Transfer of *Bacteroides splanchnicus* to *Odoribacter* gen. nov. as *Odoribacter splanchnicus* comb. nov., and description of *Odoribacter denticanis* sp. nov., isolated from the crevicular spaces of canine periodontitis patients

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Numerous novel anaerobic bacteria were isolated from the crevicular spaces of dogs with periodontitis. The phenotypic characteristics of these bacterial isolates indicated that they were similar to members of the genus *Porphyromonas*. However, comparison of the 16S rRNA gene sequences of the isolates indicated that they were related to members of the *Bacteroides splanchnicus* subgroup. A representative of the novel isolates, strain B106^T, induced alveolar bone loss in a mouse model of experimental periodontal disease. Based on biochemical, morphological, molecular phylogenetic, and pathogenic evidence, it is proposed that the taxonomic subgroup containing these novel isolates and *B. splanchnicus* should be classified in a new genus, *Odoribacter* gen. nov., within the family 'Porphyromonadaceae'. In addition, it is proposed that *B. splanchnicus* should be reclassified as *Odoribacter splanchnicus* comb. nov., and that the newly identified isolates should be classified as representing *Odoribacter denticanis* sp. nov., the type strain of which is B106^T (=ATCC PTA-3625^T=CNCM I-3225^T).

The taxonomy of the phylum *Bacteroidetes* has undergone considerable changes within the last decade. The advent of 16S rRNA gene–DNA sequence analysis has vastly improved the ability of scientists to genetically place a bacterium into an appropriate genus. Members of the families *Bacteroidaceae*, 'Porphyromonadaceae' and 'Prevotellaceae' have been found to play important roles in oral disease as well as in other anaerobic infections of

humans and companion animals (Haffajee & Socransky, 1994). Historically, the genera *Porphyromonas* and *Bacteroides* have been greatly intertwined, due, in part, to the original classification of many *Porphyromonas* isolates as belonging to the genus *Bacteroides* (Shah & Collins, 1988). *Porphyromonas* species and *Bacteroides* species both generally appear pigmented on blood agar after extended incubation. Members of these two genera are Gram-negative, non-spore-forming, non-motile rods (Holt *et al.*, 1994). At the time of writing, the genera *Porphyromonas* and *Bacteroides* comprised approximately 16 and 77 recognized species, respectively (List of Prokaryotic names with Standing in Nomenclature; <http://www.bacterio.cict.fr>).

We previously conducted an extensive field study that resulted in the collection of a large number of gingival crevicular fluid samples from companion animals with periodontitis. Analysis of these samples permitted the types and frequencies of pigmented anaerobic bacteria present in

Abbreviations: ABC, alveolar bone crest; ANI, anaerobe identification; CEJ, cemento–enamel junction.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Odoribacter denticanis* strains B106^T, B107, B113, B126, B129, B135, B140, B150, B151, B152, B155, B163, B171, B172, B174, B183 and B264 are AY560020, AY560021, AY560022, AY560023, AY560024, AY560025, AY560026, AY560027, AY560028, AY560029, AY560030, AY560031, AY560032, AY560033, AY560034, AY560035 and AY560036, respectively. The GenBank/EMBL/DDBJ accession number for the *fimA* gene of strain B106^T is AY573801.

diseased crevicular spaces to be determined (Hardham *et al.*, 2003, 2005a). Briefly, paper-point samples were taken from the crevicular spaces of dogs and cats with periodontitis. Samples were only taken from teeth with pocket depths of greater than 2 mm (cats) or 3 mm (dogs). The samples were diluted, plated on Brucella blood agar (Anaerobe Systems) and incubated at 37 °C in an anaerobic environment (5 % H₂/5 % CO₂/90 % N₂). Individual, well-isolated colonies were restreaked on Brucella blood agar in order to obtain pure cultures. A region of the 16S rRNA gene was PCR amplified directly from individual colonies using the universal primers D56 (5'-GGATTAGATACCCTGGTAGTC-3') (Rumpf *et al.*, 1999) and D57 (5'-CCCGGGAACGTATTCACCG-3') (Slots *et al.*, 1995) (Invitrogen). PCR fragments were purified and directly sequenced. The DNA sequences obtained were analysed for relatedness to DNA sequences in the available databases at the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>) using the BLASTN software (Altschul *et al.*, 1990). Nearest matches were used to presumptively classify the bacterial genus and species. We identified numerous isolates whose 16S rRNA gene sequences did not have highly similar matches in the available databases, indicating that they may represent novel isolates. One group of these isolates (Table 1) appeared to represent a novel species originally referred to as '*Porphyromonas denticanis*' (Hardham *et al.*, 2003, 2005a, b). Herein, we propose that this novel species should be placed into a new genus with the name *Odoribacter* gen. nov.

Biochemical analysis

Strain B106^T was isolated from an adult, mixed-breed, male dog of unknown age with periodontal disease. A

Table 1. Canine clinical bacterial isolates of *O. denticanis* used in this study

Strain	Tooth	Pocket depth (mm)
B106 ^T	Lower left first molar	4
B107	Lower left first molar	4
B113	Upper left pre molar no. 4	4
B126	Upper right first molar	4
B129	Upper right first molar	4
B135	Upper left pre molar no. 3	4
B140	Lower left first molar	4
B150	Lower left first molar	4
B151	Lower left first molar	4
B152	Lower left first molar	4
B155	Lower left first molar	4
B163	Upper left pre molar no. 4	4
B171	Lower left pre molar no. 4	7
B172	Lower left pre molar no. 4	7
B174	Lower left pre molar no. 4	6
B183	Lower left pre molar no. 4	3

paper-point sample was obtained from the mandibular first molar that had a periodontal pocket of 4 mm in depth and a gingival index of 1 (Harvey, 1998). The sample was processed as described above. The purified cells were subjected to biochemical analysis using a RapID ANA II clinical test kit (Remel) and the Vitek Anaerobe Identification (ANI) card system (bioMérieux). Briefly, confluent bacterial cells on Brucella blood agar plates were resuspended to McFarland no. 3 equivalency. The suspension was added to the RapID ANA II test wells or the Vitek ANI card and incubated for 4 h at 37 °C. At the end of the incubation period, the results of the biochemical tests were recorded.

Table 2 shows the results of RapID ANA II testing for strain B106^T as well as for six related bacteria. Of the 18 tests performed by using the RapID ANA II kit, only two (leucyl-glycine and indole) were positive for strain B106^T. In comparison, *Porphyromonas gingivalis* ATCC 33277^T, *Prevotella intermedia* ATCC 25611^T, *Tannerella forsythensis* ATCC 43037^T, *Bacteroides thetaiotaomicron* ATCC 29148^T, *Bacteroides fragilis* ATCC 25285^T and *Bacteroides splanchnicus* ATCC 29572^T yielded 5, 5, 10, 9 (plus 1 variable), 11 and 7 (plus 1 variable) positive tests, respectively.

The biochemical reaction patterns of strain B106^T, *Porphyromonas gingivalis* ATCC 53977, and *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 23726 were determined using the Vitek ANI card (Table 3). Strain B106^T yielded only two positive tests (indole and triphenyl tetrazolium), whereas *Porphyromonas gingivalis* ATCC

Table 2. RapID ANA II biochemical tests for strain B106^T (*Odoribacter denticanis* gen. nov., sp. nov.) and related strains

Strains: 1, B106^T; 2, *Porphyromonas gingivalis* ATCC 33277^T; 3, *Prevotella intermedia* ATCC 25611^T; 4, *T. forsythensis* ATCC 43037^T; 5, *B. thetaiotaomicron* ATCC 29148^T; 6, *B. fragilis* ATCC 25285^T; 7, *B. splanchnicus* ATCC 29572^T. Tests for urea, α-arabinosidase, and hydrolysis of leucyl-glycine, glycine and proline generated the same results for the bacteria indicated. +, Positive; –, negative; v, variable.

Biochemical test	1	2	3	4	5	6	7
β-Disaccharide	–	–	–	–	–	+	–
β-Galactosidase	–	–	–	+	+	+	–
α-Glucosidase	–	–	+	+	+	+	–
β-Glucosidase	–	–	–	+	v	+	–
α-Galactosidase	–	–	–	–	+	+	+
α-Fucosidase	–	–	–	+	+	+	+
N-Acetylglucosaminidase	–	+	–	+	+	+	+
Alkaline phosphatase	–	+	+	+	+	+	v
Hydrolysis of:							
Phenylalanine	–	–	–	+	–	+	–
Arginine	–	+	+	+	+	+	+
Serine	–	–	–	+	–	–	–
Pyrrolidonyl	–	–	–	–	–	–	+
Indole	+	+	+	–	+	–	+

Table 3. Vitek ANI biochemical tests for strain B106^T (*Odoribacter denticanis* gen. nov., sp. nov.) and related strains

Strains: 1, B106^T; 2, *Porphyromonas gingivalis* ATCC 53977; 3, *F. nucleatum* ATCC 23726. The other 25 tests in the Vitek ANI kit produced the same results for the bacteria indicated. +, Positive; –, negative.

Biochemical test	1	2	3
Gram	Negative	Negative	Negative
Alkaline phosphatase	–	+	–
N-Acetylglucosaminidase	–	+	–
Proteolytic cleavage of N-benzoyl-DL-arginine p-nitroanilide	–	+	–
Glutamyl glutamic acid arylamidase	–	–	+

53977 yielded five positive tests (indole, *p*-nitrophenyl phosphate, *p*-nitrophenyl *N*-acetyl β -D-glucosaminidase, *N*-benzoyl-DL-arginine *p*-nitroanilide and triphenyl tetrazolium). Of the 12 tests that were in common between the RapID ANA II and the Vitek ANI card, all yielded similar results. Using the database supplied by Vitek, strain B106^T was identified incorrectly as *F. nucleatum* with a confidence level of 63 % (data not shown). The misidentification may be due to a lack of veterinary pigmented anaerobic bacterial isolates in the Vitek database.

Phylogenetic analysis

The 16S rRNA gene from strain B106^T was PCR amplified in triplicate using the primers D134 (5'-GAGTTTGAT-CCTGGCTCAGG-3') and D57 (Slots *et al.*, 1995) (Invitrogen). The PCR products were pooled, purified and desalted, and subjected to direct DNA sequence analysis. BLASTN (Altschul *et al.*, 1990) searches of the non-redundant nucleotide database at the NCBI using the 16S rRNA gene sequence of strain B106^T indicated that strain B106^T was related to members of the genus *Bacteroides*. The 16S rRNA gene sequence of strain B106^T was most closely related to the 16S rRNA gene sequence of *Bacteroidetes* sp. oral clone FX069 (GenBank accession number AY134906), with 96.3 % identity over 1465 bp. *Bacteroidetes* sp. oral clone FX069 was isolated from a human patient with necrotizing ulcerative periodontitis during a study to define the bacterial species associated with necrotizing ulcerative periodontitis in HIV-positive patients (Paster *et al.*, 1994).

Phylogenetic analysis based on 16S rRNA gene sequences was performed using CLUSTAL_X version 1.81 software (Thompson *et al.*, 1997). Phylogenetic trees were generated using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap values were obtained using 1000 replicates. Fig. 1 shows the results of phylogenetic analysis for strain B106^T. The placement of the major genera (*Porphyromonas*, *Bacteroides*, *Prevotella*, *Tannerella*, etc.) is in agreement with previously published phylogenetic trees for the

phylum *Bacteroidetes* (Paster *et al.*, 1994; Sakamoto *et al.*, 2002; Shah *et al.*, 1998).

Strain B106^T is grouped in an outgroup of *Bacteroides* that includes *B. splanchnicus* (Werner *et al.*, 1975), several oral isolates and numerous uncultured strains. An 88.9 % bootstrap confidence value at the branch point of *O. denticanis* and *B. splanchnicus* indicates a moderately high degree of certainty that these two organisms are related. However, historically, there has been discussion about the placement of *B. splanchnicus* within the genus *Bacteroides* (Paster *et al.*, 1994; Shah *et al.*, 1998). Paster *et al.* (1994) suggested that *B. splanchnicus* may represent a new genus. Shah *et al.* (1998) indicated that *B. splanchnicus* shares many chemotaxonomic properties with members of the genus *Porphyromonas*. Our data are in agreement with this hypothesis, as the *B. splanchnicus*/*O. denticanis* B106^T group branches closer to the genus *Porphyromonas* than to the genus *Bacteroides* (Fig. 1).

An approximately 580 bp region of the 16S rRNA gene from 15 other canine clinical isolates of *O. denticanis* (Table 1) was PCR amplified (in triplicate), using the primers D56 and D57 described above. The PCR products were purified, desalted and pooled. The DNA sequence of the PCR products was then determined. The partial 16S rRNA gene sequences from the 16 *O. denticanis* isolates were found to all cluster together into six groups of identical sequences (I: B106^T, B107, B126, B129, B135, B140, B163, B174; II: B150, B151, B152; III: B171, B183; IV: B113; V: B155; and VI: B172). The largest divergence between these six groups was between groups II and VI, which showed 97 % identity over the 516 bp analysed. Each of these partial 16S rRNA gene sequences was found to be highly related to that of strain B106^T (Fig. 1). Recently, Elliot and others (unpublished sequence submission) have deposited in the NCBI nucleotide databases six partial 16S rRNA gene sequences from bacteria found in canine dental plaque that have homology to *O. denticanis* 16S rRNA gene sequences. Additional sequences deposited recently by Baldwin and others (unpublished sequence submission) and Eckburg *et al.* (2005) from bacteria isolated from human periodontal pockets and human intestine, respectively, also have homology to the *O. denticanis* 16S rRNA gene sequences. Based on these observations, all of these isolates appear to be varying strains of the same species.

In order to analyse further the relationship between strain B106^T and the genus *Porphyromonas*, the *fimA* gene was PCR amplified (in triplicate) using the degenerate PCR primers D122 (5'-TGGCTAARYTGACYGTAATGGTYTA-3') and D123 (5'-AGTTYACYAATACAGGRTAATAGGT-3') (Invitrogen). The PCR products were purified, desalted and pooled, and subjected to direct DNA sequence analysis. BLASTP searches (Altschul *et al.*, 1990) of the non-redundant polypeptide database at the NCBI using the FimA amino acid sequence indicated that the FimA protein of strain B106^T was related to the FimA protein from members of the genus *Porphyromonas*. Fig. 2 shows the phylogenetic

Fig. 1. Neighbour-joining phylogenetic tree for clinical isolates of *O. denticanis* gen. nov., sp. nov. and representatives of the phylum *Bacteroidetes*. The tree was generated using CLUSTAL_X version 1.81 and NJ Plot software programs (both available from <ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/>). The tree was rooted to the *Bacillus subtilis* A60 16S rRNA gene sequence. Bootstrap analysis was performed using 1000 replicates. Bootstrap values are presented graphically (●, >950; ■, >850; ○, >700; □, >500; no designation, <500). Bar, 0.02 substitutions per nucleotide position.

relationships of the various FimA protein sequences. A very strong relationship existed (bootstrap value of 100 %) between the protein of strain B106^T and several of the type I fimbrillin proteins of *Porphyromonas* species.

The FimA data support the relationship between the *B. splanchnicus*/*O. denticanis* B106^T group and the genus *Porphyromonas*. Based on the 16S rRNA gene phylogenetic data (Fig. 1) and the FimA phylogenetic data (Fig. 2), we propose that the subgroup of bacteria containing *B. splanchnicus* should be reclassified in a new genus, *Odoribacter* gen. nov., in the family 'Porphyromonadaceae' (Fig. 1). Additionally, we propose that *B. splanchnicus* should be reclassified as *Odoribacter splanchnicus* comb. nov.

Structural analysis

Strain B106^T and *Porphyromonas gulae* B 243 (Hardham *et al.*, 2003, 2005a) were subjected to scanning electron

microscopy to determine the cellular morphology. Briefly, 36-h liquid cultures in modified phytone/yeast/glucose medium (Hardham *et al.*, 2003) were centrifuged at 1800 g. The medium was replaced with 3 % electron microscopy-grade glutaraldehyde (Polysciences) in a 0.1 M phosphate buffer (pH 7.3) and incubated at 4 °C for 1 h. The cells were then washed twice in 0.1 M phosphate buffer (pH 7.3) and suspended in buffered 1 % osmium tetroxide (Polysciences) for 1 h at room temperature. The samples were dehydrated in a graded ethanol series with final dehydration done by immersion in hexamethyldisilazane for 15 min, followed by air-drying for 1 h. Samples were mounted on aluminium stubs and coated with 200 Å gold in a Polaron SEM autocoating unit (Polaron Instruments). Each sample was examined by using a DS-130 scanning electron microscope (ISI). Images were captured using a Noran 4485 digital beam control interface (Noran Instruments). Fig. 3 shows scanning electron micrographs of *Porphyromonas gulae* B 243 and strain B106^T. The short rod-shaped morphology (mean length of 1.25 µm) of

Fig. 2. Neighbour-joining phylogenetic tree for representatives from the *Porphyromonas gingivalis* FimA protein family (types I–V). The tree was generated using CLUSTAL_X version 1.81 and NJ Plot software programs. The tree was rooted to the *Escherichia coli* CFT073 fimbriin protein. Bootstrap analysis was performed using 1000 replicates. Bootstrap values are presented graphically (●, >950; ■, >850; ○, >700; □, >500; no designation, <500). Bar, 0.05 substitutions per amino acid position.

Porphyromonas gulae B 243 (Fig. 3a) is typical of members of the genus *Porphyromonas*. In contrast, strain B106^T appeared fusiform with tapered ends, with a mean cell length of 5.9 µm (Fig. 3b). The morphology of strain B106^T is analogous to that of *T. forsythensis* under the same growth conditions (Tanner *et al.*, 1986). The morphological difference between *Porphyromonas gulae* B 243 and strain B106^T further supports the placement of strain B106^T outside of the genus *Porphyromonas*.

Mouse model of periodontal disease

Several members of the genera *Bacteroides*, *Porphyromonas* and *Prevotella* are implicated as periodontal pathogens (Shah *et al.*, 1998). As the *O. denticanis* isolates described herein were isolated from the crevicular space of dogs with periodontal disease, we sought to determine whether these isolates could cause disease in an experimental model of periodontal disease. We tested the isolates in an oral mouse model of periodontal disease (Baker *et al.*, 1994). In this

model, mice that had been pre-treated with antibiotics to reduce their oral flora were orally challenged with the bacteria in question. After a 42-day infection period, the maxillary buccal surfaces of each mouse were scored for alveolar bone loss. The distance from the cemento–enamel junction (CEJ) to the alveolar bone crest (ABC) is indicative of the amount of bone loss.

Fig. 4 shows the results of alveolar bone height measurements. The negative control animals (sham infected) were used to determine the base-line CEJ–ABC distance. *Porphyromonas gingivalis* ATCC 53977 was used as a positive control. The alveolar bone loss observed following *Porphyromonas gingivalis* ATCC 53977 infection was consistent with previously published results for this isolate (Baker *et al.*, 2000; Evans *et al.*, 1992a, b). Strain B106^T was capable of eliciting alveolar bone loss as shown by the increased CEJ–ABC distances in infected animals. The bone loss elicited by strain B106^T was equal to or greater than that generated by infection with *Porphyromonas gingivalis*

Fig. 3. Scanning electron micrographs of cells of *Porphyromonas gulae* B 243 (a) and strain B106^T (b). Bars, 1000 nm.

ATCC 53977. It can be concluded that strain B106^T is capable of inducing periodontitis in infected animals.

O. denticanis may prove to be an important periodontal pathogen in companion animals. Previous work by our group (Hardham *et al.*, 2003, 2005a, b) and others (Fournier *et al.*, 2001) has indicated that the periodontal pathogens in companion animals may be slightly different from those of humans. For example, *Porphyromonas gulae* has been identified in companion animals (Fournier *et al.*, 2001; Hardham *et al.*, 2003, 2005a), but not in humans. Since vaccines for periodontitis in humans (Page, 2000) and companion animals (Hardham *et al.*, 2003, 2005b)

Fig. 4. Pathogenicity testing of strain B106^T in the oral mouse model of periodontal disease. Sixteen mice were used for each test group. Mice were treated as described previously (Baker *et al.*, 1994). Forty-two days post challenge, the mice were euthanized and the jaws were defleshed and stained. Fourteen independent measurements of the CEJ–ABC distance were taken along the buccal surface of the maxilla on each jaw. The net bone loss plus SE for each group is shown. *Statistically different from the negative control group ($P < 0.05$).

would aid in periodontal disease prevention programmes, *O. denticanis* should be considered as an important constituent in such vaccines.

We have presented data that demonstrate that a bacterium isolated from the periodontal pockets of dogs represents a previously unidentified species. Furthermore, we propose that the subgroup of bacteria in the genus *Bacteroides* containing *B. splanchnicus* should be reclassified as belonging to the family 'Porphyromonadaceae' in the genus *Odoribacter* gen. nov. We also propose that *B. splanchnicus* should be reclassified as *Odoribacter splanchnicus* comb. nov. and that the novel group of clinical isolates described herein should be classified as representing *Odoribacter denticanis* sp. nov.

All procedures in this study were approved by the Institutional Animal Care and Use Committee and were conducted in compliance with the Guide for Care and Use of Laboratory Animals, as well as with all internal company policies and guidelines.

Description of *Odoribacter* gen. nov.

Odoribacter [O.do.ri.bac'ter. L. n. *odor* smell; N.L. masc. n. *bacter* rod; N.L. masc. n. *Odoribacter* rod of (bad) smell].

Cells are anaerobic, Gram-negative, non-spore-forming, non-motile, fusiform in shape and catalase-positive. Colonies demonstrate pigmentation after >7 days of anaerobic growth at 37 °C on medium containing blood. The type species is *Odoribacter splanchnicus*.

Description of *Odoribacter splanchnicus* comb. nov.

Basonym: *Bacteroides splanchnicus* Werner *et al.* 1975 (Approved Lists).

The description is that given by Werner *et al.* (1975).

The type strain is ATCC 29572^T (=CCUG 21054^T=CIP 104287^T=DSM 20712^T=LMG 8202^T=NCTC 10825^T).

Description of *Odoribacter denticanis* sp. nov.

Odoribacter denticanis (den.ti.ca'nis. L. n. *dens* -tis tooth; L. gen. n. *canis* of a dog; N.L. gen. n. *denticanis* of the tooth of a dog).

Exhibits the following properties in addition to those given in the genus description. Colonies are punctiform and begin to show pigmentation (tan-black) after 10–12 days of incubation at 37 °C in an anaerobic environment on medium containing blood. Colonies are haemolytic on Brucella blood agar, and are resistant to kanamycin, vancomycin and colistin (antibiotic discs from Anaerobe Systems). Colonies on egg yolk agar (Anaerobe Systems) have neither lecithinase nor lipase activities. There is no evidence of bacterial swarming since distinct colonies appear on numerous media types.

The type strain is strain B106^T (=ATCC PTA-3625^T=CNCM I-3225^T). Strain B106^T is the subject of the US patent application US20030228328 and the worldwide patent application WO2003054755. The strain is now available to the public.

Acknowledgements

The authors are grateful to Bruce Paster, David Lowery and Raja Krishnan for their insight, suggestions and critical review of this manuscript.

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