Prevotella nanceiensis sp. nov., isolated from human clinical samples

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Three strains of anaerobic, non-pigmented, Gram-negative bacilli isolated from various human clinical samples were characterized in terms of phenotypic and genotypic tests, including sequence analysis of 16S rRNA and *rpoB* genes. The strains were most closely related to the type strains of *Prevotella marshii* and *Prevotella shahii* on the basis of both 16S rRNA (89.8 and 89.0 % identity, respectively) and *rpoB* gene sequences (83.1 and 82.8 % identity, respectively). Phylogenetic analysis showed that the isolates constituted a robust homogeneous group distinct from known species in the genus *Prevotella*. The *rrn* skeleton (as determined by PFGE) and the DNA G + C content, determined to be 39.4 mol% for strain LBN 293^T, distinguished the novel isolates from the type strains of *P. marshii* and *P. shahii*. The three strains were saccharolytic and produced acetic, lactic and succinic acids as major metabolic end products. Polyphasic investigations supported the proposal of a novel species, *Prevotella nanceiensis* sp. nov., with LBN 293^T (=AIP 261.03^T =CIP 108993^T =CCUG 54409^T) as the type strain.

The genus *Prevotella* includes strictly anaerobic, Gram-negative, moderately saccharolytic, bile-sensitive rods that formerly belonged to the genus *Bacteroides* (Shah & Collins, 1990). These bacteria, which are part of the human oral, intestinal and urogenital flora, may be involved in various infections of the head and neck, lower respiratory tract, central nervous system, abdominal and female genital tract and in bacteraemia (Jousimies-Somer *et al.*, 2002). The use of molecular methods, such as 16S

rRNA gene sequencing, for the identification of anaerobic bacteria has revealed that a significant proportion of strains did not correspond to species with validly published names (Jousimies-Somer, 1997; Jousimies-Somer *et al.*, 2003) and has led to a great expansion of the genus *Prevotella*, with the description of ten novel species within the last 3 years. In this study, three strains of anaerobic Gram-negative rods, isolated from different human clinical samples and affiliated to the genus *Prevotella* by presumptive identification tests, were subjected to a comprehensive range of phenotypic, genotypic, genomic and phylogenetic tests.

Strains LBN 293^T and LBN 297 were both recovered in pure culture. Strain LBN 293^T was isolated in 2003 from blood cultures from a 78-year-old man (Mory *et al.*, 2005) and strain LBN 297 was isolated in 2004 from lung abscess pus from a 67-year-old man. Strain LBN 298 was isolated in 2005 from a broncho-alveolar lavage fluid from a 66-year-old woman. The broncho-alveolar lavage fluid also contained *Pseudomonas aeruginosa* and a coagulase-negative *Staphylococcus* sp. Strains were grown at 36 °C on Brucella agar supplemented with 5 % sheep blood, haemin

Abbreviation: ML, maximum likelihood.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *rpoB* gene sequences determined in this study are respectively AY957555 and EF405531 for *P. nanceiensis* LBN 293^T, EF405529 and EF405532 for *P. nanceiensis* LBN 297 and EF405530 and EF405533 for *P. nanceiensis* LBN 298. Those for the *rpoB* gene sequences of *P. marshii* E9.34^T and *P. shahii* JCM 12083^T are respectively EF405534 and EF405535.

Transmission electron micrographs of cells of strain LBN 293^T and a comparison of its fatty acid profile with those of related type strains are available as supplementary material with the online version of this paper.

and vitamin K₁ (BBA) in an anaerobic chamber (Concept 1000; Ruskinn).

DNA was extracted by using the QIAamp DNA Mini kit (Qiagen). The 16S rRNA gene was amplified by PCR and sequenced as described previously (Carlier *et al.*, 2004). A 374 bp fragment of the gene *rpoB* was amplified using the primer pair Prev3250F (5'-AACCCGTTGGGTGTGCC-3') and Prev3623R (5'-AGIGCCCAACCTCCATCTCTCC-3') (Berger *et al.*, 2005). Nucleotide sequences were analysed by using SeqScape software (version 2.5; Applied Biosystems). The sequences were compared to those deposited in the GenBank and Ribosomal Database Project II databases using the BLAST program (Altschul *et al.*, 1997) and Seqmatch program (Cole *et al.*, 2007), respectively. The isolates displayed the highest 16S rRNA gene sequence similarity to members of the genus *Prevotella*. Maximum similarity (99.4%) was observed with the sequence of *Prevotella* sp. oral clone BI027, obtained from human subgingival plaque (Paster *et al.*, 2001). The 16S rRNA gene sequences were aligned against sequences of all known *Prevotella* type strains and that of *Prevotella* oral clone BI027 using the DIALIGN program (Morgenstern, 2002). The distance matrix constructed using the Similarity table program of the PHYLIP package (Felsenstein, 1993) showed that strains LBN 293^T, LBN 297 and LBN 298 shared more than 99.6% of their 16S

rRNA gene nucleotide positions. The best sequence matches with strains of species with validly published names were obtained with *Prevotella marshii* E9.34^T and *Prevotella shahii* JCM 12083^T, but the similarity levels (89.8 and 89.0%, respectively) were relatively low. The three isolates displayed 100% identity in *rpoB* gene sequences. Maximum similarity was again observed with the sequences of *P. marshii* E9.34^T and *P. shahii* JCM 12083^T (83.1 and 82.8%, respectively). Altogether, the sequencing results suggested that the three strains belonged to a new taxon. This prompted us to investigate the taxonomic position of these strains by a polyphasic approach.

Evolutionary trees were reconstructed using the PHYLIP suite of programs (Felsenstein, 1993) by maximum-parsimony (Kluge & Farris, 1969) and by neighbour-joining (algorithm F84 for substitution model) (Saitou & Nei, 1987; Kishino & Hasegawa, 1989). The robustness of the nodes was evaluated by 1000 bootstrap replications using SEQBOOT and CONSENSE programs (Felsenstein, 1993). The maximum-likelihood (ML) tree was reconstructed using phyML software with GTR (gamma distribution and invariable sites) as the substitution model and 100 bootstrap replications (Guindon & Gascuel, 2003). The 16S rRNA gene-based ML phylogenetic tree is shown in Fig. 1. Nodes labelled by asterisks were found by all three

Fig. 1. ML phylogenetic tree for 16S rRNA gene sequences showing the relationship between the novel strains (*Prevotella nanceiensis* sp. nov.) and the other members of the genus *Prevotella*. The sequence of *Porphyromonas gingivalis* ATCC 33277^T was used as an outgroup. Bootstrap values >50 are indicated at corresponding nodes. Consensus nodes observed in the ML, neighbour-joining and parsimony trees are labelled with asterisks. Sequence accession numbers are in parentheses. Bar, 0.02 substitutions per site.

phylogenetic methods, but other branches moved according to the phylogenetic method. Strains LBN 293^T, LBN 297 and LBN 298 formed a robust homogeneous group distinct from other species of the genus *Prevotella*.

The DNA G + C content of strain LBN 293^T, determined by HPLC at the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), was 39.4 mol%. This value is in accordance with those reported for the genus *Prevotella* (39–60 mol%) (Willems & Collins, 1995; Avguštin *et al.*, 1997) and was clearly distinct from the DNA G + C contents determined for the most closely related species, *P. marshii* (51.0 mol%; Downes *et al.*, 2005) and *P. shahii* (44.3 mol%; Sakamoto *et al.*, 2004).

Large-scale chromosome structure analysis was previously described as a sensitive indicator of phylogenetic relationships between bacteria (Liu *et al.*, 1999; Marchandin *et al.*, 2003a; Jumas-Bilak *et al.*, 2005). Chromosome size and *rrn* skeletons of the three isolates were studied in comparison with *P. marshii* DSM 16973^T and *P. shahii* DSM 15611^T using PFGE, as described previously (Jumas-Bilak *et al.*, 1998; Marchandin *et al.*, 2003a, b). All three strains studied possess a unique and circular chromosome (data not shown). The chromosome of the novel strains was 3.02 to 3.09 Mb in size, similar to the *P. marshii* chromosome (2.95 Mb) but clearly distinct from the *P. shahii* chromosome (4.01 Mb). I-CeuI profiles showed that all the strains tested possess four *rrn* operons (Fig. 2). The *rrn* skeleton distinguished the novel strains from their most closely related phylogenetic neighbours, *P. marshii* and *P. shahii*, each species exhibiting a specific I-CeuI profile (Fig. 2).

The strains were obligately anaerobic, non-spore-forming, non-motile, Gram-negative coccoid and short rods. After

Fig. 2. PFGE migration of I-CeuI-restricted DNAs. Lanes: 1, *P. nanceiensis* LBN 298; 2, *P. nanceiensis* LBN 297; 3, *P. nanceiensis* LBN 293^T; 4, *P. shahii* DSM 15611^T; 5, *P. marshii* DSM 16973^T. Lane Sc, *Saccharomyces cerevisiae* chromosomes used as molecular size marker. Sizes are indicated in kilobases.

4 days incubation on BBA, colonies were 0.5–1 mm in diameter, circular, entire, slightly convex and smooth, white, non-pigmented and surrounded by a β -haemolysis zone. For examination of the cell-wall ultrastructure of strain LBN 293^T, cells were prepared as described previously (Carlier *et al.*, 2004) and electron photomicrographs were taken with a JEOL 1010 transmission electron microscope operating at 80 kV. Rods, 0.4–0.5 $\times$ 0.5–1.0 μ m in size, were observed. Transmission electron microscope examination of ultrathin sections of strain LBN 293^T showed the presence of a typical Gram-negative cell wall composed of a thin peptidoglycan layer surrounded by an outer membrane (see Supplementary Fig. S1 in IJSEM Online).

All strains were susceptible to bile (1 mg tablet) and resistant to kanamycin (1 mg disc), vancomycin (5 μ g tablet) and colistin (10 μ g tablet).

Biochemical tests were performed in triplicate using the API 20A anaerobe identification kit (bioMérieux) as recommended by the manufacturer. Xylan fermentation was determined in trypticase/yeast extract broth as recommended by Holdeman *et al.* (1977). Metabolic end products were assayed by quantitative gas chromatography as described previously (Carlier, 1985). Enzyme profiles were generated with the Rapid ID 32A anaerobe identification kit (bioMérieux), according to the manufacturer's instructions, and performed in triplicate. The results of these tests are given in the species description.

Characteristics summarized in Table 1 for the novel strains and related *Prevotella* species showed that the novel strains were phenotypically very similar to *Prevotella buccalis* and *Prevotella veroralis*. However, the novel strains could be distinguished from *P. buccalis* by the absence of arginine arylamidase production and from *P. veroralis* by the absence of fermentation of xylan.

The cellular fatty acid composition of strain LBN 293^T was determined by using the MIDI Microbial Identification System at the Identification Service of the DSMZ (Supplementary Table S1). Analysis of cellular fatty acids showed that strain LBN 293^T had a low similarity index (0.106) to the *Prevotella loescheii* entry found in MIDI library version 3.8. The high level of summed feature 11 and smaller amount of C_{18:1} ω 9c are differential characteristics between strain LBN 293^T and closely related species.

On the basis of the above-mentioned findings, we propose that strains LBN 293^T, LBN 297 and LBN 298 should be classified within a novel species of the genus *Prevotella*, *Prevotella nanceiensis* sp. nov.

Description of *Prevotella nanceiensis* sp. nov.

Prevotella nanceiensis (nan.ce.i.en'sis. N.L. fem. adj. *nanceiensis* pertaining to Nanceium, the old name of Nancy, the French city where the strains supporting the description of the species were isolated).

Table 1. Differential phenotypic characteristics of *Prevotella nanceiensis* sp. nov. and related *Prevotella* species

Species: 1, *P. nanceiensis* sp. nov. (strains LBN 293^T, LBN 297 and LBN 298); 2, *P. bivia*; 3, *P. disiens*; 4, *P. buccalis*; 5, *P. enoea*; 6, *P. oralis*; 7, *P. oulorum*; 8, *P. veroralis*; 9, *P. shahii*; 10, *P. marshii*; 11, *P. loescheii*. +, Positive; –, negative; v, variable; ND, no data available. Data for reference species were taken from Downes *et al.* (2005), Jousimies-Somer *et al.* (2002), Sakamoto *et al.* (2004), Shah & Collins (1990), Watabe *et al.* (1983) and Wu *et al.* (1992).

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Pigment production	–	–	–	–	–	–	–	–	+	–	+
Fermentation of:											
Cellobiose	v*	–	–	+	–	+	–	+	–	–	+
Lactose	+	+	–	+	+	+	+	+	+	–	+
Mannose	+	+	–	+	v	+	+	+	+	v	+
Raffinose	+	–	–	+	–	+	+	+	+	–	+
Rhamnose	–*	–	–	–	ND	+	–	–	–	–	–
Salicin	–*	–	–	–	–	+	–	–	–	–	–
Sucrose	+	–	–	+	–	+	+	+	+	–	+
Xylan	–	–	ND	–	ND	–	–	+	ND	ND	v
Aesculin hydrolysis	+	–	–	+	v	+	+	+	–	–	+
Gelatin hydrolysis	–*	+	+	–	+	v	–	v	+	+	+
Catalase	–*	–	–	–	–	–	+	–	–	–	–

*As determined using the API 20A anaerobe identification kit.

Cells are obligately anaerobic, non-spore-forming, non-motile, Gram-negative coccoid and short rods (0.4–0.5 × 0.5–1.0 µm). After 4 days incubation on BBA, colonies are 0.5–1 mm in diameter, circular, entire, slightly convex and smooth, white, non-pigmented and surrounded by a β-haemolysis zone. Growth is inhibited in the presence of bile. Strains are saccharolytic and ferment glucose, lactose, maltose, mannose, raffinose and sucrose. Fermentation of cellobiose is variable among strains. Acid is not produced from arabinose, glycerol, mannitol, melezitose, rhamnose, salicin, sorbitol, trehalose, xylan or xylose. The major metabolic end products are acetic, lactic and succinic acids. Aesculin is hydrolysed. Gelatin and urea are not hydrolysed. Indole and catalase are not produced and nitrate is not reduced. All strains are positive for α-galactosidase, β-galactosidase, β-galactosidase-6-phosphate, α-glucosidase, N-acetyl-β-glucosaminidase, alkaline phosphatase, leucyl glycine arylamidase, alanine arylamidase, α-fucosidase and glutamyl glutamic acid arylamidase in the Rapid ID 32A identification panel, while reactions for mannose and raffinose fermentation and for β-glucosidase are variable among strains. All strains are negative for the remaining 16 tests, resulting in a Rapid ID 32A profile of 47⁰/₁ 1¹/₅/7 440222. The fatty acid profile predominantly comprises iso-C_{15:0}, anteiso-C_{15:0}, C_{16:0}, C_{16:0} 3-OH, C_{18:2}ω9,12c and summed feature 11 (iso-C_{17:0} 3-OH and/or C_{18:2} dimethylacetal). Chromosomal

genomic size ranges from 3.02 to 3.09 Mb, with four *rrn* operon copies. The DNA G + C content of the type strain is 39.4 mol%.

The type strain is LBN 293^T (=AIP 261.03^T =CIP 108993^T =CCUG 54409^T), isolated from a blood culture. Two other strains were recovered from clinical respiratory samples.

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