	12	49	29	3
<i>Prevotella multiformis</i> PPPA30		1	8	55	31	3
<i>Prevotella bivia</i> JCM 6331 ^T		5	70	23		
<i>Prevotella corporis</i> JCM 8529 ^T			5	74	20	
<i>Prevotella denticola</i> JCM 8528			6	61	32	
<i>Prevotella loescheii</i> JCM 8530 ^T			100			
<i>Prevotella melaninogenica</i> JCM 6325 ^T			61	39		
<i>Prevotella nigrescens</i> JCM 6322 ^T			22	56	22	
<i>Prevotella oralis</i> JCM 6330	2	3	36	47	8	
<i>Prevotella oris</i> JCM 8540 ^T			17	57	26	
<i>Prevotella salivae</i> JCM 12084 ^T			13	67	20	
<i>Prevotella shahii</i> JCM 12083 ^T	1	1	18	58	22	
<i>Prevotella veroralis</i> JCM 6290 ^T			49	44	6	

characteristics of strains PPA19, PPPA21^T, PPA28 and PPPA30 were similar to those of *Prevotella bivia* JCM 6331^T and *P. denticola* JCM 8528. However, these four strains could be differentiated from *P. bivia* JCM 6331^T and *P. denticola* JCM 8528 by API An-Ident tests. α -Galactosidase and arginine and glycine aminopeptidase activities were helpful in the differentiation of these species (Table 2).

The DNA G+C contents of strains PPA19, PPPA21^T, PPA28 and PPPA30 were in the range 51.1–51.3 mol%. These values are slightly higher than that of *P. denticola* JCM 8528, which was phenotypically related to the clinical isolates (Table 2). The levels of DNA relatedness among the isolates were above 81 % (81–98 %). The levels of DNA relatedness observed demonstrate the genomic distinction

Table 2. Differential characteristics of *P. multiformis* sp. nov. and some related *Prevotella* species

Species: 1, *P. multiformis* sp. nov.; 2, *P. bivia*; 3, *P. denticola*; 4, *P. melaninogenica*. Symbols: +, positive; –, negative; w, weak. v, variable.

Characteristic	1	2	3	4
Aesculin hydrolysis	–	–	+	–
API 20A:				
Sucrose	+	–	+	+
Glycerol	+	–	+	–
D-Cellobiose	+	–	–	–
D-Raffinose	+	–	+	+
API ZYM:				
α -Galactosidase	–	–	–	w
β -Galactosidase	+	+	w	+
α -Fucosidase	v	+	w	+
API An-Ident:				
β -Glucosidase	–	–	–	w
α -Galactosidase	–	w	w	+
β -Galactosidase	+	+	w	+
Arginine aminopeptidase	–	–	+	+
Histidine aminopeptidase	–	–	–	+
Glycine aminopeptidase	–	+	–	–
Predominant menaquinones	MK-10, MK-11, MK-12, MK-13	MK-9, MK-10, MK-11	MK-10, MK-11, MK-12	MK-10, MK-11
DNA G+C content (mol%)	51.1–51.3	40.0	50.5	41.1

Fig. 1. Phylogenetic tree showing the relationship between *P. multiformis* sp. nov. and related species. The tree was constructed by the neighbour-joining method based on 16S rRNA gene sequences. Numbers at nodes indicate bootstrap values for each node, out of 100 bootstrap resamplings. Bar, 0.01 substitutions per nucleotide position. An extended neighbour-joining tree is available as Supplementary Fig. B in IJSEM Online.

of these four strains from *P. denticola* JCM 8528 (relatively low DNA relatedness, $\leq 28\%$; Supplementary Table D).

Approximately 1500 bases of the 16S rRNA gene sequence were determined for each of the isolates (strains PPA19, PPA21^T, PPA28 and PPA30). For the phylogenetic analysis, 1379 bp (positions 34–1391, *Escherichia coli* numbering system) sequences of each species were used. 16S rRNA gene sequence analysis showed that each of the isolates was a species of the genus *Prevotella* (Fig. 1 and Supplementary Fig. B). These four strains were related to *P. denticola* with about 95% similarity. The levels of sequence similarity among the four strains were above 99.7% (99.7–100%). In addition, the isolates were related to *Prevotella* sp. oral clone AO036 (Paster *et al.*, 2001) with about 96% similarity. Moreover, the 16S rRNA gene sequences were determined for the different sizes of colony for each isolate. 16S rRNA gene sequence analysis revealed no differences between the cells forming the large and small colonies. Furthermore, the phenotypic and biochemical characteristics (determined using API 20A, API ZYM and API An-Ident) of colonies of different sizes were found to be the same. These findings indicated that only a single micro-organism was present.

On the basis of the above-mentioned findings and the 16S rRNA gene sequence analysis, we propose a novel *Prevotella* species, *Prevotella multiformis* sp. nov., for these four strains. Differential characteristics of *Prevotella multiformis* sp. nov. and some related *Prevotella* species are shown in Table 2.

Description of *Prevotella multiformis* sp. nov.

Prevotella multiformis (mul.ti.for'mis. L. fem. adj. *multiformis* many-shaped, multiform).

Cells are obligately anaerobic, non-spore-forming, non-motile, Gram-negative rods ($0.5\text{--}0.8 \times 1.6\text{--}6.6\ \mu\text{m}$) or cocci (coccobacilli) ($0.8 \times 0.9\text{--}1.0\ \mu\text{m}$). Colonies are 1–2 mm in diameter, grey to light brown, circular, entire, slightly convex and smooth on EG agar plates. Colonies of different sizes are detected on EG agar plates. Cells forming large colonies are cocci, whereas those forming small colonies are cocci and rods. Growth is inhibited in the presence of 20% (w/v) bile. Acid is produced from D-cellobiose, glucose, glycerol, lactose, maltose, D-mannose, D-raffinose and sucrose. Acid is not produced from L-arabinose, D-mannitol, D-melezitose, D-rhamnose, salicin, D-sorbitol, D-trehalose or D-xylose. Aesculin is not hydrolysed. Indole is not produced. Gelatin is digested. Catalase and urease are not produced. The major end-products [from 1% (w/v) peptone/1% (w/v) yeast extract/1% (w/v) glucose broth cultures] are succinic and acetic acids; small amounts of isovaleric acid are also produced. Malate dehydrogenase and glutamate dehydrogenase are present, whereas G6PDH and 6PGDH are absent. Both non-hydroxylated and 3-hydroxylated long-chain fatty acids are present. The major cellular fatty acids are anteiso-15:0, iso-15:0, iso-3-OH-17:0 and 18:1 ω 9c. The principal respiratory quinones are menaquinones MK-11 (49–55%) and MK-12 (29–31%). Minor menaquinones are MK-8 (1%), MK-9 (1%), MK-10 (8–12%) and MK-13 (2–3%). The DNA G + C content of the type strain is 51.1 mol%.

The type strain is strain PPA21^T (=JCM 12541^T=DSM 16608^T), which was isolated from subgingival plaque from a patient with chronic periodontitis.

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References

- Bailey, G. D. & Love, D. N. (1995). Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities and glucose utilization by species within the genera *Bacteroides*, *Prevotella*, and *Porphyromonas*. *Int J Syst Bacteriol* **45**, 246–249.
- Dellinger, C. A. & Moore, L. V. H. (1986). Use of the RapID-ANA system to screen for enzyme activities that differ among species of bile-inhibited *Bacteroides*. *J Clin Microbiol* **23**, 289–293.
- 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 of phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Gharbia, S. E. & Shah, H. N. (1991). Pathways of glutamate catabolism among *Fusobacterium* species. *J Gen Microbiol* **137**, 1201–1206.
- Holdeman, L. V., Cato, E. P. & Moore, W. E. C. (1977). *Anaerobic Laboratory Manual*, 4th edn. Blacksburg, VA: Virginia Polytechnic Institute and State University.
- 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.
- Komagata, K. & Suzuki, K. (1987). Lipid and cell-wall analysis in bacterial systematics. *Methods Microbiol* **19**, 161–207.
- Kuykendall, L. D., Roy, M. A., O'Neill, J. J. & Devine, T. E. (1988). Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. *Int J Syst Bacteriol* **38**, 358–361.
- Laughon, B. E., Syed, S. A. & Loesche, W. J. (1982). API ZYM system for identification of *Bacteroides* spp., *Capnocytophaga* spp., and spirochetes of oral origin. *J Clin Microbiol* **15**, 97–102.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Mayberry, W. R., Lambe, D. W., Jr & Ferguson, K. P. (1982). Identification of *Bacteroides* species by cellular fatty acid profiles. *Int J Syst Bacteriol* **32**, 21–27.
- Miller, L. T. (1982). Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. *J Clin Microbiol* **16**, 584–586.
- Miyagawa, E., Azuma, R. & Suto, T. (1979). Cellular fatty acid composition in gram-negative obligately anaerobic rods. *J Gen Appl Microbiol* **25**, 41–51.
- Paster, B. J., Boches, S. K., Galvin, J. L., Ericson, R. E., Lau, C. N., Levanos, V. A., Sahasrabudhe, A. & Dewhirst, F. E. (2001). Bacterial diversity in human subgingival plaque. *J Bacteriol* **183**, 3770–3783.
- Saito, H. & Miura, K. (1963). Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim Biophys Acta* **72**, 619–629.
- 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. (2002a). Detection of novel oral phylotypes associated with periodontitis. *FEMS Microbiol Lett* **217**, 65–69.
- Sakamoto, M., Suzuki, M., Umeda, M., Ishikawa, I. & Benno, Y. (2002b). Reclassification of *Bacteroides forsythus* (Tanner et al. 1986) as *Tannerella forsythensis* corrig., gen. nov., comb. nov. *Int J Syst Evol Microbiol* **52**, 841–849.
- Sakamoto, M., Suzuki, M., Huang, Y., Umeda, M., Ishikawa, I. & Benno, Y. (2004). *Prevotella shahii* sp. nov. and *Prevotella salivae* sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol* **54**, 877–883.
- Shah, H. N. (1992). The genus *Bacteroides* and related taxa. In *The Prokaryotes*, 2nd edn, pp. 3593–3607. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.
- Shah, H. N. & Collins, M. D. (1980). Fatty acid and isoprenoid quinone composition in the classification of *Bacteroides melaninogenicus* and related taxa. *J Appl Bacteriol* **48**, 75–87.
- Shah, H. N. & Collins, M. D. (1983). Genus *Bacteroides*. A chemotaxonomical perspective. *J Appl Bacteriol* **55**, 403–416.
- Shah, H. N. & Collins, M. D. (1988). Proposal for reclassification of *Bacteroides asaccharolyticus*, *Bacteroides gingivalis*, and *Bacteroides endodontalis* in a new genus, *Porphyromonas*. *Int J Syst Bacteriol* **38**, 128–131.
- Shah, H. N. & Collins, M. D. (1989). Proposal to restrict the genus *Bacteroides* (Castellani and Chalmers) to *Bacteroides fragilis* and closely related species. *Int J Syst Bacteriol* **39**, 85–87.
- Shah, H. N. & Collins, M. D. (1990). *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. *Int J Syst Bacteriol* **40**, 205–208.
- Slots, J. (1981). Enzymatic characterization of some oral and nonoral gram-negative bacteria with the API ZYM system. *J Clin Microbiol* **14**, 288–294.
- Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Tanner, A. C. R., Strzempko, M. N., Belsky, C. A. & McKinley, G. A. (1985). API ZYM and API An-Ident reactions of fastidious oral gram-negative species. *J Clin Microbiol* **22**, 333–335.
- 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.