Porphyromonas bennonis sp. nov., isolated from human clinical specimens

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During our investigation of the bacteriology of human wound infections and abscesses, a novel anaerobic, non-spore-forming, Gram-negative bacillus was frequently isolated. On the basis of morphological and biochemical criteria, the strains were tentatively identified as belonging to the family *Bacteroidaceae*, but they did not appear to correspond to any recognized species of this family. Comparative 16S rRNA gene sequencing showed that the 14 novel strains were genotypically homogeneous and confirmed their placement in the genus *Porphyromonas*. Sequence divergence values >10% with respect to reference *Porphyromonas* species demonstrated that the strains isolated represent a novel species. On the basis of biochemical criteria and phylogenetic considerations, it is proposed that these strains isolated from human sources should be assigned to a novel species of the genus *Porphyromonas*, named *Porphyromonas bennonis* sp. nov., with WAL 1926C^T (=ATCC BAA-1629^T =CCUG 55979^T) as the type strain.

Members of the genus *Porphyromonas* are indigenous bacterial flora in the oral cavity of humans and animals; many species are also found in the urogenital and intestinal tracts. Some species are considered true pathogens and are associated with human or animal infections. *Porphyromonas* species have been isolated from oral infections and from many infections throughout the body. *Porphyromonas* species of animal origin have been encountered in humans with animal-bite infections. During our investigation of the bacteriology of human wound infections and abscesses (Finegold *et al.*, 2008), a novel anaerobic, non-spore-forming, Gram-negative bacillus related distantly to *Porphyromonas somerae* (Summanen *et al.*, 2005) was frequently isolated. Comparative 16S rRNA gene sequencing studies demonstrated that the 14 novel strains were genotypically homogeneous, clustering within the phylogeny of the genus *Porphyromonas*. In this polyphasic taxonomic study, we describe the genotypic and phenotypic characterization of this organism, which represents a novel species of the genus *Porphyromonas*.

Fourteen strains isolated at the VA Wadsworth Medical Center were included in this study (Table 1). All strains were characterized by using 16S rRNA gene sequencing and conventional biochemical methods, unless otherwise indicated. *P. somerae* ATCC BAA-1230^T was included in the biochemical and molecular characterizations. The strains were maintained at –70 °C in double-strength skimmed milk before characterization. The strains were grown at 37 °C on Brucella blood agar (Anaerobe Systems) or CDC blood agar (Becton Dickinson) under anaerobic conditions (N₂:CO₂:H₂, 90:5:5). All tests were performed on 24–72 h pure cultures, unless otherwise indicated.

The strains were characterized by using conventional biochemical tests (Durmaz *et al.*, 1995; Jousimies-Somer, 1995; Jousimies-Somer *et al.*, 2002; Holdeman *et al.*, 1977), including the assessment of susceptibility to special potency identification discs, the fermentation of pre-reduced, anaerobically sterilized biochemicals (Anaerobe Systems), GLC of metabolic end products, API ZYM and Rapid ID 32A (bioMérieux) and Rosco Diagnostic Tablets (Rosco). Pigment production was tested on laked blood agar (Anaerobe Systems). The production of β -lactamase was assessed by using nitrocefin-impregnated discs (Cefinase;

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain WAL 1926C^T is EU414673.

Table 1. Novel strains included in this study

Strain	Isolation source (history)	Cell count (c.f.u. ml ⁻¹)
WAL 1706J	Infected pilonidal cyst (negative)	10 ⁵
WAL 1773I	Spontaneous groin abscess (negative)	10 ⁸
WAL 1804E	Axillary abscess (2 year history of lump)	10 ⁷
WAL 1860J	Buttock abscess (multiple episodes of rectal fistula)	10 ⁷
WAL 1871H	Breast abscess (diabetes mellitus)	10 ⁵
WAL 1875-12	Perirectal abscess (negative)	10 ⁸
WAL 1894-1	Thumb paronychia (diabetes mellitus)	10 ⁷
WAL 1896H	Spontaneous breast abscess (negative)	10 ⁷
WAL 1926C ^T	Spontaneous shoulder abscess (diabetes mellitus)	10 ⁷
WAL 1980J	Spontaneous buttock abscess (diabetes mellitus)	10 ⁸
WAL 1998F	Vaginal abscess (negative)	10 ⁸
WAL 2044F	Spontaneous breast abscess (diabetes mellitus)	10 ⁵
WAL 2045I	Spontaneous lower back abscess (negative)	10 ³
WAL 2083H	Groin abscess (intravenous drug abuser)	10 ⁶

Becton Dickinson). In all tests with pre-reduced, anaerobically sterilized biochemicals, supplementation with haemin (0.001%) and rabbit serum (1%) was used to obtain growth. For cellular fatty acid analyses, the cells were grown on Brucella blood agar for 48 h. Growth was collected in 0.7% (w/v) MgSO₄ and harvested by centrifugation (5000 g for 20 min); the pellets were processed immediately or kept frozen at -20 °C. Methyl ester extraction was carried out according to the manufacturer's procedure (MIDI; Microbial ID) and the analysis was performed by using a Microbial Identification System (model 5898A; Hewlett Packard) gas chromatograph, with software from MIDI. The corresponding library (ANAEROBE, version Moore 5.0) was used in successive analyses. All biochemical tests were performed in duplicate. The KOH string test was performed as described by Halebian *et al.* (1981).

Genomic DNA was extracted and purified from bacterial cells in the mid-exponential growth phase, using a QIAamp DNA Mini kit (Qiagen). 16S rRNA gene fragments were amplified by means of a PCR with universal primers. The PCR products were purified by using a QIAquick gel extraction kit (Qiagen). The purified PCR products were sequenced directly with BigDye Terminator cycle sequencing kits (Biotech Diagnostics) on an ABI 3100 Avant Genetic System (Applied Biosystems). The sequencing data were analysed by comparing the consensus sequences with sequences obtained from GenBank, using the Ribosomal Database Project (RDP-II) (Maidak *et al.*, 2001) and BLAST software (Benson *et al.*, 1999); similarity (%) with respect to other sequences was determined. Closely related sequences were retrieved from GenBank and were aligned with the newly determined sequences by using the program CLUSTAL W (Thompson *et al.*, 1994). The phylogenetic tree was constructed by using the CLUSTAL W method, with the SeqBuilder and MegAlign program modules of LASERGENE 7 (DNASTAR); bootstrap analysis was also performed with MegAlign. The G+C content (mol%) of the DNA was

determined by using HPLC according to the method of Mesbah *et al.* (1989).

The unidentified isolates were recovered from clinical infections as a part of the infecting flora together with other anaerobes or aerobes, generally at high counts (>10⁵ c.f.u. ml⁻¹) (Table 1). The mean number of accompanying aerobes for each c.f.u. of the unidentified isolates was 1.8 and that for anaerobes was 4.1. Colonies grown on anaerobic blood agar plates for 48–72 h were small (0.2–0.5 mm in diameter), circular, entire, low convex, opaque and slightly β -haemolytic. At an early stage of growth, the colonies did not show pigmentation or fluorescence under long-wave UV light (366 nm); the colour of colonies ranged from grey to cream. After more prolonged incubation (≥ 10 days), a light beige to tan pigment developed; fluorescence remained negative. On Brucella blood agar, cells were generally Gram-negative, 0.7–1.0 $\times$ 1.0–6.0 μ m in size and pleomorphic, occasionally with long filaments and swellings. Occasionally, cells appeared Gram-positive in young cultures; however, the string test was positive, indicating that they were Gram-negative. The novel isolates were all resistant to colistin and kanamycin special potency identification discs, and nine of the 14 strains tested were sensitive to the vancomycin disc. All of the isolates studied were lipase-, indole- and nitrate-negative and sensitive to bile. Catalase results were variable, with six of the 14 strains registering as catalase-positive. All strains hydrolysed gelatin. All were asaccharolytic and produced acetic acid and succinic acid, and occasionally trace amounts of iso-valeric acid and lactic acid, as metabolic end products. All strains were positive for alkaline phosphatase, esterase (weakly positive), leucine arylamidase, chymotrypsin (weakly positive), acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, α -glucosidase and *N*-acetyl- β -glucosaminidase, but were negative for cystine arylamidase, trypsin, α -galactosidase, β -glucuronidase, β -glucosidase, α -mannosidase and α -fucosidase (in the API ZYM system). Esterase lipase,

lipase and valine arylamidase activities were variable in the API ZYM test. Rosco diagnostic tablets indicated that the strains were positive for α -glucosidase, *N*-acetyl- β -glucosaminidase and ONPG, but were negative for α -fucosidase, trypsin, urease and aesculin. In the Rapid ID 32A system, β -galactosidase, β -galactosidase phosphate, α -glucosidase, *N*-acetyl- β -glucosaminidase, alkaline phosphatase, leucyl glycine arylamidase and alanine arylamidase were positive, whereas urease, arginine dihydrolase, α -galactosidase, β -glucosidase, α -arabinosidase, β -glucuronidase, mannosidase and raffinose fermentation, α -fucosidase and pyroglutamic acid arylamidase gave negative results. Other reactions with Rapid ID 32A were variably negative or weakly positive. β -Lactamase was produced by two of 13 strains (15%). The main cellular fatty acids detected in the novel organism belonged to summed feature 5 (C_{15:0} DMA and/or C_{14:0} 3-OH, which could not be separated by the method used; 23–40% of the total fatty acids). Other cellular fatty acids (detected at levels in the range 9–15% of total fatty acids) included C_{12:0}, C_{14:0} and C_{16:0}. Cluster analysis of the principal components showed a Euclidian distance of <10 between the unidentified strains, thus indicating that they belong to the same species. Biochemical tests that are useful for differentiating this novel organism from other species

and genera are listed in Table 2. The key characteristics that serve to differentiate this novel organism from other *Porphyromonas* species are the negative α -fucosidase results, the positive *N*-acetyl- β -glucosaminidase and β -galactosidase results and the production of acetic and succinic acids as metabolic end products. In addition, the fact that the novel organism produces only weakly pigmented, small colonies on blood agar is also useful for differentiation (except with respect to *Porphyromonas catoniae*, which is non-pigmented; Willems & Collins, 1995).

In this polyphasic taxonomic study, the unidentified strains were related closely to each other genetically, as demonstrated by the 16S rRNA gene sequence similarities (99–100%). Phylogenetic analysis demonstrated that the novel organism represents a distinct line of descent within the genus *Porphyromonas*, as exemplified by strain WAL 1926^T (Fig. 1). The mean interspecies similarity within the *Porphyromonas* cluster is approximately 87% (Paster *et al.*, 1994); in GenBank, the closest match to the novel organism was *P. somerae* WAL 6690^T, at 89–90% sequence similarity. There is no precise correlation between 16S rRNA gene sequence divergence (%) and species delineation, but it is generally recognized that divergence values of 3% or more

Table 2. Characteristics of *P. bennonis* sp. nov. that serve to differentiate it from recognized species of the genus *Porphyromonas*

Taxa: 1, *P. asaccharolytica*; 2, *P. endodontalis*; 3, *P. gingivalis*; 4, *P. catoniae*; 5, *P. somerae*; 6, *P. uenonis*; 7, *P. bennonis* sp. nov. (n=14). All taxa are negative for lipase activity. Data for taxa 1–4 are from Jousimies-Somer *et al.* (2002); data for taxon 5 are from Summanen *et al.* (2005) and those for taxon 6 are from Finegold *et al.* (2004). Symbols: +, $\geq 90\%$ of the strains are positive; –, $\geq 10\%$ of the strains are negative; D, 11–89% of the strains are positive; w, weakly positive reaction.

Characteristic	1	2	3	4	5	6	7
Pigment production	+	+	+	–	+	+	w
Fluorescence	+	+	–	–	D	+	–
Indole	+	+	+	–	–	+	–
Catalase	–	–	–	–	–	–	D
Enzyme activity (commercial tests)*							
α -Fucosidase	+	–	–	+	–	–	–
α -Galactosidase	–	–	–	D	–	–	–
β -Galactosidase	–	–	–†	+	+	–	+
<i>N</i> -Acetyl- β -glucosaminidase	–	–	+	+	+	–	+
Chymotrypsin	–	–	–	D	+	–	+
Trypsin	–	–	+	D	–	–	–
Fermentation of:‡							
Glucose	–	–	–	+	w	–	–
Lactose	–	–	–	+	w	–	–
Maltose	–	–	–	+	w	–	–
Metabolic end products from PYG§	A, P, ib, B, IV, s	A, P, ib, B, IV, s	A, P, ib, B, IV, s, pa	a, P, iv, l, S	A, P, ib, B, IV, s	A, P, ib, B, IV, s	A, S

*Reaction in the API ZYM system (bioMérieux) or with Rosco diagnostic tablets. Results obtained with these two systems are not always identical to each other (see footnote †).

†Negative in the API ZYM system, but positive in the Rosco ONPG test.

‡Fermentation of most other carbohydrates has been reported as negative.

§A, Acetic acid; P, propionic acid; IB, isobutyric acid; B, butyric acid; IV, isovaleric acid; V, valeric acid; L, lactic acid; S, succinic acid; PA, phenylacetic acid. Upper-case letters indicate major metabolic products from peptone–yeast extract–glucose (PYG) and lower-case letters indicate minor products.

Fig. 1. Unrooted phylogenetic tree, based on 16S rRNA gene sequences, showing the position of *P. bennonis* sp. nov. within the *Bacteroides* subgroup of the phylum *Cytophaga-Flavobacterium-Bacteroides*. Bootstrap percentages (based on 1000 replications) are given at branching points. Bar, 1% sequence divergence.

are significant (Stackebrandt & Goebel, 1994). Support for the distinctiveness of the unknown bacterium was also very evident from the phenotypic analyses.

Whilst the data from the phylogenetic analysis were consistent with the view that the unknown organism belongs to the genus *Porphyromonas*, several of the organism's phenotypic characteristics did not conform to the description of the genus (Willems & Collins, 1995). Rather like the members of the genus *Porphyromonas*, most of the novel isolates were sensitive to the special potency disc containing vancomycin. In addition, like most other human *Porphyromonas* species, the unknown organism was asaccharolytic. The organism produced very slightly pigmented colonies on laked blood agar only after prolonged incubation, whereas pigment production in most *Porphyromonas* species is evident after 2–4 days. Unlike most *Porphyromonas* species, which produce complex metabolic end-product profiles that include acetic, propionic, isobutyric, butyric and isovaleric acids, the novel organism produced moderate amounts of acetic and succinic acids, occasionally with trace amounts of isovaleric acid and lactic acid. The cellular fatty acid profile of the novel organism is unlike that of any other *Porphyromonas* species, in that predominating cellular fatty acids described for the latter are iso-C_{15:0}, iso-C_{17:0} 3-OH and anteiso-C_{15:0} (Brondz *et al.*, 1989; Shah & Collins, 1988;

Brondz & Olsen, 1991; Könönen *et al.*, 1996; Summanen *et al.*, 2005), whereas the main cellular fatty acid(s) in the former was C_{15:0} DMA and/or C_{14:0} 3-OH (23–40% of total fatty acids). In the case of iso-C_{15:0}, iso-C_{17:0} 3-OH and anteiso-C_{15:0}, the levels detected were very low (<1%) or non-existent. Furthermore, the DNA G + C content of the unknown organism (58 mol%) is somewhat higher than those of recognized *Porphyromonas* species (44–55 mol%).

With an accurate identification scheme, this hitherto-unidentified organism may prove to be more common in clinical specimens than has been appreciated. During our comprehensive microbiological evaluation of infections of skin and soft tissue (Finegold *et al.*, 2008), the novel organism was the most frequently isolated anaerobic, Gram-negative rod: 5% (19 of 394) of specimens yielded this organism. Even though the novel organism was also isolated from areas above the diaphragm, we found that the isolates were predominantly recovered from patients with chronic infections of skin or soft tissue in the perirectal/buttock/groin regions (63%). The most common predisposing condition in the patients with skin and soft-tissue infections was diabetes mellitus. There were several relatively serious infections in which the unidentified organism was part of the infecting flora at high counts (Table 1). The full extent of the virulence of this newly identified organism remains to be determined, as it was

always isolated from mixed culture. It is notable that 15 % of the novel strains were β -lactamase producers; in the genus *Porphyromonas*, this is a trait found only in *P. somerae* (Summanen *et al.*, 2005), *Porphyromonas uenonis* (Finegold *et al.*, 2004) and perhaps also *P. catoniae* (Könönen *et al.*, 1996). In mixed infections, β -lactamase-producing species may protect other penicillin-susceptible bacteria by releasing free β -lactamase into their immediate environment (Brook, 2004).

Our studies indicate that this novel organism isolated from human sources clusters phylogenetically within the genus *Porphyromonas*, but is phenotypically and genetically different from recognized *Porphyromonas* species. We therefore propose the name *Porphyromonas bennonis* sp. nov. for this novel species.

Description of *Porphyromonas bennonis* sp. nov.

Porphyromonas bennonis (ben.no'nis. N.L. masc. gen. n. *bennonis* of Benno, in honour of the contemporary Japanese microbiologist Yoshimi Benno, who has contributed significantly to our knowledge of anaerobic bacteria).

Cells are Gram-negative, non-spore-forming rods, 0.7–1.0 $\times$ 1.0–6.0 μ m in size. Obligately anaerobic. Optimum temperature for growth is 37 °C. Colonies incubated on blood agar for 2 days are small, circular, entire, convex and slightly β -haemolytic. Colonies are grey to cream; after 10 days incubation, colonies are weakly pigmented (beige to tan) and show no red fluorescence under long-wave UV light. Gelatin-positive and bile-sensitive. Indole-, nitrate- and lipase-negative. Urease- and aesculin-negative. Catalase-variable. Asaccharolytic. The major fermentation products from glucose metabolism are acetic acid and succinic acid. The major cellular fatty acids are C_{15:0} DMA and/or C_{14:0} 3-OH, C_{12:0}, C_{14:0} and C_{16:0}. Positive for alkaline phosphatase, esterase (weakly positive), leucine arylamidase, chymotrypsin (weakly positive), acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, α -glucosidase and *N*-acetyl- β -glucosaminidase, but negative for cystine arylamidase, trypsin, α -galactosidase, β -glucuronidase, β -glucosidase, α -mannosidase and α -fucosidase (API ZYM system). Esterase lipase, lipase and valine arylamidase activities are variable with the API ZYM system. With Rosco diagnostic tablets, the strains are positive for α -glucosidase, *N*-acetyl- β -glucosaminidase and ONPG, but negative for α -fucosidase, trypsin, urease and aesculin. Positive for β -galactosidase, β -galactosidase phosphate, α -glucosidase, *N*-acetyl- β -glucosaminidase, alkaline phosphatase, leucyl glycine arylamidase and alanine arylamidase, and negative for urease, arginine dihydrolase, α -galactosidase, β -glucosidase, α -arabinosidase, β -glucuronidase, mannosidase, raffinose fermentation, α -fucosidase and pyroglutamic acid arylamidase (Rapid ID 32A). β -Lactamase is produced by 15 % of the strains. The DNA G+C content of the type strain is 58 mol%.

The type strain, WAL 1926C^T (=ATCC BAA-1629^T =CCUG 55979^T), and other strains were isolated in mixed culture from various skin and soft-tissue infections of non-oral origin, mainly from abscesses in the buttock and groin areas. Probably part of the indigenous bowel flora.

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