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

Mitsuo Sakamoto,¹ Makoto Umeda,² Isao Ishikawa² and Yoshimi Benno¹

Correspondence

Mitsuo Sakamoto
sakamoto@jcm.riken.jp

¹Microbe Division/Japan Collection of Microorganisms, RIKEN BioResource Center, Wako, Saitama 351-0198, Japan

²Division of Periodontology, Department of Hard Tissue Engineering, Graduate School, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113-8549, Japan

Six bacterial strains isolated from the human oral cavity, PPPA16, PPPA20^T, PPPA24, PPPA31, EPPA6 and EPPA7, were characterized by determining phenotypic and biochemical features, cellular fatty acid profiles, menaquinone profiles and phylogenetic position based on 16S rRNA gene sequence analysis. 16S rRNA gene sequence analysis showed that the isolates represented the same species of the genus *Prevotella*. The strains were related to *Prevotella dentalis* with about 89 % similarity. In addition, the isolates were related to *Prevotella* sp. oral clone IDR-CEC-0032, which is a representative of the numerically dominant cluster VI in carious dentine lesions [Nadkarni *et al.* (2004). *J Clin Microbiol* **42**, 5238–5244], with about 99 % similarity. The strains were obligately anaerobic, non-pigmenting, non-spore-forming, non-motile, Gram-negative rods. The isolates could be differentiated from other *Prevotella* species by D-mannitol, D-melezitose, D-sorbitol and D-trehalose fermentation in API 20A tests. The cellular fatty acid composition of strains PPPA16, PPPA20^T, PPPA24, PPPA31, EPPA6 and EPPA7 was significantly different from that of other *Prevotella* species. Compared with other *Prevotella* species, only these six strains contained dimethyl acetals. The major menaquinones of the clinical isolates were MK-12 and MK-13, whereas the major menaquinones of other *Prevotella* species were MK-10 and MK-11. On the basis of these data, a novel *Prevotella* species, *Prevotella multisaccharivorax* sp. nov., is proposed, with PPPA20^T (= JCM 12954^T = DSM 17128^T) as the type strain.

Although attempts have been made to isolate novel oral phylotypes (Sakamoto *et al.*, 2002a) associated with periodontitis, such attempts have not yet been successful. In the process of this research, we isolated, from the human oral cavity, a number of strains that were obligately anaerobic, non-pigmenting, non-spore-forming, non-motile, Gram-negative rods. These isolates were provisionally identified as belonging to the genus *Prevotella*. *Prevotella* species are mainly isolated from the oral cavity, and some species are associated with periodontitis. In addition, it was reported recently that advanced dental caries provides a unique environment for a complex array of novel and uncultured

Prevotella and *Prevotella*-like bacteria (Nadkarni *et al.*, 2004). *Prevotella* species have also been isolated from the genitourinary tract, vagina and cervix. 16S rRNA gene sequence analysis has previously shown that each isolate was a member of the genus *Prevotella* and that the strains could be divided into four groups. Among these groups, we have already proposed that groups 1, 2 and 3 should be classified as representing three novel species of the genus *Prevotella*, *Prevotella shahii* sp. nov. (Sakamoto *et al.*, 2004), *Prevotella salivae* sp. nov. (Sakamoto *et al.*, 2004) and *Prevotella multiformis* sp. nov. (Sakamoto *et al.*, 2005), respectively. Group 4 (6 strains) was related to *Prevotella dentalis* with 89 % similarity. The present study was designed to determine the taxonomic status of this group. On the basis of the results presented here, we propose that the strains of group 4 should be classified as representing a novel species of the genus *Prevotella*, *Prevotella multisaccharivorax* 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 PPPA16, PPPA20^T, PPPA24, PPPA31, EPPA6 and EPPA7 were isolated, on EG agar, from

Published online ahead of print on 15 April 2005 as DOI 10.1099/ij.s.0.63739-0.

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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *P. multisaccharivorax* strain PPPA20^T is AB200414.

Phenotypic characteristics, cellular fatty acid composition and API ZYM and API An-Ident test results are available as supplementary material in IJSEM Online.

subgingival plaque of patients with chronic periodontitis. *Bacteroides* bile aesculin agar (Shah, 1992) was used to check whether the growth of the isolates was inhibited on this medium.

Physiological reactions were determined with an API 20A anaerobe test kit in duplicate, as recommended by the manufacturer (bioMérieux). The metabolic end-products were prepared as described by Holdeman *et al.* (1977), and analysed as described previously (Sakamoto *et al.*, 2004, 2005). 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 using the MIDI microbial identification system (Microbial ID). Isoprenoid quinones were extracted as described by Komagata & Suzuki (1987), and analysed as described previously (Sakamoto *et al.*, 2004, 2005). Glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), malate dehydrogenase and glutamate dehydrogenase activities were determined spectrophotometrically, as described by Gharbia & Shah (1991) and 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 as described by Marmur (1961) and Saito & Miura (1963), with some modifications. The DNA base composition was determined by using the HPLC method of Tamaoka & Komagata (1984), with some modifications. The 16S rRNA gene was analysed as described previously (Sakamoto *et al.*, 2002b). Related sequences were aligned with 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 using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap resampling analysis (Felsenstein, 1985) was performed to estimate the confidence of tree topologies.

Strains PPPA16, PPPA20^T, PPPA24, PPPA31, EPPA6 and EPPA7 were obligately anaerobic, non-spore-forming, non-motile, Gram-negative rods. Cells on EG agar were 0.8 × 2.5–8.3 µm in size and occurred singly. Colonies were 0.5–0.7 mm in diameter, grey to off-white-grey, circular, entire, slightly convex and smooth on EG agar plates. Growth of these six strains and other *Prevotella* species was inhibited on *Bacteroides* bile aesculin agar. The phenotypic characteristics are given in the species description (phenotypic characteristics of other *Prevotella* species are available as Supplementary Table S1 in IJSEM Online). The isolates could be differentiated from other *Prevotella* species by D-mannitol, D-melezitose, D-sorbitol and D-trehalose 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 reviewed for the classification of the genus *Bacteroides* (Shah & Collins, 1983). In this study, the

cellular fatty acid composition of strains PPPA16, PPPA20^T, PPPA24, PPPA31, EPPA6 and EPPA7 was found to be significantly different from those of other *Prevotella* species. Compared with other *Prevotella* species, only these six strains contained dimethyl acetals. The summarized findings are given in the species description. In addition, the cellular fatty acid compositions of other *Prevotella* species are available as Supplementary Table S2 in IJSEM Online.

The major menaquinones of the clinical isolates were MK-12 and MK-13 (Table 1). In contrast, the major menaquinones of other *Prevotella* species were MK-10 and MK-11 (Sakamoto *et al.*, 2005).

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 the 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 tested. On the other hand, malate dehydrogenase and glutamate dehydrogenase activities were both 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 other *Prevotella* species are available as Supplementary Table S3 in IJSEM Online. All strains were tested using API ZYM and API An-Ident.

The DNA G+C contents of strains PPPA16, PPPA20^T, PPPA24, PPPA31, EPPA6 and EPPA7 were 48.5–49.9 mol%.

Table 1. Menaquinone composition of strains of *Prevotella multisaccharivorax* sp. nov.

Strains: 1, PPPA16; 2, PPPA20^T; 3, PPPA24; 4, PPPA31; 5, EPPA6; 6, EPPA7. ND, Not detected; tr, trace amount. Values are percentages of total menaquinones.

Menaquinone	1	2	3	4	5	6
MK-10	ND	2	1	2	2	3
MK-11	tr	8	9	10	10	8
MK-12	55	42	41	45	45	40
MK-13	45	43	41	40	40	40

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

Taxa: 1, *P. multisaccharivorax* sp. nov.; 2, *P. baroniae* (data from Downes *et al.* 2005); 3, *P. dentalis* (Haapasalo *et al.* 1986, Willems & Collins, 1995); 4, *P. melaninogenica*. +, Positive; –, negative; NT, not tested; V, variable.

Characteristic	1	2	3	4
Aesculin hydrolysis	+	+	+	–
Acid produced from:				
D-Mannitol	+	–	–	–
Salicin	V	+	–	–
D-Xylose	+	–	–	–
L-Arabinose	V	–	+	–
Glycerol	+	NT	NT	–
D-Cellobiose	+	+	+	–
D-Melezitose	+	–	–	–
D-Sorbitol	+	–	NT	–
D-Rhamnose	+	–	–	–
D-Trehalose	+	–	NT	–
Enzyme activities:				
α -Galactosidase	–	+	+	+
β -Galactosidase	V	+	+	+
β -Glucosidase	V	+	+	V
α -Arabinofuranosidase	+	–	+	–
α -Fucosidase	–	+	–	+
Arginine aminopeptidase	+	–	–	+
Histidine aminopeptidase	–	–	–	+
Major cellular fatty acids	C _{18:1ω9c} , C _{16:0} , C _{16:0} dimethyl aldehyde	i-C _{15:0} , ai-C _{15:0} , C _{16:0} , ai-C _{17:0}	C _{16:0} 3-OH, C _{16:0} , i-C _{14:0}	ai-C _{15:0} , i-C _{17:0} 3-OH, C _{18:1ω9c}
Predominant menaquinones	MK-12, MK-13	NT	–	MK-10, MK-11
DNA G + C content (mol%)	48.5–49.9	52	56–60	41.1

These values are somewhat lower than those of *Prevotella baroniae* (Downes *et al.*, 2005) and *P. dentalis*, which were phylogenetically related (see below) to the clinical isolates (Table 2).

Approximately 1500 bases of the 16S rRNA gene sequence were determined for each isolate (strains PPPA16, PPPA20^T, PPPA24, PPPA31, EPPA6 and EPPA7). 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 the isolates represented the same species of the genus *Prevotella* (Fig. 1). The level of sequence similarity among the six strains was 100 %. The six strains were related to *P. dentalis* with about 89 % similarity. Although the level of sequence divergence (approximately 11 %) is somewhat high, this level of sequence similarity is similar to the values found when various *Prevotella* species were compared (Willems & Collins, 1995). In addition, the six strains were related to *P. baroniae*, a species that was proposed recently by Downes *et al.* (2005), with about 86 % similarity. Furthermore, the isolates were related to *Prevotella* sp.

Fig. 1. Phylogenetic tree showing the relationship between *Prevotella multisaccharivorax* sp. nov. and related species. The tree was constructed by using 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. GenBank accession numbers for the 16S rRNA gene sequences are given in parentheses.

oral clone IDR-CEC-0032 (Nadkarni *et al.*, 2004) with about 99% similarity. Nadkarni *et al.* (2004) suggested that the numerically dominant cluster VI in the carious dentine lesions, which contains *Prevotella* sp. oral clone IDR-CEC-0032, represents a novel genus, which was most closely related to uncharacterized *Prevotella*-like rumen bacteria (93–94% similarity). However, there is no simple way of subdividing the genus *Prevotella* at the present time.

Therefore, on the basis of the above-mentioned findings and the 16S rRNA gene sequence analysis, a novel *Prevotella* species, *Prevotella multisaccharivorax* sp. nov., is proposed for these six strains. Differential characteristics of *P. multisaccharivorax* sp. nov. and some related *Prevotella* species are given in Table 2. One significant difference is the absence of menaquinones in *P. dentalis*.

Description of *Prevotella multisaccharivorax* sp. nov.

Prevotella multisaccharivorax (mul.ti.sac.cha.ri.vo'rax. L. adj. *multus* many/much; L. n. *saccharum* sugar; L. adj. *vorax* liking to eat; N.L. part. adj. *multisaccharivorax* liking to eat many sugars).

Cells are obligately anaerobic, non-spore-forming, non-motile, Gram-negative rods ($0.8 \times 2.5\text{--}8.3\ \mu\text{m}$). Colonies are 0.5–0.7 mm in diameter, grey to off-white-grey, circular, entire, slightly convex and smooth on EG agar plates. Growth is inhibited in the presence of 20% (w/v) bile. Acid is produced from L-arabinose (variable), D-cellobiose, glucose, glycerol, lactose, maltose, D-mannitol, D-mannose, D-melezitose, D-raffinose, D-rhamnose, salicin (variable), D-sorbitol, sucrose, D-trehalose and D-xylose. Aesculin is 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 $C_{18:1\omega 9c}$ and $C_{16:0}$. Dimethyl acetals (mainly $C_{16:0}$ dimethyl aldehyde) are present. The principal respiratory quinones are menaquinones MK-12 (40–55%) and MK-13 (40–45%). Minor menaquinones are MK-10 (1–3%) and MK-11 (8–10%). The G+C content of the type strain is 49.9 mol%.

The type strain is PPPA20^T (= JCM 12954^T = DSM 17128^T), isolated from subgingival plaque from patients with chronic periodontitis.

Acknowledgements

We are grateful to Professor Dr H. G. Trüper, University of Bonn, Germany, for his suggestions regarding nomenclature. This work was

supported in part by a Grant-in-Aid for Scientific Research (no. 13672202) from the Japan Society for the Promotion of Science (to M. S.).

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.
- Downes, J., Sutcliffe, I., Tanner, A. C. R. & Wade, W. G. (2005). *Prevotella marshii* sp. nov. and *Prevotella baroniae* sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol* **55**, 1551–1555.
- Felsenstein, J. (1985). Confidence limits on 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.
- Haapasalo, M., Ranta, H., Shah, H., Ranta, K., Lounatmaa, K. & Kroppenstedt, R. M. (1986). *Mitsuokella dentalis* sp. nov. from dental root canals. *Int J Syst Bacteriol* **36**, 566–568.
- Holdeman, L. V., Cato, E. P. & Moore, W. E. C. (1977). *Anaerobe 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.
- Nadkarni, M. A., Caldon, C. E., Chhour, K.-L., Fisher, I. P., Martin, F. E., Jacques, N. A. & Hunter, N. (2004). Carious dentine provides a habitat for a complex array of novel *Prevotella*-like bacteria. *J Clin Microbiol* **42**, 5238–5244.
- 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.
- 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.
- 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, D. M. (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.
- Willems, A. & Collins, M. D. (1995). 16S rRNA gene similarities indicate that *Hallella seregens* (Moore and Moore) and *Mitsuokella dentalis* (Haapasalo et al.) are genealogically highly related and are members of the genus *Prevotella*: emended description of the genus *Prevotella* (Shah and Collins) and description of *Prevotella dentalis* comb. nov. *Int J Syst Bacteriol* **45**, 832–836.