***Porphyromonas gulae* sp. nov., an anaerobic, Gram-negative coccobacillus from the gingival sulcus of various animal hosts**

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A new species, *Porphyromonas gulae* sp. nov., is proposed to include strains isolated from the gingival sulcus of various animal hosts which are distinct from related strains of *Porphyromonas gingivalis* of human origin. This bacterium exhibits the following characteristics: black-pigmented colonies; asaccharolytic, obligate anaerobic growth; and Gram-negative, non-motile and non-spore-forming, rod-shaped cells. Colonies do not fluoresce under UV light. Vitamin K₁ and haemin are required for growth. Cells haemagglutinate sheep erythrocytes. Major fatty acid end products are butyric acid, isovaleric acid, succinic acid and phenylacetic acid. Strains are catalase-positive and indole is produced. Alkaline phosphatase, trypsin-like and *N*-acetyl- β -glucosaminidase activities are strong. A β -galactosidase and a glutamylglutamic acid arylamidase are also present. The G+C content of the chromosomal DNA is 51 mol%. DNA–DNA homology data and 16S rRNA gene sequence analysis provide strong evidence that strains from the animal biotype of *P. gingivalis* represent a *Porphyromonas* species that is distinct from *P. gingivalis*. The type strain of *P. gulae* is Loup 1^T (= ATCC 51700^T = NCTC 13180^T).

Keywords: *Porphyromonas gulae* sp. nov., *Porphyromonas gingivalis*, DNA–DNA hybridization, 16S rRNA sequencing, Old World monkey

INTRODUCTION

The taxonomy of the genus *Porphyromonas* has evolved quite rapidly in recent years (Jousimies-Somer, 1995, 1997). It was originally proposed (Shah & Collins, 1988) to accommodate three species found in humans, *Porphyromonas gingivalis*, *Porphyromonas asaccharolytica* and *Porphyromonas endodontalis*, all

of which are strictly anaerobic, Gram-negative, non-spore-forming and non-motile rod-shaped chemo-organotrophs. Colonies develop a brown to black pigment on laked blood agar plates within the first 48 h of growth. Subsequent studies on samples obtained from dogs led to the description of several new species: *Porphyromonas cangingivalis* and *Porphyromonas cansulci* (Collins *et al.*, 1994), *Porphyromonas gingivicanis* and *Porphyromonas crevioricanis* (Hirasawa & Takada, 1994), and *Porphyromonas canoris* (Love *et al.*, 1994). It is noteworthy that these species have all been associated with development of periodontitis in the animal. Based on 16S rRNA phylogenetic analyses, *Oribaculum catoniae* (Moore & Moore, 1994) was reassigned to the *Porphyromonas* genus (Willems & Collins, 1995). In addition, some isolates obtained from various animals have closely resembled *P. gingivalis* strains of human origin, but differed in terms of catalase activity (Laliberté & Mayrand, 1983) and antigen specificity (Parent *et al.*, 1986), suggesting the existence of two biotypes within the species *P. gingivalis*.

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Abbreviations: β -GAL, β -galactosidase; β -NAG, *N*-acetyl- β -D-glucosaminidase; PA, phenylacetic acid; RAPD, randomly amplified polymorphic DNA; UPGMA, unweighted pair group method with arithmetic averages.

The GenBank accession numbers for the 16S rRNA gene sequences of *P. gulae* sp. nov. isolates Loup 1^T (ATCC 51700^T), Chat 3.1, Ours 3.1, B 243, Chien 4.2 and Coyote 1.5.1 are AF208290, AF285871, AF285872, AF285874, AF285873 and AF287986, respectively.

Studies based on phenotypic characteristics (Fournier & Mouton, 1993), DNA–DNA reassociation (Kato *et al.*, 1997), multilocus enzyme electrophoresis (MLEE) (Loos *et al.*, 1993) and randomly amplified polymorphic DNA (RAPD) typing (Ménard & Mouton, 1993) have confirmed the existence of two biotypes within the *P. gingivalis* taxon, one of human origin and the other of animal origin. In particular, two DNA-based studies (Kato *et al.*, 1997; Ménard & Mouton, 1995) and an MLEE study (Loos *et al.*, 1993) have demonstrated sufficient genetic distance between isolates of both origins to warrant their assignation to separate species. This led us to believe that strains recovered from the gingival plaque of animals represented a cryptic species within the *P. gingivalis* taxon.

The new data we report here have been added to those previously published, and involve morphological, biochemical, physiological and genetic analyses conducted on strains representing the two biotypes and genetic clusters previously outlined. This information has led to the proposal of a new species, *Porphyromonas gulae* sp. nov.

METHODS

Bacterial strains. This investigation required a collection of strains to represent accurately the animal as a host. A total of 67 isolates of black-pigmented, Gram-negative, anaerobic coccobacilli to rod-shaped bacteria identified as *P. gingivalis* animal biotype was included in this study. This collection reflected a wide geographical and host diversity (Table 1). The isolates came from Canada, the United States, Australia and Japan. The animal reservoir included bear, cat, coyote, dog, wolf and monkey. Most strains were freshly isolated and some were originally identified by the donor laboratory and subsequently screened with our typing scheme. A collection of *P. gingivalis* human biotype and reference strains from other *Porphyromonas* species was also included (Table 1). In addition, the published characteristics for type strains of newly named species, particularly from dogs, that had not been described at the time of our experimentation have been taken into account. Those strains used for further comparison are: *P. canoris* NCTC 12835^T, *P. gingivicanis* ATCC 55562^T, *P. crevioricanis* ATCC 55563^T, *P. cangingivalis* NCTC 12856^T and *P. cansulci* NCTC 12858^T. Finally, because they are close relatives of the species under study, the cat isolate *Porphyromonas circumdentaria* VPB 3325, and a frequent colonizer of the oral cavity in children, *Porphyromonas catoniae* ATCC 51270^T (Willems & Collins, 1995), first described as *Oribaculum catoniae* (Moore & Moore, 1994), were included in our comparison.

Growth conditions. Bacteria were usually grown to mid-exponential phase in Todd–Hewitt broth (BBL Microbiology Systems) enriched with haemin (10 µg ml⁻¹; Sigma-Aldrich) and vitamin K₁ (1 mg ml⁻¹; Sigma-Aldrich). Trypticase Soy broth (BBL Microbiology Systems), enriched as above, was used specifically to grow cultures for DNA homology analysis. Otherwise, cultures were maintained on plates using the Todd–Hewitt base with 1.5% (w/v) agar (Difco) and 2% (v/v) laked human erythrocytes (Red Cross). All cultures were incubated in an anaerobic chamber (Coy Manufacturing) in a gas atmosphere composed of 80% (v/v) N₂, 10% (v/v) CO₂ and 10% (v/v) H₂ at a constant temperature of 37 °C.

Isolation and biochemical identification. The protocols used for isolation and identification of anaerobic microorganisms from the oral microflora of various animal hosts have been described previously (Fournier & Mouton, 1993). Our complete phenotyping scheme, run in duplicate, included colony fluorescence, catalase, trypsin-like and haemagglutinating activities, biochemical tests from the RAPID ID 32A (formerly ATB 32A) kit (bioMérieux) and GLC analysis of metabolic end products. GLC analysis was carried out using a gas-liquid chromatograph (model 5830A; Hewlett Packard) equipped with a hydrogen flame-ionization detector as outlined in the Virginia Polytechnic Institute Manual (Holdeman *et al.*, 1977). Peaks were recorded and integrated with a Hewlett Packard GC terminal, model 18850A (chart speed 1.0 cm min⁻¹). The carrier gas was helium (80 ml min⁻¹) and the injection port temperature was 120 °C (for volatile fatty acids) or 125 °C (for non-volatile fatty acids). Ten-microlitre aliquots of ether-extracted or methylated and chloroform-extracted samples from Todd–Hewitt broth supernatants were injected into a coiled glass column packed with 10% Fluorad FC 431 (Chromatographic Specialties) plus 1% H₃PO₄ on acid-washed Chromosorb W (80–100 mesh; Johns-Manville) (for volatile fatty acids) or with 10% (w/w) Resoflex LAC-1-R296 (Chromatographic Specialties) on acid-washed Chromosorb AW (45–50 mesh; Johns-Manville) (for non-volatile fatty acids). The results were compared with those obtained using standard 10 mM solutions of acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, lactate, succinate and phenylacetate (Sigma-Aldrich).

An additional method, using 4-methylumbelliferone derivatives (Moncla *et al.*, 1991), was used for the detection of α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, α -fucosidase and neuraminidase (sialidase) activities and *N*-carbobenzoxyl-L-arginine-7-amido-4-methylcoumarin for trypsin-like activity. Briefly, Whatman no. 2 strips (~6 × 60 mm) were soaked with substrate solutions; a loopful of bacterial growth was then smeared on each strip and the strips incubated for 15 min at 37 °C in the dark. The paper strips were then examined under a long-wavelength (366 nm) hand-held UV lamp (UltraViolet Products). The enzymic activity was visualized by the appearance of a fluorescent blue spot around the smear on the filter paper strip.

RAPD fingerprinting (Ménard & Mouton, 1995). We examined 98 strains recovered from 79 human subjects and 32 more recovered from 30 animals of nine different species. RAPD was carried out on purified DNA using modifications (Ménard *et al.*, 1992) of the original method (Williams *et al.*, 1990). To assess overall genetic relatedness (genetic affinity), a data matrix scoring major amplicons as 1 (present) and 0 (absent) was first compiled for each isolate. Similarities between all tested strains were then estimated using the Nei coefficient (Nei & Li, 1979): $F = (2n_{xy}/n_x + n_y)$ where n_{xy} is the number of bands common to strains *x* and *y*, and n_x and n_y are the total number of bands in each strain. These data were clustered according to the unweighted pair group method with arithmetic averages (UPGMA) (Sneath & Sokal, 1973) yielding a dendrogram. Data handling was performed with the R package (Legendre & Vaudor, 1991) and we used the PHYLIP program (Felsenstein, 1993) for reconstruction.

DNA homology methods and determination of G + C content (Kato *et al.*, 1997). Chromosomal DNA was extracted and purified by the method of Marmur (1961). Excluding the

Table 1. Clinical *Porphyromonas* strains used in this study

Strain	Infection or diagnosis	Host (no. carrier animals/no. sampled animals)	Geographic origin
<i>P. gingivalis</i> animal biotype			
(<i>P. gulae</i> sp. nov.)			
Received isolates (15)			
3318, 3492	Healthy gingival sulcus	Cats	Dr D. N. Love, Australia
A 1834, B 243, G 251, I 372, I 433	Experimental periodontitis	Monkeys*	Dr W. P. McArthur, FL, USA
Bas beagle PZ4T+, DogID12	Healthy gingival sulcus	Dogs	Dr S. A. Syed, MI, USA
Chat 1, Chat 3(7)	Healthy gingival sulcus	Cats	Dr D. Mayrand, Quebec
Chien 5B, Chien N9	Healthy gingival sulcus	Dogs	Dr D. Mayrand, Quebec
D2-9, D2-10	Healthy gingival sulcus	Dogs	Dr K. Okuda, Japan
Freshly recovered isolates (52)			
Ours 3.1	Healthy gingival sulcus to gingivitis	Bears (1/2)	Own isolates, Quebec
Chat strains (8): 1.3, 2.4, 2.5, 3.1, 3.31, 3.3S1, 6.21, 6.32	Healthy gingival sulcus to gingivitis	Cats (6/6)	Own isolates, Quebec
Coyote strains (12): 1.4S2, 1.4S3, 1.43f, 1.43p, 1.5S1f, 1.5S1p, 1.5S2, 1.5.1, 1.5.2, 1.53, 1.55, 1.56	Gingivitis	Coyote (1/1)	Own isolates, Quebec
Chien strains (21): 4.2, 5.3S1, 5.41, 5.42, 5.43, 6.31, 6.32, 6.41, 6.51, 7.2S1, 7.2S2, 7.3S1, 8.31, 8.32, 8.3S1, 9.3S1, 9.4S1, 9.51, 10.21, 10.2S1, 10.3S1	Gingivitis	Dogs (3/3)	Own isolates, Quebec
Ateles strains (5): 5.3S1, 5.4S1, 5.51, 5.52, 5.5S1	Periodontitis	Monkeys (1/3)†	Own isolates, Quebec
Loup strains (5): 1 (= ATCC 51700 [†]), 1.2S1, 2.2S1, 2.3S1, 2.4S1	Healthy gingival sulcus to gingivitis	Wolves (2/2)	Own isolates, Quebec
<i>P. gingivalis</i> human biotype (13)			
ATCC 33277 [‡] , ATCC 53977, ATCC 53978, ATCC 49417	Healthy gingival sulcus, mellitus diabetes, clinical isolate, periodontal pocket	Human	ATCC, VA, USA
381	Periodontal pocket	Human	Dr S. S. Socransky, MA, USA
OMG 406	Unknown	Human	Dr G. Dahlén, Kenya
16-1	Juvenile periodontitis	Human	Dr K. Okuda, Japan
HW24D-5	Periodontal pocket	Human	Dr D. Mayrand, Quebec
I-2	Unknown	Human	Dr K. Okuda, Japan
R113	Periodontal pocket	Human	Dr J.-C. Robert, France
JKG-1	Buccal cavity	Human	Dr S. A. Syed, MI, USA
Cerco 1.5.2	Gingivitis	Monkeys‡	Own isolates, Quebec
Manga 1.41	Gingivitis	Monkeys§	Own isolates, Quebec
<i>P. endodontalis</i> (1)			
ATCC 35406 [†]	Infected root canal	Human	ATCC, VA, USA
<i>P. asaccharolytica</i> (2)			
ATCC 25260 [†] , ATCC27067	Empyema, leg wound	Human	ATCC, VA, USA
<i>P. circumdentaria</i> (1)			
VPB 3325	Normal margin	Cat	Dr D. N. Love, Australia
<i>P. macacae</i> (2)			
ATCC 33141 [†] , ATCC 49407	Periodontal pocket, normal margin	Monkey , cat	ATCC, VA, USA, Dr D. N. Love, Australia
<i>P. levii</i> (1)			
ATCC 29147 [†]	Rumen	Cow	ATCC, VA, USA

* Isolated from *Saimiri sciureus* (common squirrel monkey).

† Isolated from *Ateles paniscus* (black spider monkey).

‡ Isolated from *Cercocebus torquatus lemniscatus* (white-collared mangabey).

§ Isolated from *Cercopithecus nictitans* (greater white-nosed monkey).

|| Isolated from *Macaca arctoides* (stump-tailed macaque).

coyote, a representative strain was selected for each host for this part of the study. DNA purity was assessed by the A_{260}/A_{280} ratio and by agarose gel electrophoresis. To prepare labelled DNA *in vitro*, the nick translation system [3H]- (Du Pont NEN Products), which is an adaptation of the procedure described by Rigby *et al.* (1977), was used.

DNA similarity values were determined by means of the S1 nuclease procedure as described previously (Kato *et al.*, 1987). A reaction mixture containing 0.01 µg labelled DNA and 10 µg unlabelled DNA was incubated for 24 h at 65 °C. S1 nuclease digestions were carried out using 0.5 U of the enzyme (Seikagaku Kogyo). After 20 min incubation at

37 °C, the remaining dsDNA segments were precipitated with cold 10% TCA and collected on a membrane filter (type HA; Millipore). The membranes were dried and the radioactivity was measured. DNA similarity values were calculated by dividing the counts per minute of the heterologous S1 nuclease-resistant DNA by the counts per minute of the homologous S1 nuclease-resistant DNA and multiplying by 100. A correction was made for self-reassociation of labelled DNA using calf thymus DNA as control. The thermal stabilities of the DNA–DNA hybrids were estimated using the S1 nuclease procedure described by Johnson (1981).

Table 2. Positions of the primers used for PCR amplification and sequencing of the 16S rDNA

Primer	Position*	Sequence	Reference
SSU 27	8–27	5'-AGAGTTTGATCMTGGCTCAG-3'	Dorsch & Stackebrandt (1992)
SSU 536	515–536	5'-GTGCCAGCMGCCGCGGTAATAC-3'	Dufresne <i>et al.</i> (1996)
SSU 1175'	1175–1195	5'-GACGTCATTCCCCTCCTTCCTC-3'	Dufresne <i>et al.</i> (1996)
SSU 926	908–926	5'-AAACTYAAAKGAATTGACGC-3'	Dufresne <i>et al.</i> (1996)
SSU 1492'	1492–1513	5'-TACGGYTACCTTGTTACGACTT-3'	Dorsch & Stackebrandt (1992)

* Relative to *E. coli* 16S rDNA sequence (Brosius *et al.*, 1978).

Table 3. Phenotypic differences among species of the genus *Porphyromonas*

1, *P. gulae* Loup 1^T + 66 isolates; 2, *P. gingivalis* ATCC 33277^T + 12 isolates; 3, *P. endodontalis* ATCC 35406^T; 4, *P. asaccharolytica* ATCC 25260^T + ATCC 27067; 5, *P. circumdentaria* VPB 3325; 6, *P. macacae* ATCC 33141^T + ATCC 49407; 7, *P. canoris* NCTC 12835^T; 8, *P. gingivicanis* ATCC 55562^T; 9, *P. crevioricanis* ATCC 55563^T; 10, *P. cangingivalis* NCTC 12856^T; 11, *P. cansulci* NCTC 12858^T; 12, *P. catoniae* ATCC 51270^T; 13, *P. levii* ATCC 29147^T. Data from this study except where indicated. —, No activity; +, activity (97% positive); ND, not determined.

Phenotypic feature	1	2*	3	4	5	6	7†	8‡	9‡	10§	11§	12	13
UV fluorescence	—	—	+	+	+	+(1/2)	+	ND	ND	—	+	ND	+
Catalase	+	—	—	—	+	+	+	+	—	+	+	—	+
Trypsin-like activity	+	+	—	—	—	+	—	—	—	—	—	—	—
Haemagglutination	+	+	—	—	—	—	—	—	+	—	—	ND	—
α -Galactosidase	—	—	—	—	—	+	—	ND	ND	—	—	—	—
β -Galactosidase	+	—	—	—	—	+	+	ND	ND	—	—	+	+
<i>N</i> -Acetyl- β -glucosaminidase	+	+	—	—	—	+	+	—	—	—	—	+	+
Indole production	+	+	+	+	+	+	+	+	+	+	+	—	—
Arginine arylamidase	+(64/66)	+	—	—	—	—	ND	ND	ND	ND	ND	ND	—
α -Fucosidase	—	—	—	+	—	—	—	—	—	—	—	+	—
Glutamylglutamic acid arylamidase	+	—	—	—	+	—	ND	ND	ND	ND	ND	ND	+
Propionic acid production	—	—	—	—	—	+	+	—	+	+	+	+	—
Phenylacetic acid production	+	+	—	—	—	+	—	—	+	—	+	—	—

* Within the taxon *P. gingivalis*, two strains representing the Old World monkeys subgroup as hosts were also included.

† Data from Love *et al.* (1994).

‡ Data from Hirasawa & Takada (1994).

§ Data from Collins *et al.* (1994).

|| Data from Könönen *et al.* (1996).

Thermal melting points were used to determine the G+C content of the DNA preparations (Johnson, 1981).

16S rDNA phylogenetic analysis. DNA was extracted from six *P. gulae* sp. nov. strains: Loup 1^T (ATCC 51700^T), Chat 3.1, Chien 4.2, B 243, Ours 3.1 and Coyote 1.5.1, and from three *P. gingivalis* strains: ATCC 33277^T, Cerco 1.5.2 and Manga 1.41 using the G NOME Kit (Bio101) according to the manufacturer's instructions. DNA purity was assessed by calculating the A_{260}/A_{280} ratio measured on a spectrophotometer, and by agarose gel electrophoresis, comparing with *Escherichia coli* genomic DNA high-molecular-mass standards (Sigma-Aldrich).

Genes encoding 16S rRNA (rDNA) fragments corresponding to positions 8–1510 of *E. coli* (Brosius *et al.*, 1978) were amplified in three tubes by using primer pairs SSU 27 and SSU 1492' (Table 2). The 40 μ l PCR sequencing reaction mixtures contained 0.4 mM (each) of the selected primers, 200 μ M of all four deoxyribonucleoside triphosphates (Pharmacia Biotech), 10 mM Tris/HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl₂, 0.5 U *Taq* DNA polymerase (Promega) combined with the TaqStart anti-

body (Clontech). The PCR mixtures were subjected to thermal cycling (3 min at 95 °C; and then 35 cycles of 60 s at 95 °C, 60 s at 50 °C and 1 min at 72 °C; with a 7 min final extension at 72 °C) using a PTC-200 DNA Engine (MJ Research). The PCR product was resolved by electrophoresis through 1% (w/v) agarose gels at 4 V cm⁻¹ for 90 min, and visualized with a white light box after staining with methylene blue (Flores, 1992) to avoid interference in the sequencing reaction. Bands were recovered from the gel by using the QIAquick Gel Extraction Kit (Qiagen). The amplicons were then used directly in the sequencing protocol. Both strands of amplified DNA were sequenced with the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit using an Applied Biosystems 377 sequencer (PE Applied Biosystems). The sequence was amplified in overlapping fragments with the primers described in Table 2. The reactions were performed by using the same amplification primers and 100 ng per reaction of the nucleic acid product. To exclude the possibility of sequencing errors attributable to misincorporations by the *Taq* DNA polymerase, each strand of the amplicon was sequenced from two different PCR reactions.

The 16S rRNA sequences from the six *P. gulae* sp. nov. (former *P. gingivalis* animal biotype) strains, i.e. Loup 1^T (ATCC 51700^T), Chat 1, Ours 3.1, B 243, Chien 4.2 and Coyote 1.5.1, were aligned with those from five *P. gingivalis* (human biotype), nine other *Porphyromonas*, five phylogenetically related species of *Prevotella*, *Bacteroides* and *Flavobacterium* genera, and two *Bacillus* species as outgroup. The resulting alignments of 1417 nucleotides with ambiguous regions removed (positions 1–109, 230–240, 481–502, 589, 647–675, 833–887, 1027–1075, 1315 and 1463–1568) were subjected to phylogenetic analysis. Pairwise sequence identities were estimated and transformed in rate of substitution by the two-parameter method of Kimura (1980), and the phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) with PAUP version 4.0b4 (Swofford, 2000). A bootstrap analysis with 500 replicates was conducted to confer a confidence level in the tree topology.

Nucleotide sequence accession numbers. The 16S rDNA sequences obtained in this study have been deposited in the GenBank nucleotide sequence database under the following accession numbers: *P. gulae* sp. nov. Loup 1^T (ATCC 51700^T), AF208290; Chat 3.1, AF285871; Ours 3.1, AF285872; B 243, AF285874; Chien 4.2, AF285873; Coyote 1.5.1, AF287986; *P. gingivalis* ATCC 33277^T, AF285870; *P. gingivalis* Cerco 1.5.2, AF287987; *P. gingivalis* Manga 1.41, AF285869. The reference sequences used in the phylogenetic analysis were as follows: *Bacteroides fragilis* ATCC 25285^T, X83935; *Bacteroides vulgatus* ATCC 8482^T, M58762; *Bacillus coagulans* IAM 12463, D16267; *Bacillus subtilis* NCDO 1769, X60646; *Flavobacterium uliginosum* ATCC 14397^T, M62749; *P. asaccharolytica* ATCC 25260^T, L16490; *P. cangingivalis* UPB 4874, X76259; *P. cansulci* UPB 4875, X76260; *P. catoniae* ATCC 51270^T, X82823; *P. circumdentaria* NCTC 12469^T, L26102; *P. endodontalis* ATCC 35406^T, L16491; *P. gingivalis* (W83), TIGR genome project, not yet released; *P. gingivalis* DSM 20709, X73964; *Porphyromonas macacae* ATCC 33141^T, L16494; *P. macacae* NCTC 11632, L26103; *Prevotella intermedia* ATCC 25611^T, L16468; *Prevotella nigrescens* ATCC 33563^T, L16471.

RESULTS

Isolation and growth of strain collection

Table 1 shows the geographical and host diversity of the *P. gingivalis* animal biotype received strains and fresh isolates included in this study. Only those from monkeys could be associated with an alteration of the tooth-supporting tissues. The others were usually isolated from healthy subgingival sites, or from sites with a minor presence of calculus. Brown- to black-pigmented colonies were obtained from subgingival plaques sampled from 17 animals, of which 7 gave a primary culture with more than one type of black-pigmented colony. After presumptive identification, a total of 86 isolates was classified *Porphyromonas* or *Prevotella* (data not shown).

Fig. 1. Dendrogram constructed from RAPD data indicating relationships among 129 isolates of *P. gingivalis*. A distance matrix was calculated by using the Nei and Li coefficient. The tree was generated from the distance matrix by UPGMA. (reprinted with permission from Ménard & Mouton, 1995).

Table 4. Levels of DNA–DNA relatedness (%; data from Kato *et al.*, 1997) and sequence similarity of 16S rRNAs among strains of *P. gingivalis*, *P. gulae* sp. nov. and related species

Source of unlabelled DNA	<i>P. gingivalis</i> ATCC 33277 ^T (human biotype)		<i>P. gingivalis</i> Loup 1 ^T (animal biotype) (<i>P. gulae</i> sp. nov.)		<i>P. gingivalis</i> Chat 3.1 (animal biotype) (<i>P. gulae</i> sp. nov.)	
	DNA–DNA	16S rDNA	DNA–DNA	16S rDNA	DNA–DNA	16S rDNA
<i>P. gingivalis</i>						
human biotype						
ATCC 33277 ^T	(100)*	100	58	98.1	62	98.1
W83	–	99.4	–	97.7	–	97.7
DSM 20209	–	99.6	–	98.0	–	98.0
Cerco	–	99.7	–	98.1	–	98.1
Manga	–	99.7	–	98.1	–	98.1
ATCC 53977	78	–	54	–	55	–
ATCC 49417	85	–	62	–	60	–
381	85	–	54	–	68	–
OMG 406	91	–	63	–	58	–
16-1	84	–	54	–	61	–
HW24D5	75	–	63	–	63	–
I-2	83	–	68	–	60	–
R113	84	–	55	–	60	–
JKG-1	82	–	60	–	55	–
<i>P. gingivalis</i>						
animal biotype (<i>P. gulae</i> sp. nov.)						
Loup 1 ^T	65	98.1	(100)	100	100	98.8
Chat 3.1	58	98.1	83	98.8	(100)	100
Chien 4.2	63	98.1	90	99.9	96	98.8
B 243	60	97.9	96	99.4	90	98.5
Ours 3.1	53	98.0	90	98.8	85	99.9
Chat 1.1	60	–	85	–	80	–
Coyote 1.5.1	–	97.9	–	99.2	–	98.6
<i>P. cansulci</i>						
VPB 4875	–	89.5	–	89.6	–	89.6
<i>P. catoniae</i>						
ATCC 51270 ^T	–	89.6	–	89.7	–	89.7
<i>P. asaccharolytica</i>						
ATCC 25260 ^T	10	–	5	–	12	–
<i>P. endodontalis</i>						
ATCC 35406 ^T	19	–	13	–	15	–
<i>P. macacae</i>						
VPB 3313	18	–	8	–	13	–
ATCC 33141 ^T	11	–	3	–	10	–
<i>P. levii</i>						
ATCC 29147 ^T	15	–	10	–	16	–

* Value normalized to 100 %.

Physiological and biochemical characteristics

The use of new substrate analogues has definitely helped our presumptive identification of fresh isolates. According to some published work, the 4-methyl-umbelliferone derivatives could be a good alternative, especially for their sensitivity and accuracy (Moncla

et al., 1991). Furthermore, this method has been specifically defined for black-pigmented organisms.

The typing scheme was employed and delimited a distinct group of 52 strains of animal origin. These strains were phenotypically similar to *P. gingivalis* but still remained unique (Table 3). The distinguishing

criteria, also shared with the 15 strains received from other laboratories, were a positive catalase reaction, β -galactosidase activity (β -GAL) and glutamylglutamic acid arylamidase activity (Fournier & Mouton, 1993). All strains and fresh isolates haemagglutinated sheep erythrocytes, produced phenylacetic acid (PA) as a metabolic end product, possessed a trypsin-like activity and an *N*-acetyl- β -D-glucosaminidase activity (β -NAG) as did *P. gingivalis* human biotype. No fluorescence of the pigmented colonies under UV light was observed (Table 3). Strains from wolves and dog strains from Japan (D2-9, D2-10) also expressed a β -galactosidase-6-phosphate enzyme. Additionally, the coyote isolates all showed a pyroglutamic acid arylamidase activity (data not shown).

P. canoris, the newly named bacterial species associated with developing periodontitis in dogs, remained distinct from the above, in that it failed to haemagglutinate or to produce PA and showed no trypsin-like activity. β -GAL and β -NAG were detected, and an orange emission from colonies was seen under UV light. Haemagglutination, production of PA and trypsin-like activity were negative for *P. gingivicanis*. *P. crevioricanis*, which produced PA and haemagglutinated erythrocytes, showed no catalase or trypsin-like activity. *P. cangingivalis* was unique in its inability to produce measurable β -GAL, β -NAG, PA or haemagglutination activity. *P. cansulci* was distinguished by fluorescent colonies on blood agar, and by its lack of trypsin-like or haemagglutinating activities. Although β -GAL and β -NAG activities were present in *P. catoniae*, this species was quite distinct due to its lack of pigmentation and absence of catalase or trypsin-like activity, and failure to produce indole or PA (Table 3).

The colonies of *P. macacae* were rather brown-pigmented, cells did not haemagglutinate, they showed α -galactosidase activity and propionic acid appeared to be the major volatile metabolic end product. The discriminating criteria for *P. circumdentaria* were a positive UV fluorescence, no trypsin-like nor haemagglutinating activities, a negative enzymic detection for trypsin-like, β -GAL and β -NAG activities, and the absence of PA production.

Summary of RAPD observations (Ménard & Mouton, 1995)

Four arbitrary primers were used to analyse the extent and nature of polymorphism in the *P. gingivalis* taxon. A first dendrogram obtained by hierarchical clustering of a data matrix of 66 discrete characters (major amplicons) revealed that 129 out of the 130 study isolates clustered in two major phylogenetic groups in which a total of 101 clonal types could be recognized (Fig. 1). A third group consisted of a single strain that was not used in the present study. Group I included all 98 human isolates, four of non-human primate (Old World monkeys) origin and one from dog (Chien N9). Group II consisted exclusively of animal strains,

Table 5. Thermal stability upon reassociation with labelled DNA from *P. gingivalis* ATCC 33277

Modified from Kato *et al.* (1997).

DNA from strain	ΔT_m (e) value (°C)
<i>P. gingivalis</i> human biotype	
ATCC 33277 ^T	0
HW24D5	0.5
ATCC 53977	0.5
<i>P. gingivalis</i> animal biotype (<i>P. gulae</i> sp. nov.)	
Loup 1 ^T	5.5
Chien 4.2	5.5

including three non-human primate (New World monkey) isolates. Group III consisted of one animal strain isolated from a sheep with broken mouth disease (not included in the present study). The average genetic distances between groups were as follows: between groups I and II, 0.58; between groups I and III, 0.59; and between groups II and III, 0.78. The average genetic distance between isolates of group II (0.43) was higher than for group I (0.32), the latter reflecting the great diversity of hosts in group II. Interestingly, isolates from a carrier species, i.e. dog, cat, etc., were never seen to group in a given subcluster. This presumably reflected the higher diversity of the animal population studied (nine mammalian species). From our data, the existence of two phyla can be inferred.

Summary of DNA homology data (Kato *et al.*, 1997)

By comparison with the reference strain ATCC 33277^T, the animal isolates shared a DNA homology of only 53–65% (Table 4). In turn, using strain Loup 1^T as a reference, the relatedness within the animal biotype was more than 83%, but decreased to around 60% by comparison to strains from human origin. Another strain of the animal biotype, Chat 3.1, gave similar observations. These values are too low for *P. gingivalis* human biotype and *P. gingivalis* animal biotype (*P. gulae* sp. nov.) to be considered members of the same species. The intraspecies level of *P. gulae* sp. nov. DNA–DNA relatedness is 80% and higher. The similarity is lowered if the hybridization is done with *P. gingivalis* (~60%), *P. endodontalis* (14%), *Porphyromonas levii* (13%), *P. asaccharolytica* (10%) and *P. macacae* (9%) (Table 4) (Kato *et al.*, 1997). In addition, thermal stability experiments of hybrids were carried out using strains from the human biotype with the lowest percentage hybridization with the reference ATCC 33277^T, i.e. ATCC 53977 and HW24D5; and amongst the animal biotype strains, those with the highest values, i.e. Loup 1^T and Chien 4.2 (Table 5). The hybrids of strains HW24D5/ATCC 33277^T and

Fig. 2. 16S rDNA sequence phylogenetic tree showing the position of *P. gulae* sp. nov. within the *Porphyromonas* genus and related taxa. The branching pattern was generated by the neighbour-joining method and the bootstrap values, shown at the nodes, were calculated from 500 replicates. The scale bar indicates 1 inferred nucleotide substitution per 100 nucleotides.

ATCC 53977/ATCC 33277^T, which showed below 80% DNA homology values, had a $\Delta T_m(e)$ of 0.5 °C. In contrast, the hybrids Loup 1^T/ATCC 33277^T and

strains Chien 4.2/ATCC 33277^T, which gave relatively the highest percentage of reassociation values, had a $\Delta T_m(e)$ higher than 5 °C (Table 5).

Phylogenetic analysis by 16S rRNA comparison

For each target strain, the sequencing results for the two PCR reactions were identical. In addition, the sequences of both strands were 100% complementary, thus confirming the high accuracy of the data. The G+C content of the 16S rDNA was 53.8 mol% for proposed *P. gulae* sp. nov. type strain i.e. Loup 1^T, and the strain shared 98.1% identity with *P. gingivalis* of human origin and only 89.5% with the other nearest *Porphyromonas* species, i.e. *P. cansulci* (Table 4, Fig. 2). *P. canoris*, associated with developing periodontitis in dogs, remained distinct from *P. gulae*. The identity between the *P. gingivalis* strains of human origin was 99.7%.

DISCUSSION

The results presented in this paper reveal a paradox in bacterial systematics that is not restricted to the *P. gingivalis* taxon (Fox *et al.*, 1992). We compared a set of strains of animal origin, closely resembling *P. gingivalis* found in humans, which can be distinguished phenotypically and exhibit sufficient genotypic differences from the latter (Loos *et al.*, 1993; Ménard & Mouton, 1993, 1995) to be correctly assigned to a different genospecies on the basis of accepted criteria. Data on DNA–DNA similarity (Table 4) (Kato *et al.*, 1997) confirmed the heterogeneity found between the two biotypes of *P. gingivalis* and delineated *P. gulae* sp. nov. according to standard criteria (Wayne *et al.*, 1987). However, when we compared sequences in genes encoding 16S rRNA to clarify the phylogenetic position of the new species in the genus *Porphyromonas*, the difference between *P. gulae* sp. nov. and *P. gingivalis* was found to be sufficient to define a new genospecies but appeared tenuous.

The recognition of these two distinct phyla in monkey strains suggests the intriguing possibility that the most recent ancestors of human strains of *P. gingivalis* are closely related to the Old World monkey isolates or are those themselves. This recent divergence of the ancestral phylum may explain a less efficient resolution by the 16S rDNA sequence analysis. This possibility needs to be investigated for a larger sample of strains from a variety of many non-human primate species indigenous to different parts of the world.

What is the range of *P. gulae* sp. nov.? Using the WEE-TAB System for biochemical identification, Hudspeth *et al.* (1997) found conflicting results between *P. gingivalis* ATCC 33277^T and clinical strains isolated from infected dog and cat bites in humans. *P. gingivalis* ATCC 33277^T was catalase and β -GAL negative, clinical strains were all catalase-positive and 7 out of 10 (70%) were β -GAL positive. These two biochemical activities suggest that the clinical isolates are likely to be authentic *P. gulae* sp. nov. strains. In contrast, Citron *et al.* (1996) found weak β -GAL activity in *P. gingivalis* ATCC 33277^T and clinical strains isolated from infected dog and cat bites in humans, while *P. gingivalis* ATCC 33277^T demonstrated no β -GAL

activity in current enzymic profiling (Durmaz *et al.*, 1995). From published data it would appear that the bas beagle isolates known as 'canine *Bacteroides*' (former classification) (Shah & Collins, 1988; Syed, 1980) and those studied by Yamasaki *et al.* (1990), as well as the cat strains that Love *et al.* (1990) introduced as *P. gingivalis* subsp. *felis*, most likely belong to the species *P. gulae*.

Allaker *et al.* (1997a, b) detected *P. gingivalis* in the supragingival dental plaque of 68% of healthy dogs. In contrast, the recently described *P. canoris*, *Porphyromonas salivosa*, *P. cangingivalis*, *P. cansulci* and *P. crevioricanis* were isolated in less than 9% of dogs. Moreover, *P. gingivicanis* was not isolated from any of the animals sampled (Allaker *et al.*, 1997a, b). All *P. gingivalis* isolated from supragingival dental plaque of dogs in our laboratory, and not included in the present study, have been re-identified with our extended typing scheme. They all shared the biochemical characteristics and genetic markers of *P. gulae* (data not shown). Since the identification of *P. gingivalis* in the studies of Allaker *et al.* (1997a, b) was based on a shorter set of phenotypic tests, we can infer that these strains truly belong to the new species *P. gulae*.

Wild animals were a good source of *P. gulae* sp. nov. In the present study, most of them had spent a significant portion of their life in captivity. The coyote was an exception because it was recovered during winter from a trap set by poachers, brought to the Québec City Zoo and sampled the same day. The primary culture of its dental plaque gave black-pigmented colonies, most of them being *P. gulae* sp. nov. The diet is known to play a selective role on the dental flora. In this case, protein shortage during winter may have altered the composition of the normal flora, thus allowing bacterial overgrowth of *P. gulae* sp. nov. in the oral cavity of this mammal.

According to previous results (Fournier & Mouton, 1993; Kato *et al.*, 1997; Ménard & Mouton, 1993, 1995; Parent *et al.*, 1986) and the present study, it can be inferred that *P. gingivalis* cannot be found in dogs and other animals, with the exception of non-human primates from the Old World (Ménard & Mouton, 1995). Moreover, it would appear that *P. gulae* sp. nov. is the most prominent species of the genus *Porphyromonas* to be found in the oral cavity of mammals.

Description of *Porphyromonas gulae* sp. nov.

Porphyromonas gulae (gu'lae. L. n. gula [animal] mouth; N.L. gen. n. *gulae* from the animal mouth, referring to its isolation from subgingival plaque of various animal hosts).

Cells are obligately anaerobic, non-spore-forming, non-motile, Gram-negative rods or coccoid forms. From Todd–Hewitt broth cultures or blood agar plates, the following cellular morphologies can be

observed. The size of cells ranges from 0.5 to 0.9 µm by 1.0 to 4.0 µm. Cells are arranged singly, in short chains or in groups; some filaments up to 10 µm are occasionally seen. After 48 h growth on solid media, colonies are 0.5–3.5 mm in diameter, with a circular form, an entire margin, a convex elevation and a mucoid aspect. A brown to black pigment is produced on ageing of the cultures. Moreover, colonies do not fluoresce under UV light ($\lambda = 366$ nm). Vitamin K₁ and haemin are required for growth. Mannose and raffinose are not fermented.

The major metabolic end products in Todd–Hewitt broth are butyric acid, isovaleric acid, succinic acid and phenylacetic acid. Strains are all catalase-positive and indole is produced. Alkaline phosphatase, trypsin-like and β -NAG activities are strong. β -GAL and glutamylglutamic acid arylamidase activities are present. Urea is not used and nitrate is not reduced. Cells significantly haemagglutinate sheep erythrocytes and are resistant to kanamycin (50 µg ml⁻¹).

All isolates came from subgingival plaque samples of various mammals, including cat, dog, coyote, wolf, bear and non-human primate from the New World i.e. squirrel monkey and black spider monkey. The sites were mainly healthy, or with a minor presence of calculus. The G + C content of the DNA is 51 mol %. The average level of intraspecies DNA–DNA homology is 80 % and higher. The DNA–DNA relatedness with related species is *P. gingivalis* (~60 %), *P. endodontalis* (14 %), *P. levii* (13 %), *P. asaccharolytica* (10 %) and *P. macacae* (9 %). The type strain of *P. gulae* sp. nov. is Loup 1^T (= ATCC 51700^T = NCTC 13180^T).

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