Porphyromonas canoris sp. nov., an Asaccharolytic, Black-Pigmented Species from the Gingival Sulcus of Dogs

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A new species, *Porphyromonas canoris*, is proposed for black-pigmented asaccharolytic strains isolated from subgingival plaque samples from dogs with naturally occurring periodontal disease. This bacterium is an obligately anaerobic, nonmotile, non-spore-forming, gram-negative, rod-shaped organism. On laked rabbit blood or sheep blood agar plates, colonies are light brown to greenish brown after 2 to 4 days of incubation and dark brown after 14 days of incubation. Colonies on egg yolk agar and on nonhemolyzed sheep blood agar are orange. The cells do not grow in the presence of 20% bile and have a guanine-plus-cytosine content of 49 to 51 mol%. The type strain is VPB 4878 (= NCTC 12835). The average levels of DNA-DNA hybridization between *P. canoris* strains and other members of the genus *Porphyromonas* are as follows: *Porphyromonas gingivalis* ATCC 33277^T (T = type strain), 6.5%; *Porphyromonas gingivalis* cat strain VPB 3492, 5%; *Porphyromonas endodontalis* ATCC 35406^T, 1%; *Porphyromonas salivosa* NCTC 11362^T, 5%; and *Porphyromonas circumdentaria* NCTC 12469^T, 6%. The level of hybridization between *P. canoris* NCTC 12835^T DNA and *Porphyromonas asaccharolytica* ATCC 25260^T DNA is 3%. *P. canoris* cells produce major amounts of acetic, propionic, isovaleric, and succinic acids and minor amounts of isobutyric and butyric acids as end products of metabolism in cooked meat medium. The major cellular fatty acid is 13-methyltetradecanoic acid (iso-C_{15:0}). Glutamate and malate dehydrogenases are present, as are glucose-6-phosphate dehydrogenase activity (65.7 nmol mg of protein⁻¹ min⁻¹) and 6-phosphogluconate dehydrogenase activity (63.0 nmol mg of protein⁻¹ min⁻¹). *P. canoris* cells do not agglutinate sheep erythrocytes but exhibit brick red fluorescence at 265 nm and produce catalase.

During our investigation of the subgingival flora of 16 family-owned dogs with naturally occurring periodontitis, strictly anaerobic, nonsporing, gram-negative, asaccharolytic, rod-shaped organisms were found frequently (10, 19, 20). The results of morphological, biochemical, and API ZYM enzyme analyses placed several groups of these organisms in the genus *Porphyromonas* and DNA-DNA hybridization tests performed by using dot blot assays (12) showed that some of these organisms exhibited homology with either *Porphyromonas gingivalis* or *Porphyromonas salivosa* (unpublished data). However, the members of one group, which included 12 of the 259 phenotypically characterized isolates, did not hybridize in the DNA-DNA dot blot assay with any of the currently recognized species in the genus *Porphyromonas*. However, these strains had a range of characteristics which suggested that they could legitimately belong to the genus *Porphyromonas*. In this paper, we describe the results of DNA homology studies, a cellular fatty acid analysis, and an allozyme electrophoresis analysis and propose a new species, *Porphyromonas canoris*.

MATERIALS AND METHODS

Bacterial strains. A total of 5 of the 12 strains described previously (10) as members of group C2 were examined in more detail in this study. These were strains AHN 4393^T (= VPB 4878^T) (T = type strain) (AHN, Anaerobe Reference Laboratory, Helsinki Collection; (VPB, Veterinary Pathology and Bacteriology Collection), AHN 4425 (= VPB 4879), AHN

4489 (= VPB 4880), AHN 4446 (= VPB 4881), and AHN 4812 (= VPB 4882); these strains originated from four gingival sulcal sites of three dogs. Strains VPB 4880 and VPB 4882 were isolated from the same site of a dog during different visits 3 months apart. Strains VPB 4879 and VPB 4881 were isolated from two different sites on another dog during the same visit. Strain VPB 4878^T was isolated from a third dog. In addition, DNAs were isolated for hybridization studies from feline strains *P. salivosa* NCTC 11632^T (= VPB 157^T) and VPB 3313, *Porphyromonas circumdentaria* NCTC 12469^T (= VPB 3329^T), VPB 3325, and VPB 3497, and *P. gingivalis* VPB 3492, VPB 3318, and VPB 3498 and human strains *Porphyromonas endodontalis* ATCC 35406^T, *P. gingivalis* ATCC 33277^T, and *Porphyromonas asaccharolytica* ATCC 25260^T.

Growth conditions and biochemical methods. The general methods used for growth and biochemical characterization have been described previously (3, 13–15). Cells for cellular fatty acid and allozyme assays were grown anaerobically for 2 days on 5% sheep blood agar plates (Oxoid blood agar base no. 2; Oxoid, Ltd., Basingstoke, England) supplemented with additional hemin-menadione, formate-fumarate (7), and proteose peptone (1.5%, wt/vol; Difco Laboratories, Detroit, Mich.) in the presence of a streak of *Staphylococcus epidermidis* (to enhance the growth rate and pigmentation of the colonies).

DNA hybridization assays. The cells used for DNA isolation were grown as described above and harvested in 0.15 M NaCl–0.01 M EDTA (pH 8.0). The cells were lysed by adding sodium dodecyl sulfate to a final concentration of 1% (wt/vol). DNAs from the feline strains were isolated by a hydroxyapatite procedure (13), while DNAs from the human type strains and the canine periodontal organisms were isolated essentially by the method of Marmur and Doty (16).

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TABLE 1. Levels of DNA-DNA hybridization for *Porphyromonas* type strains, feline strains, and *P. canoris* sp. nov. strains isolated from canine periodontal pockets

Source of unlabeled DNA		G+C content (mol%)	% Hybridization with labeled DNA from ^a :					
Species or group	Strain		Strain VPB 3492	<i>P. circumdentaria</i> NCTC 14329 ^T	<i>P. salivosa</i> NCTC 11632 ^T	<i>P. canoris</i> NCTC 12835 ^T	<i>P. endodontalis</i> ATCC 35406 ^T	<i>P. gingivalis</i> ATCC 33277 ^T
<i>P. gingivalis</i> cat strains	VPB 3492	50 ^b	100	<1	16	7	4	85
	VPB 3318	51	91	6	21	5	6	73
	VPB 3498	51	84	5	15	4	3	84
<i>P. gingivalis</i>	ATCC 33277 ^T	49	95	7	8	4	5	100
<i>P. circumdentaria</i>	NCTC 12469 ^T	42	15	100	17	4	5	15
	VPB 3325	40	3	92	4	4	3	<1
	VPB 3497	40	15	93	5	2	3	19
<i>P. salivosa</i>	NCTC 11632 ^T	42	18	12	100	4	2	19
	VPB 3313	42	19	13	100	4	4	24
	NCTC 12835 ^T	50 ^c	7	8	7	100	<1	10
<i>P. canoris</i> sp. nov.	VPB 4879	49 ^c	7	6	5	91	2	5
	VPB 4880	49 ^c	3	5	1	85	<1	5
	VPB 4882	51 ^c	3	5	7	84	<1	6
	ATCC 35406 ^T	ND ^d	6	9	4	2	100	6
<i>P. endodontalis</i>	ATCC 35406 ^T	ND ^d	6	9	4	2	100	6
<i>P. asaccharolytica</i>	ATCC 25260 ^T	48	2	4	<1	3	ND	9

^a The reference preparation was normalized to a value of 100%.

^b DNA was extracted by the hydroxyapatite procedure; the thermal melting point was determined with a Gilford spectrophotometer and a thermoprogrammer.

^c DNA was extracted by the method of Marmur and Doty (16).

^d ND, not determined.

G+C content of DNA. Thermal melting points were used to determine the guanine-plus-cytosine (G+C) contents of the DNA preparations as described previously (13). Two preparations were made for each organism, and each preparation was used for two separate determinations.

Preparation of labeled nucleic acids. Fragmented, denatured DNA was labeled with ¹²⁵I by using a variation of the thallium chloride method (21, 28).

DNA homology methods. Fragmented, denatured DNAs were prepared by three passages through a French pressure cell at 16,000 lb/in², followed by heating in a boiling water bath for 5 min; the concentration of each preparation was adjusted to 0.4 mg/ml. DNA homology values were determined by using an S1 nuclease procedure as described previously (9). Each reassociation mixture contained 10 µl of denatured unlabeled DNA (0.01 to 0.03 µg), 50 µl of denatured unlabeled DNA (20 µg), 25 µl of 5.28 M NaCl–2 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.0), and 25 µl of deionized formamide. The reassociation vials were incubated for 20 h at 58°C. To measure background reassociation values and the amounts of nuclease S1-resistant material in the labeled preparations, the denatured unlabeled DNA was replaced with 50 µl (20 µg) of sheared native salmon sperm DNA.

Allozyme electrophoresis. Cells grown for 2 days were harvested from plates with a bent glass rod, suspended in sterile water, and stored at –140°C until they were used. Electrophoresis on Cellologel (Chemetron, Milan, Italy) was performed with supernatant fluids from sonicated cell preparations (Wellcome tube 5 density) by using the method of Richardson et al. (18) to determine the presence of characteristic metabolic pathway enzymes. End points of reactions were determined no later than 15 min after addition of substrate.

Preparation and analysis of fatty acids of whole bacterial cells. Cells were harvested from plates after 2 days with a glass rod (1) and were treated immediately essentially as described by Ghanem et al. (6). A model HP 5890 series II gas chromatograph and a model HP 7673 injector (Hewlett-Packard) were used for detection. The organic phase was chromatographed by using a fused silica capillary column (type

19091J-102-HP). The column running conditions used have been described previously (6). For fatty acid identification a bacterial acid methyl ester CP mixture (catalog no. 4-7080; Supelco, Bellefonte, Pa.) was chromatographed at the beginning of each run and after each four samples. The total area of each compound and the area percentage of each peak were determined by using a computer program. Equivalent chain lengths were calculated as described in the MIS system instructions (Microbial ID, Inc., Newark, Del.).

Spectrophotometric determination of dehydrogenase activity. Portions of the preparations used for allozyme electrophoresis of strains VPB 4878^T, VPB 4879, and VPB 4882 were assayed by using a standard method for glucose-6-phosphate dehydrogenase incorporating maleimide (5). The 6-phosphogluconate dehydrogenase assay was performed like the glucose-6-phosphate dehydrogenase assay except that maleimide was omitted from the assay mixture and 6-phosphogluconate was the substrate. For these assays *Bacteroides fragilis* ATCC 25285^T was used as a species with known dehydrogenase activity (25, 27). The activities reported below are the averages of the values obtained in three separate assays per preparation. The Bio-Rad protein assay (Bio-Rad, Richmond, Calif.) was used as recommended by the manufacturer to determine the protein concentration in each preparation.

RESULTS AND DISCUSSION

Table 1 shows the results of a DNA hybridization assay performed with 4 of the 12 group C2 strains (10) used in this study; in this assay these strains were compared with a representative of each of the previously described species of the genus *Porphyromonas*. The data in Table 1 show that members of group C2 are distinct from the previously described species of the genus *Porphyromonas*. The results of the cellular fatty acid analyses of the group C2 strains are compared with the results obtained for previously described members of the genus *Porphyromonas* in Table 2. The presence of 13-methyltetradecanoic acid (iso-C_{15:0}) as the major acid in these strains is consistent with membership in the genus *Porphyromonas*. It is

TABLE 2. Distribution of cellular fatty acids in *Porphyromonas* strains as determined by gas-liquid chromatography after extraction of methylated whole cells

Strain	Relative % of ^a :														
	C _{14:0}	iso-C _{15:0}	anteiso-C _{15:0}	C _{16:0}	iso-C _{17:0}	C _{18:0}	ECL 12.6	ECL 12.9	ECL 13.6	ECL 16.1	ECL 16.6	ECL 17.5	ECL 17.7	ECL 17.8	ECL 18.15
<i>P. canoris</i> NCTC 12835 ^T	0.4	37.2	2.9	3.5	4.7	1.6	0.4	0.6	8.0	5.1	0.9	1.8	2.7	3.5	12.8
<i>P. canoris</i> VPB 4879	0.5	38.8	3.0	3.6	3.6	1.6	0.3	0.8	9.3	5.1	1.0	1.8	2.5	3.4	20.6
<i>P. canoris</i> VPB 4880	0.6	56.7	1.4	2.8	1.3	1.0	0.3	0.5	15.1	1.8	1.6	0.5	0.9	1.4	11.2
<i>P. canoris</i> VPB 4881	0.7	38.6	3.1	3.5	3.4	1.6	0.3	0	5.3	5.9	1.1	2.2	2.5	3.5	26.1
<i>P. canoris</i> VPB 4882	0.5	52.5	4.1	2.1	2.1	1.0	0.9	0	3.1	6.0	0.6	1.2	1.6	2.2	20.2
<i>P. gingivalis</i> ATCC 33277 ^T	2.5	29.3	9.2	9.7	2.1	1.2	2.0	1.7	11.2	2.1	0	3.8	0	0	12.8
<i>P. salivosa</i> NCTC 11632 ^T	2.3	41.9	9.8	8.0	1.4	1.5	3.9	2.6	17.2	0	1.5	1.3	1.7	3.3	2.9
<i>P. circumdentaria</i> NCTC 12469 ^T	1.4	55.7	1.8	10.9	6.7	1.5	0.4	0	3.2	2.5	0	0.7	1.4	2.3	9.8
<i>P. asaccharolytica</i> NCTC 25260 ^T	1.4	63.4	1.6	4.3	2.6	0.8	1.9	0.5	11.3	1.3	1.7	0.2	0.4	1.3	3.3
<i>P. endodontalis</i> ATCC 35406 ^T	0	31.5	2.6	6.6	0.4	1.1	0.2	0.4	12.5	5.4	1.8	1.1	1.1	1.7	29.0

^a The relative amount of each fatty acid is expressed as the area of its peak as a percentage of the total area of the peaks of all of the fatty acids detected. ECL, equivalent chain length. There was no C_{15:0} in any strain.

generally accepted that the presence of iso-C_{15:0} as the major branched-chain cellular fatty acid is a characteristic of the genus *Porphyromonas* (1, 2, 11, 14, 17, 22–24), and this characteristic is considered by Brondz et al. (1) to constitute a true taxonomic marker of the genus *Porphyromonas*, although iso-C_{15:0} also is a major acid in the genera *Tissierella* and *Reikenella* (17a). However, allozyme electrophoresis of four metabolic enzymes showed that malate and glutamate dehydrogenases were present as required for members of the genus *Porphyromonas* (24) but that glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were also present. In order to check that the latter results were true enzymatic reaction results, the spectrophotometric assay described above was carried out. The average glucose-6-phosphate dehydrogenase activity for the three strains examined was 54.8 nmol mg of protein⁻¹ min⁻¹ (compared with 96.2 nmol mg of protein⁻¹ min⁻¹ for *B. fragilis*), and the average 6-phosphogluconate dehydrogenase activity was 54.4 nmol mg of protein⁻¹ min⁻¹ (compared with 35.9 nmol mg of protein⁻¹ min⁻¹ for *B. fragilis*). The latter results are at variance with the results obtained for other members of the genus

Porphyromonas. However, if the key features of the genus *Bacteroides* (which is the only other genus of anaerobic, gram-negative, nonmotile, nonsporing, rod-shaped bacteria known to have this set of metabolic enzymes) are examined, the group C2 cluster cannot be considered a member of this genus as it has been defined (8, 25, 26). Members of the genus *Bacteroides* are nonpigmented, highly fermentative, rod-shaped organisms which produce major amounts of acetic and succinic acids. Characteristically, they have 12-methyltetradecanoic acid (anteiso-C_{15:0}) as their major long-chain cellular fatty acid. In addition, *Bacteroides* strains contain pentadecanoic acid (C_{15:0}), which is not present in all *Porphyromonas* strains that have been described. Nor are the characteristics of the group C2 strains consistent with the characteristics of other nonfermentative gram-negative anaerobes that have been re-defined recently (4). For example, members of the genus *Tissierella* are motile rod-shaped organisms which do not produce pigment, are indole negative, and have a DNA G+C content of 28 mol%. Table 3 shows biochemical and enzyme reactions which may be helpful in differentiating species within the genus *Porphyromonas*. While the group C2 strains do not fit

TABLE 3. Selected characteristics of members of the genus *Porphyromonas* which may enable differentiation of species: comparison of canine strains of *P. canoris* sp. nov. with feline and human members of the genus

Strain	Catalase activity	Colonies fluoresce at 265 nm	Lipase activity	Hemagglutination of erythrocytes	Phenylacetic acid production	Trypsin-like activity	Chymotrypsin activity	α-Galactosidase activity	β-Galactosidase activity	N-acetyl-β-glucosaminidase activity	α-Fucosidase activity ^a
<i>P. asaccharolytica</i> ATCC 25260 ^T	— ^b	+	—	—	—	—	—	—	—	—	+
<i>P. endodontalis</i> ATCC 35406 ^T	—	+	—	—	—	—	—	—	—	—	—
<i>P. gingivalis</i> ATCC 33277 ^T	—	—	—	+	+	+	—	—	—	+	—
<i>P. gingivalis</i> VPB 3492	+	—	—	+	+	+	—	—	—	+	—
<i>P. salivosa</i> NCTC 11632 ^T	+	— ^c	+	—	+	+	+	+	—	+	—
<i>P. circumdentaria</i> NCTC 124969 ^T	+	+	—	—	+	—	—	—	—	—	—
<i>P. canoris</i> NCTC 12835 ^T	+	+	— ^d	—	—	—	+	—	+	+	—

^a Detected by API ZYM tests (3).

^b +, activity; —, no activity.

^c The type strain does not fluoresce; other strains may fluoresce (13).

^d A yellowish orange pigment is produced on egg yolk agar.

clearly into the genus *Porphyromonas* as currently defined, the available data (for example, comparable G+C contents, non-fermentative metabolism, fatty acid composition, dark pigmentation) suggest that this cluster belongs in an emended genus *Porphyromonas* whose description does not contain a requirement for a lack of glucose-6 phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities.

Description of *Porphyromonas canoris* sp. nov. *Porphyromonas canoris* (can' or. is. L. n. *canis*, dog; L. gen. n. *oris*, of the mouth; N.L. gen. n. *canoris*, of a dog's mouth). Cells are obligately anaerobic, nonsporing, nonmotile, gram-negative rods or coccoid forms. In cooked meat carbohydrate medium (14) and on sheep blood agar plates, cells are 0.3 to 0.6 by 0.8 to 1.5 μm and are arranged singly and in groups; filaments up to 16 μm long are seen occasionally. On sheep blood agar plates, surface colonies after 48 h are 1.5 mm in diameter, circular, and rough and have orange pigmentation. Colonies exhibit brick red fluorescence (265 nm) after 3 and 6 days, and after 13 days the fluorescence is light orange. On laked rabbit or sheep blood medium, colonies are light brown to greenish brown after 2 to 4 days and black after 12 days of incubation. Vitamin K and hemin are required for growth. After incubation for 5 days, the pH values in media containing carbohydrates (uninoculated pH, 6.8 to 7.4) generally range from 6.3 to 6.5. Carbohydrate fermentation is not detected in the presence of adonitol, cellobiose, fructose, glucose, glycogen, inositol, lactose, maltose, mannitol, rhamnose, salicin, starch, sucrose, trehalose, and xylose. Ammonia is produced from cooked meat medium (14).

The short-chain fatty acids produced in cooked meat medium are acetate (average concentration, 36.7 $\mu\text{mol/ml}$), propionate (average concentration, 42.5 $\mu\text{mol/ml}$), isovalerate (average concentration, 10.1 $\mu\text{mol/ml}$), and succinate (average concentration, 15.6 $\mu\text{mol/ml}$); in addition, minor amounts of isobutyrate (average concentration, 2.8 $\mu\text{mol/ml}$) and butyrate (average concentration, 4.3 $\mu\text{mol/ml}$) are produced. No phenylacetic acid is detected. Lactate is converted to propionate, but pyruvate is not utilized, and threonine is not converted to propionate. Strains are catalase positive and indole positive, liquefy gelatin, and are alkaline and acid phosphatase positive, but do not exhibit trypsin-like activity or produce lipase or lecithinase. They produce chymotrypsin, *N*-acetyl- β -glucosaminidase, and β -galactosidase but do not produce α -galactosidase or α -fucosidase. Cells do not hemagglutinate sheep erythrocytes. Nitrate is not reduced, urea is not split, and esculin is not hydrolyzed. Malate, glutamate, glucose-6-phosphate, and 6-phosphogluconate dehydrogenases are present. The predominant cellular fatty acid is 13-methyltetradecanoic acid (iso-C_{15:0}); smaller amounts of the acids shown in Table 2 are also present.

Strains are susceptible to penicillin (2 U/ml), amoxicillin (2.5 $\mu\text{g/ml}$), carbenicillin (100 $\mu\text{g/ml}$), and erythromycin (3 $\mu\text{g/ml}$).

All strains were isolated from subgingival pockets of dogs with naturally occurring periodontitis. The G+C content of the DNA is 49 to 51 mol% as determined by the thermal denaturation method (16). The average level of intraspecies DNA-DNA hybridization is 90%. The average levels of DNA-DNA hybridization between strain VPB 4878^T (= NCTC 12835^T) and other members of the genus *Porphyromonas* are as follows: *P. gingivalis* ATCC 33277^T, 4%; *P. salivosa*, 4%; cat strains of *P. gingivalis*, 5%; *P. endodontalis* ATCC 35406^T, 2%; *P. circumdentaria*, 3%; and *P. asaccharolytica* ATCC 25260^T, 3%.

The type strain is NCTC 12835 (= VPB 4878).

Characteristics which distinguish *P. canoris* sp. nov. from other members of the genus *Porphyromonas* are summarized in Table 3.

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