Confirmation of *Bacteroides gingivalis* as a Species Distinct from *Bacteroides asaccharolyticus*

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We divided the asaccharolytic, black-pigmented *Bacteroides* strains into two groups on the basis of deoxyribonucleic acid (DNA) base ratios, DNA hybridization (S1 nuclease method) results, and direct hemagglutination. One homologous group of strains, which included the type strain of *Bacteroides asaccharolyticus* and had guanine-plus-cytosine contents of 52 to 54 mol%, contained only nonoral isolates. Another DNA homology group contained all of the strains from periodontal pockets and some nonoral isolates. These strains appeared to belong to the recently described new species *Bacteroides gingivalis* Coykendall et al. *B. gingivalis* strains had guanine-plus-cytosine contents of 48 to 50 mol%, showed high DNA homology values, and shared hemagglutinating activity. No DNA homology was observed between the two groups. Using a different method of analysis for DNA homology (S1 nuclease method), we confirmed the conclusion of Coykendall et al., who separated these two groups into different species, *B. asaccharolyticus* and *B. gingivalis*. Two strains from infected root canals could not be placed in either of these two species. On the basis of the DNA homology results, all asaccharolytic strains were distinguished clearly from the saccharolytic, black-pigmented *Bacteroides* strains, which at present are classified in the species *Bacteroides melaninogenicus*.

The asaccharolytic, black-pigmented strains originally identified as members of *Bacteroides melaninogenicus* have been reclassified as members of *Bacteroides asaccharolyticus* (6). Recently, Coykendall et al. (2) proposed separating these strains into the following two different species: *B. asaccharolyticus* and a new species, *Bacteroides gingivalis*. *B. asaccharolyticus* strains can be isolated from many different kinds of infection (5), whereas high numbers of *B. gingivalis* are isolated from periodontal pockets (19, 22). The genetic heterogeneity within the group of asaccharolytic, black-pigmented *Bacteroides* strains is shown by the deoxyribonucleic acid (DNA) base contents of these organisms; one group has low guanine-plus-cytosine (G+C) values, and another has high G+C values (17, 24). In a preliminary report (25), we stated that these two groups showed no DNA-DNA hybridization. These groups were also heterogeneous with respect to polypeptide content (23), electrophoretic mobility of the enzyme malate dehydrogenase (17), fatty acid composition, and isoprenoid quinone composition (16). Differences between oral and nonoral asaccharolytic, black-pigmented *Bacteroides* strains were demonstrated by direct hemagglutination (20), by morphological and immunochemical methods (11), by antigenic studies (13, 15), and by the

production of phenylacetic acid (9). In this paper we present the results of studies on DNA hybridization and G+C contents and hemagglutination by asaccharolytic, black-pigmented *Bacteroides* strains. Our results confirm the proposal by Coykendall et al. of a new species, *B. gingivalis*, which is different from *B. asaccharolyticus* (2).

MATERIALS AND METHODS

Bacterial strains. The strains which we studied are listed in Table 1.

Isolation of strains and maintenance of cultures. To isolate black-pigmented *Bacteroides* strains from periodontitis, plaque samples from deep periodontal pockets were inoculated directly onto BM blood agar plates (24), which were then incubated anaerobically at 37°C for at least 1 week. Black-pigmented colonies were subcultured repeatedly on the same medium until pure cultures were obtained. Strains were maintained on blood agar plates, and study cultures were grown in liquid BM medium as described previously (24).

Biochemical characteristics. Fermentation of glucose was determined by measuring the final pH in BM medium. Other biochemical characteristics were determined by using the API 20A system (Analytab Products, Inc.).

Hemagglutination test. Direct hemagglutination of sheep and horse erythrocytes was determined by the slide test described by Slots and Genco (21).

TABLE 1. *Strains used in this study*

Strain	Source	Site of isolation
<i>B. asaccharolyticus</i>		
ATCC 25260 (VPI 4198, B 440)	Shah ^a	Empyema
ATCC 27067 (VPI 5834)	ATCC ^b	Human leg wound
NCTC 9337 (VPI 8945)	Shah	Infected hemorrhoids
VPI 4199 (B 477)	Shah	Human feces
B 536	Shah	Human feces
<i>B. gingivalis</i>		
W 83	Shah	Clinical specimen
2848	Shah	Perforated appendix
W 50	Shah	Clinical specimen
381	Socransky ^c	Periodontitis
376	Socransky	Periodontitis
382	Socransky	Periodontitis
H 184	This laboratory	Periodontitis
H 185	This laboratory	Periodontitis
H 186	This laboratory	Periodontitis
Asaccharolytic, black-pigmented <i>Bacteroides</i> strains		
H 11 a-e	Sundqvist ^d	Infected root canal
BN 11 a-f	Sundqvist	Infected root canal
<i>B. melaninogenicus</i> subsp. <i>melaninogenicus</i>		
ATCC 25845 (VPI 4196, B 282)	Shah	Sputum
WAL 2728	Shah	Fecal fluid
<i>B. melaninogenicus</i> subsp. <i>intermedius</i>		
ATCC 25611 (VPI 4197, B 422)	ATCC	Empyema
LH 100	Shah	Dental plaque
" <i>B. melaninogenicus</i> subsp. <i>levii</i> "		
Lev	Shah	Bovine rumen
JP 2	Shah	Unknown

^a H. N. Shah, London Hospital Medical College, London, England.

^b ATCC, American Type Culture Collection, Rockville, Md.

^c S. S. Socransky, Forsyth Dental Center, Boston, Mass.

^d G. Sundqvist, University of Umeå, Umeå, Sweden.

Isolation of unlabeled DNA. Unlabeled DNA was isolated from sodium dodecyl sulfate-lysed cells as described previously (24) or from cells lysed mechanically with a Vibrogen cell mill (Bühler, Tübingen, Germany) by the method of Meyer and Schleifer (12).

Isolation of ³H-labeled DNA. Strains were grown anaerobically at 37°C for 24 h in BM medium (17). Then the bacteria were diluted 1:100 in 10 ml of BM medium containing 10 µCi of [³H]thymidine per ml or 10 µCi of [³H]deoxyadenosine (Radiochemical Centre, Amersham, England) per ml. After each culture was grown for an additional 24 h, its purity was checked by aerobic and anaerobic incubations on blood agar plates. The cells were harvested by centrifugation, washed in 50 mM tris(hydroxymethyl)aminomethane-5 mM ethylenediaminetetraacetate-50 mM CaCl₂ (pH 8.0) buffer (TES buffer), and resuspended in 3 ml of TES buffer. The cells were lysed by adding 10 mg of Sarkosyl (Ciba/Geigy) per ml and 50 µg of proteinase K (Merck) per ml, and the mixture was incubated for 1 h at 37°C. To the lysate, we added 6 g of CsCl, 2.8 ml of TES buffer, and 0.2 ml of an ethidium bromide solution (10 mg/ml in TES buffer). Density gradient centrifugation was performed with an International Equipment Co. model B 60 ultracentrifuge and a 20 × 14 rotor for 48 h at 36,000 rpm and 15°C. Fractions were collected with a tube piercer (Hoefer Scientific Instruments, San Francisco, Calif.); after trichloro-

acetic acid precipitation 10-µl samples were counted with a Berthold liquid scintillation counter. The fractions containing the ³H-labeled DNA were pooled, extracted with CsCl-saturated isopropanol to remove the ethidium bromide, and treated ultrasonically for 5 s with an ultrasonic disintegrator (Measuring & Scientific Equipment, Ltd.) to shear the DNA into fragments of about 7S, as determined by sucrose gradient centrifugation. The DNA was dialyzed against 0.42 M NaCl. The concentration of the DNA was measured, and the purity was checked with a Gilford spectrophotometer.

DNA-DNA hybridization. A 0.013-µg amount of [³H]thymidine-labeled DNA and 20 µg of unlabeled DNA were mixed in 200 µl of a 0.42 M NaCl solution, denatured for 10 min at 100°C, and renatured for 90 h at 60°C to obtain a C₀t of 100 mol·s/liter (1). The amount of reassociation was determined by the S1 nuclease method described by Crosa et al. (3), using 100 U of S1 nuclease (Boehringer Mannheim Corp.) per ml in a 30 mM sodium-acetate buffer (pH 4.5) containing 0.1 mM ZnSO₄, 0.15 M NaCl, and 20 µg of sheared denatured calf thymus DNA (type V; Sigma Chemical Co.) per ml. The mixture was incubated for 20 min at 50°C, precipitated in 5% trichloroacetic acid, and filtered on membrane filters (type HA; Millipore Corp.). The DNA was counted in a PPO (2,5-diphenyloxazole)-POPOP [1,4-bis-(5-phenyloxazolyl)ben-

zene]-toluene scintillation mixture. Hybridization was determined as a percentage of the homologous reaction. Values were corrected for self-reassociation of the tritiated DNA; calf thymus DNA was used as a control.

Determination of DNA base composition. DNA base composition was determined both by the buoyant density method and by the thermal denaturation method. The buoyant density was determined from a CsCl density gradient obtained in an analytical ultracentrifuge by a previously described method (24). The midpoint of thermal denaturation was determined in a 0.15 M sodium chloride-0.015 M sodium citrate buffer by the method of Mandel and Marmur (10). The temperature absorbance profile was measured with a Gilford model 250 spectrophotometer equipped with a Gilford thermoprogrammer. The G+C contents of the DNA samples were calculated by using the formula of De Ley (4).

RESULTS

To isolate asaccharolytic, black-pigmented *Bacteroides* strains, plaque samples were obtained from deep periodontal pockets in 16 adult patients undergoing periodontal therapy. From

these samples, we isolated 15 black-pigmented strains, 3 of which did not ferment glucose or other sugars.

Some properties of these asaccharolytic isolates and of other black-pigmented *Bacteroides* strains (obtained from different laboratories) are listed in Table 2. With the asaccharolytic strains, the final pH in BM medium ranged from 6.7 to 7.3, whereas the cultures of the saccharolytic strains of the different subspecies of *B. melaninogenicus* had terminal pH values of 5.0 to 5.4. All of the asaccharolytic strains from periodontal pockets agglutinated both sheep and horse erythrocytes. The three nonoral asaccharolytic isolates (isolates W 83, 2848, and W 50) were also hemagglutination positive. The other nonoral asaccharolytic strains, the two asaccharolytic strains from infected root canals (strains H 11 a-e and BN 11 a-f), and all of the *B. melaninogenicus* strains tested showed no hemagglutination. All strains were catalase negative. The G+C contents of the DNAs of the oral and nonoral, hemagglutination-positive strains varied between 48 and 50 mol%. These strains were

TABLE 2. Properties of the black-pigmented *Bacteroides* strains studied

Strain	Final pH (glucose medium)	Hemaggluti- nation	G+C content (mol%) of DNA	
			Buoyant density method	Melting point method
<i>B. asaccharolyticus</i>				
ATCC 25260	6.7	—	53.0	53.8
ATCC 27067	6.5	—	54.1	52.2
NCTC 9337	6.6	—	53.0	52.3
VPI 4199	7.0	—	54.1	53.7
B 536	7.1	—	54.2	ND ^a
<i>B. gingivalis</i>				
W 83	7.1	+	48.2	50.2
2848	6.5	+	48.0	49.0
W 50	6.9	+	48.6	48.8
381	6.7	+	47.8	49.5
376	6.5	+	48.7	47.3
382	7.0	+	49.8	ND
H 184	6.8	+	49.7	ND
H 185	7.0	+	50.1	ND
H 186	6.9	+	49.9	ND
Asaccharolytic, black-pigmented <i>Bacteroides</i> strains				
H 11 a-e	6.9	—	50.6	ND
BN 11 a-f	7.0	—	51.1	ND
<i>B. melaninogenicus</i> subsp. <i>melaninogenicus</i>				
ATCC 25845	5.0	—	41.9	43.3
WAL 2728	5.3	—	41.9	41.8
<i>B. melaninogenicus</i> subsp. <i>intermedius</i>				
ATCC 25611	5.3	—	44.6	42.9
LH 100	5.2	—	41.6	42.9
" <i>B. melaninogenicus</i> subsp. <i>levii</i> "				
Lev	5.3	ND	46.6	46.0
JP 2	5.4	ND	45.0	46.2

^a ND, Not determined.

identified as members of *B. gingivalis* (2). The asaccharolytic strains H 11 a-e and BN 11 a-f had G+C contents of about 51 mol%, whereas the asaccharolytic, hemagglutination-negative strains had G+C contents of 53 to 54 mol%. The latter group could be identified as strains of *B. asaccharolyticus*. The G+C contents of the DNAs of the saccharolytic strains of the different subspecies of *B. melaninogenicus* were much lower; these values were about 42 to 44 mol% for *B. melaninogenicus* subsp. *melaninogenicus* and *B. melaninogenicus* subsp. *intermedius* and about 46 mol% for "*B. melaninogenicus* subsp. *levii*" (names in quotation marks are not on the Approved Lists of Bacterial Names [18]). There were no significant differences between the G+C values obtained by the buoyant density and the melting point methods.

In the DNA-DNA hybridization studies, the DNAs of the two *B. gingivalis* strains (strains W 83 and 2848) were labeled with tritiated thymidine. The type strain of *B. asaccharolyticus* (strain ATCC 25260) and strain ATCC 27067 did not incorporate [^3H]thymidine into their DNAs;

however, the DNAs of these strains could be labeled with tritiated deoxyadenosine.

Table 3 shows the results of the hybridization experiments with these strains. The recovery of label in the homologous reaction was between 70 and 95% for strains W 83 and 2848, and this value was between 40 and 60% for strains ATCC 25260 and ATCC 27067. These results demonstrated the existence of two main groups of asaccharolytic, black-pigmented *Bacteroides* strains. The five nonoral strains, including ATCC 25260, form a rather homogeneous group, which has DNA homology values of 64 to 100%. The DNA hybridization values with *B. gingivalis* and *B. melaninogenicus* strains were less than 20%. The second homology group, corresponding to *B. gingivalis*, included the nonoral strains W 83, 2848, and W 50 and all of the asaccharolytic strains isolated from periodontal pockets; these strains showed DNA homology values of 67 to 100% with each other and of 22% or less with saccharolytic and other asaccharolytic strains. Strains H 11 a-e and BN 11 a-f could not be placed in either of these two groups.

TABLE 3. Relative hybridization of DNAs from black-pigmented *Bacteroides* strains with reference DNAs from four asaccharolytic strains

Source of unlabeled DNA	Mean G+C content of DNA (mol%)	Relative % hybridization to labeled reference DNA from:			
		ATCC 25260	ATCC 27067	W 83	2848
<i>B. asaccharolyticus</i>					
ATCC 25260	53	100	70	22	8
ATCC 27067	53	72	100	ND ^a	4
NCTC 9337	53	66	72	1	6
VPI 4199	54	72	64	1	20
B 536	54	72	ND	0	6
<i>B. gingivalis</i>					
W 83	49	9	8	100	86
2848	48	19	ND	67	100
W 50	49	17	6	98	100
381	49	7	2	81	92
376	48	0	ND	85	81
382	50	0	ND	83	79
H 184	50	ND	ND	85	87
H 185	50	10	ND	81	72
H 186	50	20	ND	88	80
Asaccharolytic, black-pigmented <i>Bacteroides</i> strains					
H 11 a-e	51	6	ND	0	5
BN 11 a-f	51	20	ND	0	15
<i>B. melaninogenicus</i> subsp. <i>melaninogenicus</i>					
ATCC 25845	43	2	0	0	ND
WAL 2728	42	ND	ND	0	20
<i>B. melaninogenicus</i> subsp. <i>intermedius</i>					
ATCC 25611	44	0	ND	1	ND
LH 100	42	0	18	1	ND
" <i>B. melaninogenicus</i> subsp. <i>levii</i> "					
Lev	46	10	3	3	ND
JP 2	46	ND	ND	ND	1

^a ND, Not determined.

DISCUSSION

There has been considerable confusion concerning the taxonomy of black-pigmented *Bacteroides* strains. In *Bergey's Manual of Determinative Bacteriology*, 8th ed., the following three different subspecies of *B. melaninogenicus* were described (8): *B. melaninogenicus* subsp. *melaninogenicus*, *B. melaninogenicus* subsp. *intermedius*, and "*B. melaninogenicus* subsp. *asaccharolyticus*." Subsequently, two other subspecies were added, "*B. melaninogenicus* subsp. *levii*" and *B. melaninogenicus* subsp. *macacae* (7, 21). The asaccharolytic strains have been reclassified as a separate species, *B. asaccharolyticus* (Holdeman and Moore) Finegold and Barnes (6). Recently, Coykendall et al. (2) proposed separating these asaccharolytic strains into the following two species on the basis of DNA homology relationships: *B. asaccharolyticus*, containing the nonoral strains, and *B. gingivalis*, containing the oral strains, mostly from periodontal pockets. In the study reported here, we confirmed the existence of these two groups of strains on the basis of DNA-DNA hybridization, DNA base contents, and hemagglutination activity.

Hemagglutination by 29 of 59 black-pigmented *Bacteroides* strains was reported by Okuda and Takazoe (14); however, these authors did not identify these strains at the subspecies level. Slots and Genco (20) reported that oral asaccharolytic strains agglutinate sheep erythrocytes, whereas nonoral strains do not. Our results generally agree with this observation; i.e., all of the strains from periodontal pockets which we studied were hemagglutination positive. However, we also found three nonoral strains that agglutinated erythrocytes. Therefore, we conclude that hemagglutination is a property of *B. gingivalis* that can be used as an easy and valuable differential character for this species. If one assumes that dental plaque is the ecological niche for *B. gingivalis*, this could mean that the nonoral hemagglutinating strains of *B. gingivalis* originate from the oral cavity. Strains H 11 a-e and BN 11 a-f, which were isolated from infected root canals, were hemagglutination negative and did not belong to either *B. gingivalis* or *B. asaccharolyticus*. From these observations, we conclude that in order to identify an isolate, a worker should not rely merely on the site of isolation (either oral or nonoral), for obviously there are different ecological niches within the oral cavity which harbor different organisms; besides, it is possible that strains migrate to different parts of the body.

In general, the G+C contents which we found confirm previous observations (2, 6, 17, 24). Con-

trary to the results of Coykendall et al. (2), we did not find a low (with respect to other *B. asaccharolyticus* strains) G+C content in the DNA of strain B536. Because of differences between laboratories, it is difficult to compare the G+C values obtained by different workers for the same strains. In this study, both the melting point method and the buoyant density method were used to determine the G+C contents of the DNAs; however, we consider the values obtained by the buoyant density method more reliable because this method is less sensitive to impurities in DNA preparations (24).

We have described previously the existence of two DNA homology groups of asaccharolytic strains (25). Johnson and Moore reported the existence of the same two main DNA homology groups of asaccharolytic, black-pigmented *Bacteroides* strains (J. L. Johnson and W. E. C. Moore, J. Dent. Res. 59A:511, 1980).

Recently, Coykendall et al. (2) proposed the species *B. gingivalis* as a species distinct from *B. asaccharolyticus*. Our results, which were obtained by using a different method to detect DNA-DNA homology, essentially confirm this proposal. Furthermore, our DNA hybridization studies strengthen the conclusion that all asaccharolytic strains should be separated from strains of *B. melaninogenicus*.

In this work, only a limited number of strains were studied. We expect that additional studies of more asaccharolytic, black-pigmented *Bacteroides* strains will reveal additional groups. The strains which were isolated from infected root canals and which we were unable to identify as either *B. asaccharolyticus* or *B. gingivalis* probably represent such a new group of strains, and very likely they will have to be placed into a new species.

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REPRINT REQUESTS

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