Genetic Heterogeneity in *Bacteroides asaccharolyticus* (Holdeman and Moore 1970) Finegold and Barnes 1977 (Approved Lists, 1980) and Proposal of *Bacteroides gingivalis* sp. nov. and *Bacteroides macacae* (Slots and Genco) comb. nov.

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Strains of *Bacteroides asaccharolyticus* (Holdeman and Moore 1970) Finegold and Barnes 1977 (Approved Lists, 1980) which had been isolated from human mouths, most often from periodontal pockets, showed remarkably little deoxyribonucleic acid base sequence homology with the deoxyribonucleic acid of strains of the same species which were isolated from nonoral sites. In addition, four strains of catalase-positive *Bacteroides melaninogenicus* subsp. *macacae* Slots and Genco 1980 isolated from periodontal pockets in monkeys were distinct from *B. asaccharolyticus*. With deoxyribonucleic acid immobilized on membrane filters and index deoxyribonucleic acid made radioactive by translation repair with [³H] thymidine triphosphate, we found that the deoxyribonucleic acid homology among the three groups could readily be distinguished by their deoxyribonucleic acid base contents. The nonoral strains had 51 to 52 mol% guanine plus cytosine, the human oral strains had 46.5 to 48.5 mol% guanine plus cytosine, and the monkey catalase-positive strains had 42 to 43 mol% guanine plus cytosine in their deoxyribonucleic acids. Some strains which fit the species description of *B. asaccharolyticus* were not genetically related to the three groups. A new species, *Bacteroides gingivalis* sp. nov., which comprises those strains that resemble *B. asaccharolyticus* but have 46.5 to 48.5 mol% guanine plus cytosine, is described. These strains can also be distinguished serologically and by their production of phenylacetic acid. The type strain is Slots' strain 2561 (=ATCC 33277). *Bacteroides melaninogenicus* subsp. *macacae*, which is comprised of the monkey isolates which resemble *B. asaccharolyticus* but which have a guanine plus cytosine content of 42 to 43 mol% and give a positive catalase reaction, is elevated to species status. Its name is thus *Bacteroides macacae* (Slots and Genco) comb. nov. The type strain of this species is Slots' strain 7728-L6C (=ATCC 33141).

Bacteroides asaccharolyticus (Holdeman and Moore 1970) Finegold and Barnes 1977 (Approved Lists, 1980) (12) was established as a species distinct from *Bacteroides melaninogenicus* (Oliver and Wherry 1921) Roy and Kelly 1939 (Approved Lists, 1980) (12) because of metabolic (3), serological (5), and genetic (20) differences. The latter is often recovered from normal and infected sites in humans (see reference 5), and it has become of great interest to oral microbiologists because of its occurrence in diseases of the periodontium. Slots (13) found that asaccharolytic strains of *B. melaninogenicus* were the predominant gram-negative rods in four of six human periodontal pockets surveyed, and similar organisms were recovered from monkeys (*Macaca arctoides*) during the develop-

ment of experimental periodontal disease (16). Evidence suggests that this species can be correlated with the severity of certain types of periodontal disease, i.e., the resorption of alveolar bone (17).

Despite its distinction from *B. melaninogenicus*, *B. asaccharolyticus* itself may be heterogeneous. Determination of the guanine-plus-cytosine (G+C) contents of the deoxyribonucleic acids (DNAs) of several strains of *B. melaninogenicus* by Shah et al. (11) and by van Steenberg et al. (19) showed a range of values which was greater than would be expected for a species. To ascertain the extent of homogeneity among *B. asaccharolyticus* strains, we determined the base contents (as mol% G+C) and base sequence homologies in DNA extracted from strains iso-

lated from various human and other animal sources. Also included were the DNAs from five strains of *Bacteroides melaninogenicus* subsp. *macacae* Slots and Genco 1980 (15).

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1 together with their sources. Most of the strains were received from J. Slots, State University of New York at Buffalo. Some were received as frozen pellets of cells which had been grown by Slots. Others were sent as live cultures which were then grown in large volumes at the University of Connecticut School of Dental Medicine. The remaining strains were donated by K. Kornman, Department of Periodontology, University of Connecticut School of Dental Medicine. All cultures were identified as to species at the State University of New York at Buffalo

or the University of Connecticut School of Dental Medicine or both. We accepted as *B. asaccharolyticus* only gram-negative, strictly anaerobic rods which produced black or dark brown colonies, did not depress the pH of the broth to below 6.3, and produced acetic, propionic, *n*-butyric, *iso*-butyric, and *iso*-valeric acids but no or little succinic acid. In addition, cultures were checked for the production of indole, the lack of esculin hydrolysis, the lack of nitrate reduction, and the production of catalase (negative for *B. asaccharolyticus*, positive for *B. melaninogenicus* subsp. *macacae*).

Bacteriological tests. Cells were stained with Koppeloff modified Gram stain (4). Cultures were grown in an atmosphere of 5% CO₂-10% H₂-85% N₂ in a plastic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.) and tested for microaerophilic growth in a candle jar. Strains were maintained and checked for purity on 5% sheep blood agar plates containing 1% (wt/vol) Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 1% (wt/vol) peptone (Difco Laboratories, Detroit, Mich.), 0.5% (wt/vol)

TABLE 1. *Strains used in this study*

Strain	Origin	Source ^a
<i>B. asaccharolyticus</i>		
Strains from humans		
2561	Subgingival plaque	J. Slots
W83	Clinical specimen	J. M. Hardie
K110	Gingival debris	R. J. Gibbons
1432	Subgingival plaque	J. Slots
W	Oral cavity	K. Kornman
1021	Subgingival plaque	J. Slots
1312	Subgingival plaque	J. Slots
381	Subgingival plaque	S. S. Socransky
1112	Subgingival plaque	J. Slots
25260 ^b	Empyema	J. Slots
In4	Unknown	H. R. Ingham
536B	Human feces	J. Slots
VPI 4199 (=B477)	Stools	J. Slots
14015	Buttock abscess	W. Miethaner
Cervix A	Cervix abscess	K. J. Wicher
Cervix B	Cervix abscess	K. J. Wicher
785-75A	Mastoid tissue	D. W. Lambe
783-75A	Left flank abscess	D. W. Lambe
750-75A	Ear tissue	D. W. Lambe
Strains from dogs		
720.1	Dog subgingival plaque	K. Kornman
754.1	Dog subgingival plaque	K. Kornman
<i>B. melaninogenicus</i> subsp. <i>macacae</i>		
7728-L6C ^c	Monkey subgingival plaque	J. Slots
7511-L6C	Monkey subgingival plaque	J. Slots [*]
7511-R6B	Monkey subgingival plaque	J. Slots
MD1-17	Monkey subgingival plaque	J. Slots
735.2	Dog subgingival plaque	K. Kornman

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^b Type strain of *B. asaccharolyticus* (received from the American Type Culture Collection, Rockville, Md.).

^c Type strain of *B. melaninogenicus* subsp. *macacae* (15).

yeast extract (BBL), 0.05% NaCl, and 0.5% (wt/vol) glucose supplemented with 5 μ g of hemin per ml and 0.05 μ g of menadione per ml. A peptone-yeast-Trypticase broth of identical composition but without blood or agar was used for routine culture. For the indole, esculin, and nitrate tests, cells were grown in the medium described by Syed and Loesche (18). Acid end products produced in peptone-yeast-Trypticase broth were determined with a Capco 700 gas chromatograph (Clinical Analysis Products Co., Sunnyvale, Calif.) and by the methods described by Holdeman and Moore (4).

Extraction of DNA. DNA was extracted from cells grown in 1-liter peptone-yeast-Trypticase cultures which were harvested by centrifugation, washed twice with distilled water, and then suspended in about 25 ml of saline sodium citrate (0.15 M NaCl plus 0.015 M sodium citrate; SSC). To this suspension was added 1 ml of 25% (wt/vol) sodium dodecyl sulfate. This treatment lysed the cells and seemed to release not only the DNA but also an agent with properties of a deoxyribonuclease because the viscous, lysed material often decreased in viscosity shortly after lysis, and DNA could not be recovered. This deoxyribonuclease effect was countered by adding an equal volume of phenol to the lysed material 1 to 2 min after the addition of sodium dodecyl sulfate. The extraction and purification of the DNA then proceeded essentially as described by Marmur (8) and detailed elsewhere (1). The DNA was quantitated by the absorbance of ultraviolet light at 260 nm and then frozen at -35°C until needed. The purity of the 1-liter cultures was always determined by Gram stain, gas-liquid chromatography, and biochemical tests.

Labeled DNA. The *B. asaccharolyticus* and *B. melaninogenicus* subsp. *macacae* cells did not incorporate exogenous thymidine. Therefore, labeled DNA was produced by the in vitro method outlined by Maniatis et al. (7). Essentially, DNA was sheared by ultrasound (50 W for four 15-s periods), and the resulting nicks and singlestranded ends were repaired with DNA polymerase (EC 2.7.7.7) in the presence of nucleotide triphosphates. The reaction mixture contained 100 μ g of DNA, 3.75 μ g of each nucleotide triphosphate, with the exception of the thymidine triphosphate, which was 2.25 μ g plus 1.5 μ g (about 80 μCi) of [*methyl*- ^3H]thymidine triphosphate (New England Nuclear Corp., Boston, Mass.), 33 U of DNA polymerase in 0.75 ml of 50 mM tris-(hydroxymethyl)aminomethane, 5 mM MgCl_2 , 1 mM dithiothreitol buffer (pH 7.8), and 30 μ g of bovine serum albumin. The reaction proceeded for 3 h at 15°C . The mixture was washed with phenol and chloroform and finally with ether. Then the DNA was separated from unreacted nucleotides by chromatography through a Sephadex G-50 column. The activity of these tritiated DNAs ranged from 0.4×10^5 to 1.2×10^5 CMP/ μ g. The labeled DNAs were analyzed for base content as a test of their resemblance to their unsheared, unlabeled predecessors. Each labeled strain, *B. asaccharolyticus* 25260, *B. asaccharolyticus* 2561, and *B. melaninogenicus* subsp. *macacae* 7728-L6C, denatured at a temperature within 1°C of the denaturation temperature of the native DNAs.

Determination of DNA base composition. The

base composition of the DNA (as mol% G+C) was determined by its thermal denaturation in SSC (9). A Gilford model 2527 programmable cuvette heater was used to heat the sample while the absorbance was continuously recorded on a Gilford model 6051 recorder. Each DNA sample was analyzed at least three times.

DNA hybridization. DNA was hybridized on membrane filters by the dimethyl sulfoxide method of Legault-Démare et al. (2, 6). Unlabeled DNA was denatured by heating at 102 to 105°C for 15 min. This DNA was chilled in ice and diluted to 10 μ g/ml in $6\times$ SSC (i.e., 0.90 M NaCl plus 0.09 M sodium citrate). A total of 50 μ g of this DNA was applied to 25-mm-diameter HAWP filters (Millipore Corp., Bedford, Mass.) which had been soaked in water overnight and for 30 min in $6\times$ SSC and then prerinsed with 5 ml of $6\times$ SSC. After the DNA was applied, the filter was rinsed with 4 ml of $6\times$ SSC and then with 1 ml of $2\times$ SSC (0.30 M NaCl plus 0.03 M sodium citrate). The filters were dried quickly with a heat lamp and then baked at 70°C for 4 h. The filters were then immersed in 1.1 ml of 30% (vol/vol) dimethyl sulfoxide in $2\times$ SSC. After 30 min, 2.5 μ g of denatured ^3H -labeled index DNA contained in $2\times$ SSC was added and allowed to reassociate with the unlabeled filter-borne DNA for 16 h at $\sim 52^{\circ}\text{C}$. The exact temperature was determined from the mol% G+C of the index DNA by the formula of De Ley (2). The filters were then rinsed with $2\times$ SSC and dried, and the hybridized [^3H]DNA was quantitated by liquid scintillation. Filters bearing salmon sperm DNA and *Proteus mirabilis* DNA served as negative controls. The homologous controls were filters bearing DNA from the strain whose DNA served as the radioactive index DNA.

RESULTS

Analysis of the DNA of the *B. asaccharolyticus* strains studied showed clear groupings. Strains from the oral cavity were distinct from those isolated from nondental infections, and the *B. melaninogenicus* subsp. *macacae* strains were also distinct. These divisions were apparent merely by examination of the G+C contents of their DNAs. As seen in Table 2, the nonoral strains, including the type strain of *B. asaccharolyticus* (ATCC 25260) (12), had between 51.6 and 53.4 mol% G+C, with the exception of strain 536B, which had 49.4 mol% G+C. The oral strains had between 46.7 and 49.1 mol% G+C, and the *B. melaninogenicus* subsp. *macacae* strains isolated from monkeys had 43.2–44.5 mol% G+C. Two isolates from dogs, one of which was catalase positive, had base contents below 42 mol% G+C.

These groupings were confirmed by our DNA homology experiments (Table 2). The strains within groups showed a high level of DNA hybridization with the labeled index DNA. DNA of most of the strains hybridized above 70%, relative to the homologous control, although some of the values were slightly lower. Some

TABLE 2. *Extent of DNA hybridization among B. asaccharolyticus and B. melaninogenicus subsp. macacae strains*^a

Source of unlabeled DNA	% Hybridization of index (labeled) DNA from strain:			DNA base content ^b
	2561	25260	7728-L6C	
Human oral genotype strains				
2561	100	9	10	47.1 ± 0.67
W83	108	ND ^c	ND	46.9 ± 0.25
K110	93	13	9	47.7 ± 0.90
1432	97	ND	ND	47.3 ± 0.40
W	110	13	11	49.1 ± 0.83
1021	112	ND	ND	47.6 ± 0.66
1312	141	13	ND	47.5 ± 0.29
381	105	ND	11	46.7 ± 0.80
1112	161	14	ND	47.2 ± 0.51
754.1	98	12	11	48.2 ± 0.56
Nonoral genotype strains				
25260	9	100	11	52.1 ± 0.58
In4	9	110	ND	52.3 ± 0.31
536B	10	67	7	49.4 ± 0.32
VPI 4199	9	43	8	51.6 ± 0.49
14015	7	117	ND	52.8 ± 0.40
Cervix A	10	60	ND	53.4 ± 0.25
Cervix B	10	101	ND	52.3 ± 0.12
785-75A	8	70	ND	52.8 ± 0.53
750-75A	12	106	ND	52.7 ± 0.78
Macacae genotype strains				
7728-L6C	8	9	100	43.7 ± 0.73
7511-L6C	10	ND	119	44.2 ± 0.35
7511-R6B	10	ND	119	43.2 ± 0.68
MD1-17	9	11	66	43.8 ± 0.40
Unrelated strains				
735.2	7	13	10	41.3 ± 0.29
720.1	9	ND	12	41.7 ± 0.35
783-75A	7	12	9	45.2 ± 1.10
Salmon sperm DNA	6	9	11	
<i>P. mirabilis</i>	8	10	11	

^a Expressed as hybrid duplex formation compared with homologous duplex formation.^b Values represent mol% G+C ± the standard deviation.^c ND, Not determined.

DNAs hybridized at very high levels (above 100%, such as strain 1112 DNA, which was most likely an artifact). Strain VPI 4199 and possibly strain Cervix A were of low homology and may represent members of a somewhat divergent group.

The lack of DNA hybridization between strains of different groups was striking and indicates extremely low base-sequence homology. Intergroup hybridization was not significantly greater than hybridization with negative controls.

Three strains failed to join any group. Of these, two were isolated from dogs. One (720.1) was originally thought to be *B. asaccharolyticus*, and the other (735.2) was thought to be *B. melaninogenicus* subsp. *macacae*, based on its positive catalase reaction. The third strain (783-

75A) had been isolated from a nonoral abscess. All three were suspect because each had a G+C content lower than those of others with the same origin or characteristics or both.

DISCUSSION

In a study of strains of the three then recognized subspecies of *B. melaninogenicus* isolated from various sources, Shah et al. (11) found that the strains now called *B. asaccharolyticus* formed two groups when they were arranged by either their DNA base contents or the electrophoretic mobilities of their malate dehydrogenases. The base contents of some were about 50.5 mol% G+C, and those of the others were about 45.8 mol% G+C. This 4.7% difference is predictive of genetic heterogeneity. Later, van Steenberg et al. (19) found a similar dichotomy in

B. asaccharolyticus strains and suggested that genetic heterogeneity existed in the species. When the sources of the strains used in these studies are compared, there is a suggestion that oral strains have lower DNA base contents. The recent discoveries that only oral strains of *B. asaccharolyticus* would agglutinate sheep erythrocytes (14) and that there are serological differences between nonoral and oral strains (M. J. Reed, J. Slots, C. Mouton, and R. J. Genco, manuscript in preparation) further suggest molecular differences between the oral and nonoral strains.

The results reported here show that the difference between oral and nonoral strains of *B. asaccharolyticus* is very large and that two distinct genotypes exist within this species. The hybridization experiments indicate surprisingly little similarity between the central informational molecules (DNA) of these two genotypes. Similarly, the *B. melaninogenicus* subsp. *macacae* strains are genetically quite distinct from the *B. asaccharolyticus* strains. It should be pointed out that the division of oral from nonoral strains does not preclude the existence of the oral genotype in other sites or in nonoral infections and vice versa, but it does appear that the oral genotype favors the mouth as its ecological niche.

The fact that the two *B. asaccharolyticus* genotypes and the *B. melaninogenicus* subsp. *macacae* genotype did not account for all of our strains suggests that even more genotypes will be found. The human nonoral strain 783-75A, which had all the phenotypic features of *B. asaccharolyticus*, proved to be completely unrelated to all other such strains. Its base content was noticeably lower. Although *B. asaccharolyticus* strain 754.1, from the mouth of a dog, proved to be homologous with human oral strains, strain 720.1 from the same habitat was clearly different. A third canine strain, 735.2, which was catalase positive, was not related to the catalase-positive *B. melaninogenicus* subsp. *macacae* strains from monkeys. Perhaps several genotypes of these asaccharolytic organisms exist, each with a preferred host.

The human oral strains of *B. asaccharolyticus* are clearly different from the nonoral strains. The catalase-positive strains isolated from monkeys are distinct from both oral and nonoral human strains. It seems useful and appropriate to consider these two groups of microbes as two species separate from *B. asaccharolyticus*. Therefore, we propose the name *Bacteroides gingivalis* sp. nov. for the strains isolated from human periodontal areas. *Bacteroides melaninogenicus* subsp. *macacae* is elevated to species rank, and its name thus becomes *Bacteroides*

macacae (Slots and Genco) comb. nov. Descriptions of these two species follow.

***Bacteroides gingivalis* sp. nov.** (L.n. *gingiva* gum; L. gen. n. *gingivalis* of the gums). Gram-negative rods which occur as coccobacilli or as long rods (0.5 by 1.0 to 2.0 μ m). Strictly anaerobic. On blood agar containing hemin and menadione, cells form small (1 to 2-mm), convex colonies which become brownish and then black in 7 to 10 days, although the pigment rate can vary among strains. Sugars are not fermented. Cultures do not reduce nitrate, hydrolyze esculin, or produce catalase. Indole is produced. The end products of metabolism are *n*-butyric, *iso*-butyric, propionic, *iso*-valeric, and acetic acids; succinic acid is either not produced or is produced in small quantities. The DNA base composition is 46.5 to 48.4 mol% G+C.

Type strain. Strain Slots 2561 is designated as the type strain. It was isolated by Slots from a human gingival sulcus. Its description conforms to that of the species, and it has a DNA base content of 47.1 mol% G+C. This strain has been deposited in the American Type Culture Collection. Its accession number is ATCC 33277.

Additional comments. *B. gingivalis* is distinguished from *B. asaccharolyticus* by its base composition, which is 4.6 mol% lower than that of *B. asaccharolyticus*. Slots and Genco have found that the strains here named *B. gingivalis* do not react with a commercial fluorescent antibody staining system (Fluoretec M., Pfizer Inc., New York, N.Y.), a difference which may aid in discriminating *B. asaccharolyticus* from *B. gingivalis*. With an indirect fluorescence technique, they could also distinguish the two species by specific antisera (Reed et al., in preparation). Slots and Genco (14) also found that oral *B. asaccharolyticus* strains agglutinated sheep erythrocytes, whereas nonoral strains did not. A potentially useful and simple test for differentiating these strains may be the production of phenylacetic acid by the oral strains (10). Although we have detected phenylacetic acid in our cultures of *B. gingivalis*, we have consistently failed to find this product in (nonoral) *B. asaccharolyticus* cultures (F. S. Kaczmarek and A. L. Coykendall, manuscript in preparation).

***Bacteroides macacae* (Slots and Genco) comb. nov.** (Basonym: *Bacteroides melaninogenicus* subsp. *macacae* Slots and Genco) (ma.cá cae, M.L. fem. n. *Macaca* genus name of the macaque; M.L. gen. n. *macacae* of the macaque). Gram-negative, coccoid to rod-shaped cells, 0.50 $\times$ 1.0 to 1.5 μ m. Strictly anaerobic. On blood agar containing hemin and menadione, cells form small (0.5 to 1.5-mm), colonies which become grayish-green and then black after prolonged incubation. Catalase and indole are pro-

duced, and arginine is hydrolyzed. Nitrate is not reduced. Starch is not hydrolyzed. The pH of glucose broth is reduced by about 1.1 units. The end products of metabolism are acetic and propionic acids and small amounts of *iso*-butyric, butyric, *iso*-valeric, and succinic acids. The DNA base content is 43 to 44.5 mol% G+C.

Type strain. Strain Slots 7728-L6C is the type strain of the species. The strain has been deposited in the American Type Culture Collection as ATCC 33141. The description of this strain conforms to that of the species. Its DNA base content is 43.7 mol% G+C.

Additional comments. The strains that are described here as *B. macacae* were originally isolated and described by Slots and Genco (15). Their paper details the conditions under which these bacteria were grown and reports the results of serological experiments that showed that *B. macacae* is serologically distinct from other black-pigmenting species and subspecies.

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REPRINT REQUESTS

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