Taxonomic Study of *Bacteroides oralis* and Related Organisms and Proposal of *Bacteroides veroralis* sp. nov.

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The genetic and phenotypic properties of 18 strains which were considered to be *Bacteroides oralis* or related organisms were examined. These strains were differentiated into several groups on the basis of deoxyribonucleic acid homology, physiological properties, guanine-plus-cytosine content of the deoxyribonucleic acid, and electrophoretic patterns of cell proteins. The groups were considered to constitute separate species. Strains of group I (strains VPI 7570A, WAL 3030, and 1221) belonged to the newly described species *Bacteroides denticola*. Strain VPI 8906D (group II) has been designated a new species, *Bacteroides buccalis*, by Shah and Collins. Strain VPI D27B-24 (group III) is the type strain of *B. oralis*. However, the biological properties of none of the three groups described above fit the original description of *B. oralis* (Loesche et al. 1964). Strain VPI D22A-7^T did not belong to any of the three groups but had phenotypic properties that matched the phenotypic properties given in the first description of *B. oralis*. We propose the name *Bacteroides veroralis* for this organism and have deposited strain VPI D22A-7 in the American Type Culture Collection (= ATCC 33779) as the type strain.

Bacteroides oralis was first described by Loesche et al. (16) as a gram-negative anaerobic bacillus from the oral cavity of humans. The biochemical characteristics of *B. oralis* reportedly are similar to those of *Bacteroides melaninogenicus* subsp. *melaninogenicus* and *Bacteroides ruminicola* (6, 16, 25), except that *B. melaninogenicus* produces a black pigment (7, 15) and *B. ruminicola* requires hemin for growth and ferments pentose (1). The type strain of *B. oralis* designated by Loesche et al. (16) apparently has been lost, and the strain now designated 7CM has been reclassified as *Bacteroides vulgatus* (23). A second strain deposited by Loesche et al. as representative of *B. oralis* (strain Loesche 8B [= ATCC 15930]) was reclassified as *B. melaninogenicus* subsp. *melaninogenicus* (4) and is now the type strain of *Bacteroides loescheii* (8). *B. oralis* VPI D27B-24 (= ATCC 33269) has been designated the type strain of *B. oralis*.

The present study was undertaken to clarify the relationships of strains which are similar to *B. oralis* (5, 23, 27, 29); this was done by examining the physiological and biochemical characteristics, deoxyribonucleic acid (DNA) base compositions, DNA homologies, and protein electrophoretic mobility patterns of these strains.

MATERIALS AND METHODS

Bacterial strains. We studied the following strains of *B. oralis* and similar organisms: VPI 7570A, from T.

Hofstad; WAL 3030, from S. M. Finegold; 1221, from S. S. Socransky; WPH 179, from J. G. Collee; 5540 and 7880, from H. Werner; VPI 8906D, VPI D22A-7^T, VPI E1S-8, VPI D25B-5, VPI 5832, VPI 9958, VPI D27B-24^T (= ATCC 33269^T [type strain of *B. oralis*]), VPI 6822^T (= ATCC 29303^T [type strain of *B. bivius*]), and VPI 6318, from W. E. C. Moore; HS4^T (= NCDO 2354^T [type strain of *B. buccalis*]), from J. M. Hardie; B56020 (*B. ruminicola*-*B. oralis* group III), from our collection; and ATCC 25845^T (type strain of *B. melaninogenicus* subsp. *melaninogenicus*), from the American Type Culture Collection.

Maintenance of cultures. Strains were grown on modified Eggerth-Gagnon (EG) agar (19) and in peptone yeast extract Fildes solution glucose broth (13) at 37°C in anaerobic steelwool jars under an atmosphere containing 100% CO₂. All strains were maintained on prereduced EG agar liver slants with H₂CO₃-CO₂ buffer (19) and stored at 4°C. New transfers were made at monthly intervals.

Cultural and biochemical tests. The methods used in this study for cultural and biochemical characterization of the strains have been described previously (11). For fermentation tests, the pH after 7 days of incubation was measured with a glass electrode. A pH decrease of 0.4 to 0.5 U below the pH values of control cultures grown without fermentable carbohydrate was interpreted as weak fermentation, and greater decreases were considered positive. Fatty acid production was analyzed by the method of Holdeman et al. (6). Colonies on EG agar supplemented with 5% laked rabbit or human blood were examined for pigmentation. Colonies were held for 7 days before recording absence of pigmentation.

Preparation of DNA. For the preparation of DNA, cells were grown in peptone yeast extract Fildes

solution glucose broth. Cells were harvested in the late logarithmic phase of growth and washed twice with 0.15 M NaCl–0.1 M ethylenediaminetetraacetic acid (pH 8.0). For the preparation of tritium-labeled DNA, the organisms were grown in prereduced EG broth containing [*methyl*-³H]thymidine (1 μ Ci/ml; Radiochemical Centre Ltd., Amersham, England). DNA was isolated by a modification of the procedures of Marmur (17, 22). DNA purity was assessed by the ratio of extinction at 230 nm to extinction at 260 nm. The amount of DNA was estimated by measuring the hyperchromic shift during thermal denaturation (3).

DNA base composition. The guanine-plus-cytosine (G+C) contents of the DNA preparations were determined by the thermal melting point method (18), using an automatic recording spectrophotometer (Komatsu Electronics, Tokyo, Japan). Calf thymus DNA was included in each test as a standard.

DNA homology experiments. The methods used for immobilizing DNA on nitrocellulose filters and for DNA competition experiments were the procedures of Johnson and Cummins (11), with some modification. Native DNA (50 μ g/ml) was denatured by boiling for 10 min in 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0), and the denatured DNA was immobilized on 4.9-mm nitrocellulose membrane filters (Toyo Roshi Ltd., Tokyo, Japan) by slow filtration. At the time of an experiment, small filters (3 by 9 mm) were cut from a large filter, and each small

filter contained 4 to 8 μ g of DNA. The labeled DNA and competitor DNA were sonicated twice (30 s each time) in 0.1 $\times$ SSC and denatured by heating at 100°C for 10 min. Then 10 μ l (3,000 cpm/10 μ l) of labeled DNA fragments homologous to the DNA on the filter and 100 μ l (containing 100 μ g of DNA) of competitor DNA in 2.2 $\times$ SSC were added to small incubation vials. The vials were incubated for 24 h at a temperature 25°C below the thermal melting point of the reference DNA in 1 $\times$ SSC. The amount of DNA bound to the filter DNA was estimated by counting the radioactivity of the filter, using a liquid scintillation counter (Packard model 3330). DNA homology experiments were also performed by using the S1 nuclease procedure, as described by Johnson et al. (12). S1 nuclease digestion was conducted with 0.5 U of S1 nuclease (Seikagaku Kogyo Co., Tokyo, Japan). After incubation for 15 min at 37°C, an equal volume of 10% trichloroacetic acid was added to each tube. The tubes were cooled in a refrigerator at 4°C for at least 1 h, and the precipitates were collected on nitrocellulose membrane filters (type HA; Millipore Corp., Bedford, Mass.). The membranes were dried, and the radioactivity was measured.

Preparation of cell-free extracts. Cells were grown anaerobically in peptone-yeast extract-Fildes solution-glucose broth for 24 h at 37°C. Soluble enzyme extracts and acid-phenol-soluble proteins were prepared from the same batch of cells. Cells were harvested by

FIG. 1. Morphology of cells from 2-day-old surface colonies on EG agar. Bar = 10 μ m. (A) *B. denticola* VPI 7570A. (B) *B. buccalis* VPI 8906D. (C) *B. oralis* VPI D27B-24^T. (D) *B. veroralis* VPI D22A-7^T. (E) *B. bivia* 7880. (F) Strain VPI E1S-8. (G) Strain VPI D25B-5. (H) Strain WPH 179. (I) Strain B56020.

centrifugation at $8,000 \times g$ for 10 min and washed twice with 0.05 M phosphate buffer (pH 7.4) containing 0.04% (vol/vol) 2-mercaptoethanol. The cells were resuspended in the same buffer (150 mg [wet weight] per ml) and were subjected to ultrasonic treatment for 1 to 3 min at 0°C . After centrifugation at $20,000 \times g$ for 30 min, the supernatants were used for electrophoretic enzyme assays; 1 ml of fluid was mixed with 0.15 ml of 0.1% bromophenol blue in 40% sucrose, and the preparation was stored at -20°C until it was used.

The cell pellets were washed twice in 0.25 M NaCl and suspended in the same solution. The amount of protein was determined and adjusted to give 10 mg of protein per ml. The cell proteins then were extracted with phenol-acetic acid-water (2:1:0.5, wt/vol/vol), as described by Rottem and Razin (21).

Polyacrylamide gel electrophoresis. Gels to detect enzymes were prepared by a modification of the method of Davis (2, 20). The separation gels contained 7.5% acrylamide, and the pH of the gels was adjusted to 8.9. Electrode buffer [6.0 g of tris(hydroxymethyl)-aminomethane and 28.8 g of glycine in 1,000 ml of water] was diluted 1:10 before use. The lower electrode served as the anode, and samples were electrophoresed at 15 mA for 30 min. The current was then increased to 35 mA and held constant at this level until the bromophenol blue front reached the bottom of the gel. The gel was stained specifically to reveal malate dehydrogenase by using a solution containing 20 ml of

0.1 M tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 7.4), 40 mg of sodium malate, 2 mg of nicotinamide adenine dinucleotide, 4 mg of Nitro Blue Tetrazolium, and 1 mg of phenazine methosulfate.

Gels to separate phenol-acetic acid-soluble proteins were prepared by the methods of Rottem and Razin (21) and Zola et al. (30). These gels contained 7.5% acrylamide, 35% acetic acid, and 5 M urea; 10% acetic acid was used as the electrode buffer, and the lower electrode served as the cathode. Electrophoresis was carried out at a constant current of 15 mA. The gels were stained with 0.05% Coomassie blue in 9% acetic acid–40% methanol.

RESULTS

Cultural characteristics. The colonial and cellular appearances of the strains studied are shown in Fig. 1 and 2. All strains were gram-negative rod-shaped organisms. Gram stains of pure cultures showed predominantly short or long rod-shaped cells in pairs or, occasionally, short chains. After incubation for 2 days on EG agar, colonies of most strains were circular, entire, convex, translucent, smooth, and shiny. Strain VPI D25B-5 formed eroded semiopaque colonies. Strains VPI 7570A, WAL 3030, VPI

FIG. 2. Two-day-old surface colonies of *Bacteroides* strains on EG agar. Bar = 5 mm. (A) *B. denticola* VPI 7570A. (B) *B. buccalis* VPI 8906D. (C) *B. oralis* VPI D27B-24^T. (D) *B. veroralis* VPI D22A-7^T. (E) *B. biviaus* 7880. (F) Strain VPI E1S-8. (G) Strain VPI D25B-5. (H) Strain WPH 179. (I) Strain B56020.

TABLE 1. Characteristics differentiating strains similar to *B. oralis*^a

Characteristic	Group I (<i>B. denticola</i>)			Group II (<i>B. buccalis</i>)		Group III (<i>B. oralis</i>)			<i>B. veroralis</i>		<i>B. bivius</i>			Other strains		
	VPI 7570A	WAL 3030	1221	VPI 8906D	HS4 ^r	VPI 5832	VPI 9958	VPI D27B-24 ^r	VPI D22A-7 ^r	7880	5540	VPI 6822 ^r	VPI E1S-8	VPI D25B-5	WPH 179	B56020
Final pH in PYG ^b	4.6	4.6	4.6	5.3	5.3	4.8	4.8	4.8	4.7	5.0	5.3	5.0	5.4	5.1	4.9	5.3
Pigment formation	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+
Isovaleric acid from PYG	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-	+
Acid from:																
Rhamnose	-	-	-	-	-	w	w	-	-	-	-	-	-	w	-	-
Ribose	+	+	-	-	-	-	+	w	+	-	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	w	-	-	-	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+
Cellobiose	-	-	-	+	+	+	+	+	+	-	-	-	+	+	-	+
Melibiose	-	-	-	+	+	+	+	+	w	-	-	-	+	+	w	+
Raffinose	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Glycogen	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Inulin	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Glycerol	+	+	+	-	-	w	-	-	+	-	w	+	+	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Sorbitol	-	-	-	-	-	+	+	+	w	-	-	-	+	-	-	+
Esculin	-	-	-	-	-	+	+	+	-	-	-	-	+	w	-	-
Salicin	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-
Amygdalin	-	-	-	w	w	+	+	+	-	-	-	-	-	+	-	-
Xylan	-	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-
Starch hydrolysis	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	-	-	-	-	-	-	-	-	-	w	+	+	-	-	-	+

^a +, Positive reaction; -, negative reaction; w, weak reaction.^b PYG, Peptone-yeast extract-glucose medium.

E1S-8, VPI D25B-5, and B56020 formed brown or black pigments on laked blood agar.

Biochemical reactions. The biochemical properties of the strains studied are shown in Table 1. In addition, all strains produced acetic acid and succinic acid on peptone yeast extract glucose medium and produced acid from glucose, mannose, galactose, maltose, and lactose. No strain produced acid from arabinose, xylose, trehalose, melezitose, adonitol, dulcitol, inositol, erythritol, or gum arabic, produced gas, indole, or H₂S, reduced nitrate, or grew in 20% bile.

G+C contents and DNA homologies. Table 2 shows the G+C contents and DNA homologies among strains, as determined by the membrane competition method. Although DNA preparations of group II and III strains had similar G+C contents (42.8 to 45.4 mol%), only low levels of DNA homology were found between these two groups. Strains VPI E1S-8 and VPI D25B-5 displayed levels of relatedness to each other of 70 and 68%, indicating that these two strains may be members of the same species. Approximately 60% homology was obtained with strain VPI E1S-8 and competitor DNAs from strains VPI 5832, VPI 9958, and VPI D27B-24^T but not with the related strain VPI D25B-5. Thus, the relationships of these strains are not clear. The DNAs from strains WPH 179, B56020, and VPI D22A-7^T showed low relatedness to each other and the other bacteroides strains used.

Table 3 shows the DNA homologies among some of the strains as determined by the S1 nuclease method. With this method, strains VPI E1S-8 and VPI D25B-5 showed only 17.2 and 18% homology to each other and little or no relatedness to other strains.

Electrophoretic analysis. The range of the electrophoretic mobilities of malate dehydrogenase in the strains examined is shown in Fig. 3. The mobilities of the malate dehydrogenases obtained from the strains within each group were similar to each other. Strains VPI E1S-8 and VPI D25B-5 had similar protein bands, and strains VPI D22A-7^T and B56020 also were similar. The malate dehydrogenase of each of the other strains was distinct.

The electrophoretic patterns of the phenol-acetic acid-soluble proteins of the strains are shown in Fig. 4. The patterns within each group (groups I, II, and III) were identical, and each was different from any other bacteroides species examined. The strains not in group I, II, or III had different protein electrophoretic patterns.

DISCUSSION

On the basis of phenotypic and genotypic characteristics, the strains which were similar to *B. oralis* were differentiated into three major groups and five unrelated strains. Because all of these organisms are obligately anaerobic, non-motile, gram-negative rod-shaped bacteria that produce succinic acid and acetic acid, they belong to the genus *Bacteroides* (6). As van Steenberg et al. (28) have shown, our results also indicate that strains similar to *B. oralis* are very heterogenous and fall into several different groups on the basis of G+C content and DNA homology. The levels of DNA homology observed among strains of *B. denticola*, *B. buccalis*, and *B. oralis* are consistent with distinct species (10). Phenotypic characteristics and the electrophoretic protein patterns of the strains also differentiate these species.

TABLE 2. DNA homologies among strains of *B. oralis* and similar species

Unlabeled DNA from strain:	Homology group and/or species	% Homology with reference DNA from strain:									G+C content (mol%)
		VPI 7570A	VPI 8906D	VPI 5832	VPI D22A-7 ^T	VPI 6822 ^T	VPI E1S-8	VPI D25B-5	WPH 179	B56020	
VPI 7570A	I (<i>B. denticola</i>)	100	27	10	25	10	41	46	19	21	50.6
WAL 3030	I (<i>B. denticola</i>)	102	20	14	10	10	43	31	21	26	51.4
1221	I (<i>B. denticola</i>)	89	38	35	32	11	44	33	28	20	50.1
VPI 8906D	II (<i>B. buccalis</i>)	11	100	38	14	18	28	24	29	21	45.3
HS4 ^T	II (<i>B. buccalis</i>)	15	96	26	14	20	29	42	32	24	45.4
VPI 5832	III (<i>B. oralis</i>)	12	32	100	40	10	61	34	24	15	43.1
VPI 9958	III (<i>B. oralis</i>)	20	38	107	42	36	61	36	27	26	42.8
VPI D27B-24 ^T	III (<i>B. oralis</i>)	29	16	90	30	ND ^a	62	53	21	21	43.1
VPI D22A-7 ^T	<i>B. veroralis</i>	40	35	33	100	ND	42	42	21	21	42.1
7880	<i>B. bivia</i>	24	36	36	29	88	35	29	21	24	37.5
5540	<i>B. bivia</i>	32	24	25	29	84	26	24	18	32	37.5
VPI 6822 ^T	<i>B. bivia</i>	31	36	14	24	100	28	31	23	21	40.0
VPI 6318	<i>B. bivia</i>	24	36	34	31	98	31	33	17	18	40.0
VPI E1S-8	Other	20	42	42	17	ND	100	70	30	29	43.9
VPI D25B-5	Other	32	29	32	28	ND	68	100	18	32	46.4
WPH 179	Other	25	31	20	31	21	41	41	100	15	45.1
B56020	Other	18	44	23	11	10	32	32	29	100	50.0

^a ND, Not done.

TABLE 3. DNA homologies among strains as determined by the S1 nuclease method

Unlabeled DNA from strain:	% Homology with reference DNA from strain:		
	VPI D22A-7 ^T	VPI D25B-5	VPI E1S-8
<i>B. oralis</i> group			
VPI D22A-7 ^T	100	4.7	7.1
VPI D25B-5	0	100	17.2
VPI E1S-8	11.8	18	100
VPI D27B-24 ^T	9.1	2.7	5.5
VPI 5832	0	5.5	6.3
VPI 7570A	0	0	0
WPH 179	0	0	0
B56020	2.5	0.4	0
<i>B. melaninogenicus</i> subsp. <i>melaninogenicus</i> ATCC 25845 ^T	9.9	0.7	0

Strain VPI D27B-24 was listed as the type strain of *B. oralis* on the Approved Lists of Bacterial Names (26); this strain was identical to strains VPI 5832 and VPI 9958 (group III) phenotypically and genetically. Strains of this group fermented salicin, whereas according to the original description of the species (16), *B. oralis* should not ferment salicin.

van Steenberg et al. (28) reported that strains VPI 8906D and HS4^T fit the original phenotypic description of *B. oralis*. However, strain VPI 8906D was isolated from umbilicus drainage, and strain HS4^T was isolated from a monkey mouth. Surface colonies of these two strains on blood agar were less 0.5 mm in diameter, whereas *B. oralis* was derived from a healthy human oral cavity and the colonies of *B. oralis* were reported to be 1 to 2 mm in diameter. We believe that strains VPI 8906D and HS4^T differ from the first description of *B. oralis*. Recently, Shah and Collins (24) described these strains as a new species, *Bacteroides buccalis*.

Of the strains examined, only strain VPI D22A-7^T possessed physiological, biochemical, and ecological characteristics which completely fit the original description of *B. oralis*. However, inasmuch as strain VPI D27B-24 was designated the type strain of *B. oralis* on the Approved Lists of Bacterial Names, this strain is a priori the type strain of *B. oralis* (14). Therefore, we propose the name *Bacteroides veroralis* sp. nov. for the strains that accurately fit the original description of *B. oralis* and designate strain VPI D22A-7 the type strain. A description of this species is given below.

Description of *Bacteroides veroralis*. *Bacteroides veroralis* sp. nov. (ver'o.ral.is. L. adj. *verus* true; L. adj. *oralis* pertaining to the mouth; N.L. masc. adj. *veroralis* the true oral-).

FIG. 3. Polyacrylamide gel stained for *Bacteroides* malate dehydrogenases. Lane a, *B. denticola* VPI 7570A; lane b, *B. buccalis* VPI 8906D; lane c, *B. oralis* VPI 5832; lane d, strain VPI E1S-8; lane e, strain VPI D25B-5; lane f, *B. veroralis* VPI D22A-7^T; lane g, strain WPH 179; lane h, strain B56020; lane i, strain 7880; lane j, *B. melaninogenicus* subsp. *melaninogenicus* ATCC 25845^T.

Cell characteristics. Obligately anaerobic, nonmotile, nonsporeforming, gram-negative, rod-shaped cells 0.5 μ m wide by 1.0 to 1.5 μ m long. Gram strains of pure cultures show predominantly short rod-shaped cells in pairs or short chains of long rod-shaped cells (Fig. 1D).

Colonies. After incubation for 2 days on EG agar, colonies were 1.0 to 2.0 mm in diameter, circular, entire, convex, translucent, smooth, and shiny (Fig. 2D).

Cultural characteristics. Cultures in pre-reduced peptone yeast extract broth were moderately turbid, sometimes with a slight, smooth sediment. Growth was enhanced in broth supplemented with a fermentable carbohydrate. The optimal temperature for growth was about 37°C. After good growth was observed in peptone-yeast extract-glucose medium cultures, the pH was 4.7. The pH of cultures in broth media without fermentable carbohydrate was usually 6.1.

Biochemical characteristics. Some biochemical reactions of this species are shown in Table 1. Nitrate was not reduced. Neither indole nor hydrogen sulfide was produced. Bile did not stimulate growth. Little or no gas was formed from glucose. Acid and clots were produced in milk. Lecithinase was not produced in egg yolk agar.

Source. This organism was isolated from a human oral cavity.

G+C content. The G+C content of the DNA was 42.1 mol%.

Type strain. The type strain is VPI D22A-7^T (= ATCC 33779^T).

FIG. 4. Electrophoretic analysis of phenol-acetic acid-soluble proteins of *Bacteroides* strains. Lane a, *B. denticola* VPI 7570A; lane b, *B. oralis* VPI 5832; lane c, *B. oralis* VPI D27B-24^T; lane d, strain VPI E1S-8; lane e, strain VPI D25B-5; lane f, strain B56020; lane g, *B. buccalis* VPI 8906D; lane h, *B. buccalis* HS4^T; lane i, *B. veroralis* VPI D22A-7^T; lane j, strain WPH 179; lane k, *B. bivius* 7880.

Characteristics which differentiate *B. veroralis* from related organisms. *B. veroralis* is rather similar to the other organisms which we studied in colony size and biochemical characteristics. Table 1 shows the biochemical characteristics which are most useful for distinguishing *B. veroralis* from some members of the genus *Bacteroides* that are similar. *B. veroralis* produces acid from inulin, cellobiose, melibiose (weak), esculin (weak), and xylan, but not from glycerol and salicin; this species also hydrolyzes starch, but does not liquefy gelatin.

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