Bacteroides heparinolyticus sp. nov. Isolated from Humans with Periodontitis

KATSUJI OKUDA,^{1*} TETSUO KATO,¹ JIRO SHIOZU,¹ ICHIRO TAKAZOE,¹ AND TAKESHI NAKAMURA²

Department of Microbiology, Tokyo Dental College, Masago, Chiba City, Japan¹ and Department of Oral Microbiology, Matsumoto Dental College, Shiojiri City, Nagano Prefecture, Japan²

We describe a new species of nonpigmented, saccharolytic *Bacteroides*, *Bacteroides heparinolyticus*, which is frequently isolated from human periodontitis lesions. This species produces an enzyme that hydrolyzes heparin. The major product from glucose is succinic acid. *B. heparinolyticus* strains have membranes typical of gram-negative cells and external surface structures that stain with ruthenium red and glutaraldehyde-osmium. The guanine-plus-cytosine content of the deoxyribonucleic acid is 47 to 49 mol%. Strains of this species have negligible deoxyribonucleic acid homology with other *Bacteroides* species described previously. Strain HEP (= ATCC 35895) is the type strain. *B. heparinolyticus* strains are serologically distinct from other *Bacteroides* species on the basis of cell agglutination and gel diffusion tests.

Recent studies of the subgingival microflora in human periodontitis lesions indicate the predominance of gram-negative, anaerobic, rod-shaped bacteria (19, 22). Special emphasis has been put on black-pigmented *Bacteroides* species as possible etiological agents of periodontal disease. However, frequent isolation of nonpigmented, saccharolytic *Bacteroides* strains from subgingival lesions of patients with periodontitis and from pus of mixed infections in guinea pigs induced by human dental plaque also has been reported (14, 20).

Nakamura et al. found that some of these *Bacteroides* strains hydrolyzed heparin (14, 15). The mucopolysaccharidase activities of these *Bacteroides* strains from human periodontal lesions did not conform to the description of any previously described species (1-6, 8, 9, 11, 18, 23). In this paper we describe the characteristics of strains isolated from human periodontal sites and propose a new species, *Bacteroides heparinolyticus*. Strain HEP (= ATCC 35895) is the type strain of this species.

MATERIALS AND METHODS

Bacterial strains. Six heparinase-producing *Bacteroides* strains were isolated from five humans with adult periodontitis. These patients had multiple sites with alveolar bone loss. Microbiological samples from deep periodontal pockets yielded predominantly anaerobic rod-shaped bacteria, including black-pigmented *Bacteroides* and spirochetes. The biochemical characteristics and heparin-hydrolyzing activity of some isolates have been described previously by Nakamura et al. (14-16).

Bacteroides denticola ATCC 33185, *Bacteroides oralis* ATCC 33269^T (T = type strain), *Bacteroides oris* ATCC 33573^T, *Bacteroides buccae* ATCC 33574^T, *Bacteroides capillus* (*B. buccae*) ATCC 33690, *Bacteroides veroralis* ATCC 33779^T, and *Bacteroides pentosaceus* (*B. buccae*) NCDO 2353 were used for comparisons of deoxyribonucleic acid (DNA) composition and biological, physiological, and serological characteristics. For serological characterization, *Bacteroides loescheii* ATCC 15930^T and *Bacteroides melaninogenicus* ATCC 25845 also were used.

Media for growth. Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 10%

defibrinated horse blood, 5.0 µg of hemin per ml, and 0.5 µg of menadione per ml was used to maintain the strains. For characterization of strains, the peptone-yeast extract medium described in the *Anaerobic Laboratory Manual* (3) generally was used. To extract DNA and antigen and to obtain immunogens, a diffusate broth was used as described below. A 34-g portion of Trypticase peptone (BBL), 6 g of Phytone peptone (BBL), and 6 g of yeast extract (Difco Laboratories, Detroit, Mich.) were dissolved in 150 ml of distilled water. This preparation was dialyzed against 850 ml of distilled water for 2 days at 4°C. After the volume of diffusate was adjusted to 1 liter, 5 g of NaCl, 2.5 g of K₂HPO₄, 2.5 g of glucose, 0.2 g of L-tryptophane, 0.2 g of L-asparagine, and 0.2 g of L-cystine monohydrochloride were added. The pH was adjusted to 7.4, and the medium was autoclaved; then a membrane filter-sterilized solution containing 5 mg of hemin and 0.5 mg of menadione was added aseptically.

Methods used for characterizations. The phenotypic reactions of the strains were studied by using the methods described in the *Anaerobic Laboratory Manual* (3). Collagenase activity was determined by the gel method described by Yamamoto and Takazoe (24). Mucopolysaccharidase activities, including heparinase activity, were determined colorimetrically as described by Taniguchi et al. (21). The API ZYM system was used to examine enzyme activities (API Produits de Laboratoire Ltee., Toho Chemicals, Tokyo, Japan).

Determination of DNA base composition and DNA homology. DNA was isolated by the method of Marmur (12) from cells grown in diffusate broth. DNA purity was assessed by determining the ratio of extinction at 230 nm to extinction at 260 nm. The guanine-plus-cytosine content of DNA was determined by the methods described by Schildkraut et al. (17).

DNA from "*Micrococcus lysodeikticus*" (*Micrococcus luteus*) was used as the standard reference DNA in the study. A DNA solution in CsCl was centrifuged in a Beckman model L8 centrifuge at 40,000 rpm and 25°C. After 24 h of centrifugation, the ultraviolet adsorption of the preparation was recorded.

Labeled DNA was prepared in vitro by nick translation (7). DNA homology values were determined by using the S1 nuclease procedure, as described by Watabe et al. (23).

Polyacrylamide gel electrophoresis. The polyacrylamide gel

* Corresponding author.

electrophoretic patterns of cellular proteins were determined as described by Moore et al. (13).

Morphological study. Cells were harvested from blood agar plates that were incubated for 3 days. The cells were washed twice with 0.15 M NaCl. For electron microscopy, washed cells were prefixed in a mixture containing equal volumes of freshly prepared 4% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.0) and a 0.2% ruthenium red solution in distilled water for 2 h at 4°C. The prefixed cells were washed three times with 0.1 M cacodylate buffer and postfixed overnight in a solution consisting of equal volumes of 4% OsO₄ in 0.2 M cacodylate buffer and a 0.2% ruthenium red solution in distilled water. The cells were washed with cacodylate buffer, dehydrated in a graded series of alcohol solutions, placed into propylene oxide, and embedded in Epon 812 (Polysciences, Warrington, Pa.). Ultrathin sections were stained with uranium acetate and lead citrate and observed with an electron microscope (Hitachi type HS).

Serological studies. Cells grown in the diffusate medium were harvested and washed with phosphate-buffered saline (pH 7.2). A solution containing 10 mg (wet weight) of cells per ml in phosphate-buffered saline was used as the immunogen. Antiserum was produced in New Zealand white male rabbits by injecting them intravenously with 1 to 3 ml of the immunogen. Blood was drawn by heart puncture once a week after the sixth or seventh injection.

To prepare antigens for the immunodiffusion test, the intact bacterial cells were suspended in phosphate-buffered saline at a concentration of 100 mg (wet weight) per ml and subjected to ultrasonic treatment. Sonic supernatants were obtained by centrifuging the preparation at 12,000 × g for 20 min.

The cell-agglutinating titer against antiserum to heparinase-producing *Bacteroides* strains was also determined. Serial dilutions of antiserum were made in phosphate-buffered saline by using microtiter plates and 25-μl diluters (Dynatech Laboratories, Inc., Alexandria, Va.). One drop of cell suspension was added to each well. The plate was rocked gently for 10 min at 37°C on a platform shaker and then placed at room temperature overnight before reading.

RESULTS AND DISCUSSION

The heparinase-positive strains of *Bacteroides* isolated from human periodontal lesions formed a distinct homology group with mutual DNA homology values of 76% or more (Table 1). When *B. denticola*, *B. buccae*, and *B. pentosaceus* (*B. buccae*) DNA preparations were labeled, we found little homology with DNAs from the heparinase-producing *Bacteroides* strains. From these results we concluded that the heparinase-producing strains should be placed in a separate species, for which we propose the name *Bacteroides heparinolyticus* (he.pa'ri.no.ly.ti.cus. Gr.n.heparin heparin; Gr.adj.lyticus dissolving; *heparinolyticus* heparin dissolving). A description of this species is given below.

After incubation for 4 days on blood agar, colonies of the type strain and five other strains of *B. heparinolyticus* are circular, entire, convex, smooth, translucent, and gray. The strains do not grow under aerobic conditions or in air supplemented with 10% CO₂. No dark brown or black colonies are produced on hemolyzed rabbit blood agar. Broth cultures are turbid and have a dense sediment. Harvested cells are markedly viscous. Cells grown in blood agar are gram-negative, nonmotile, nonsporeforming rods 0.5 to 0.7 by 0.8 to 2.0 μm, with occasional cells 10 μm long or longer. Thin sections of *B. heparinolyticus* strain HEP^T (T = type strain) and strain HR-6 are shown in Fig. 1. The cell

TABLE 1. Guanine-plus-cytosine contents and relative levels of hybridization of DNAs from *B. heparinolyticus* strains and reference *Bacteroides* strains

Source of unlabeled DNA	Guanine-plus-cytosine content (mol%)	% Homology with DNA from <i>B. heparinolyticus</i> strains:	
		HEP ^T (= ATCC 35895 ^T)	HR-6
<i>B. heparinolyticus</i>			
HEP ^T (= ATCC 35895 ^T)	47	100	96
HR-6	48	100	100
KHR-1	47	88	76
T-2-C	49	77	104
KHR-6	48	81	85
<i>B. denticola</i> ATCC 33185	51	4	5
<i>B. oralis</i> ATCC 33269 ^T	44	4	9
<i>B. oris</i> ATCC 33573 ^T	42	0	4
<i>B. buccae</i> ATCC 33574 ^T	42	3	1
<i>B. capillus</i> (<i>B. buccae</i>) ATCC 33690	52	1	2
<i>B. veroralis</i> ATCC 33779	42	12	19
<i>B. pentosaceus</i> (<i>B. buccae</i>) NCDO 2353	50	3	2

wall structure is typical of gram-negative bacteria. The cytoplasmic and outer membranes exhibit a trilamellar structure.

The outer wall of the strains is separated from the outer membrane by an electron-lucent layer. By using phase-contrast microscopy with India ink negative staining, a distinctive capsular structure surrounding the cell was detected in all strains of *B. heparinolyticus*.

After incubation for 5 days, the pH in broth containing 1% glucose is 5.4 to 5.6. All strains produce indole. The type strain and five other strains produce acid from glucose, fructose, cellobiose, lactose, maltose, mannose, sucrose, salicin, xylose, starch, and esculin. None of the strains ferments raffinose mannitol or sorbitol. None reduces nitrate. Sheep blood is not hemolyzed. Growth is inhibited by 20% bile. No strain produces H₂S, catalase, or gelatinase. All strains hydrolyze starch and esculin.

The acid products in PYG broth cultures are succinic acid and small amounts of acetic, propionic, and isovaleric acids. All strains produce heparinase, alkaline phosphatase, esterase, esterase-lipase, acid phosphatase, phosphoamidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. None of the strains produces collagenase, lipase, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin, chymotrypsin, α-galactosidase, α-glucosidase, or α-mannosidase.

The guanine-plus-cytosine content of the DNA is 47 to 49 mol% (Table 1). The type strain of *B. heparinolyticus* is strain HEP (= ATCC 35895).

The sole characteristic that reliably differentiates the new species from other nonpigmented saccharolytic *Bacteroides* species is the heparin-degrading activity.

The polyacrylamide gel electrophoretic patterns of sonicated supernatants are shown in Fig. 2. The patterns were quite consistent among the *B. heparinolyticus* isolates, but the patterns of the *B. heparinolyticus* strains were distinctively different from those of the other *Bacteroides* species studied.

Gel diffusion results are shown in Fig. 3. Antiserum to *B. heparinolyticus* was in each trough, and the wells between

FIG. 1. Thin sections of *B. heparinolyticus* strains. The cytoplasmic membrane (CM) and outer membrane (OM) are stained intensely. The outer layer (OL) structure is separated from the outer membrane by an electron-lucent layer. (A) Strain HR-6. (B) strain HEP^T (= ATCC 35895^T).

the troughs contained sonicated antigens from the strains. The upper and lower wells contained the sonicated antigens from reference *Bacteroides* species. The antiserum reacted with homologous and heterologous antigens from *B. heparinolyticus*. No antigenic relationship was observed between *B. heparinolyticus* and other *Bacteroides* species. Sonicated antigens from *B. loescheii* and *B. melaninogenicus* did not react with either antiserum.

The cell agglutination titers between antiserum to *B.*

heparinolyticus and cell suspensions of test strains showed that strains of *B. heparinolyticus* were distinguished serologically from other *Bacteroides* species used in this study (Table 2).

Many characteristics of *B. heparinolyticus* are similar to those of other *Bacteroides* species isolated from human oral cavities. The colony morphology, Gram stain morphology, and fermentation patterns were not significantly different among the species studied. However, the guanine-plus-

FIG. 2. Polyacrylamide gel electrophoretic patterns of strains of *B. heparinolyticus* and reference *Bacteroides* species. Lane 1, *B. heparinolyticus* KHR-1; lane 2, *B. heparinolyticus* HEP^T; lane 3, *B. heparinolyticus* HR-6; lane 4, *B. denticola* ATCC 33185^T; lane 5, *B. oralis* ATCC 33573^T; lane 6, *B. veroralis* ATCC 33779^T; lane 7, *B. buccae* ATCC 33574^T; lane 8, *B. capillus* (*B. buccae*) ATCC 33690.

cytosine contents of DNAs from *B. heparinolyticus* were 2 to 4 mol% lower than the guanine-plus-cytosine contents of reference strains of *B. denticola*, *B. buccae*, *B. capillus*, and *B. pentosaceus* and 4 to 8 mol% higher than the guanine-

TABLE 2. Cell agglutination titers between antiserum to *B. heparinolyticus* and reference *Bacteroides* species

Strain	Agglutinating titer with <i>B. heparinolyticus</i> strain:	
	HEP ^T = ATCC 35895 ^T)	HR-6
<i>B. heparinolyticus</i>		
HEP ^T (= ATCC 35895 ^T)	160	80
HR-6	160	80
KHR-1	80	80
T-2-C	80	40
KHR-6	80	80
HEP-A	80	80
<i>B. denticola</i> ATCC 33185	— ^a	—
<i>B. oralis</i> ATCC 33269 ^T	—	—
<i>B. oralis</i> ATCC 33573 ^T	—	—
<i>B. buccae</i> ATCC 33574 ^T	—	—
<i>B. capillus</i> (<i>B. buccae</i>) ATCC 33690	—	—
<i>B. veroralis</i> ATCC 33779 ^T	—	—
<i>B. pentosaceus</i> (<i>B. buccae</i>) NCDO 2353	—	—
<i>B. loescheii</i> ATCC 15930 ^T	—	—
<i>B. melaninogenicus</i> ATCC 25845 ^T	—	—

^a —, No cell agglutination detected in 10-fold dilution serum.

plus-cytosine contents of *B. oralis*, *B. veroralis*, and *B. oralis*. The definitive characteristic of *B. heparinolyticus* is its heparin-splitting activity. In addition, obvious differences in the antigenic structure of *B. heparinolyticus* were determined by immunodiffusion and cell agglutination studies.

The outer layer, which is separated from the outer membrane by an electron-lucent layer, was confirmed by ruthenium red staining. The outer layer structure is similar to that observed in *Bacteroides melaninogenicus* subsp. *intermedius* (10), *B. capillus* (1, 9), and *B. pentosaceus* (9). The

FIG. 3. Immunodiffusion of ultrasonicated antigens reacted with antisera to whole cells of *B. heparinolyticus* strains. The troughs contained antisera against *B. heparinolyticus* strains HEP^T (= ATCC 35895^T) and HR-6. The wells between the troughs contained sonicated antigens from strains of *B. heparinolyticus* and phosphate-buffered saline (PBS). The upper and lower wells contained sonicated antigens from *B. denticola* ATCC 33185, *B. oralis* ATCC 33269^T, *B. oralis* ATCC 33573^T, *B. buccae* ATCC 33574^T, *B. capillus* (*B. buccae*) ATCC 33690, *B. veroralis* ATCC 33779^T, and *B. pentosaceus* (*B. buccae*) NCDO 2353.

presence of a capsular structure may play a role in protecting the cells against host defense mechanisms, such as phagocytic killing.

ACKNOWLEDGMENTS

We thank Tomotari Mitsuoka and Tomohiko Fujisawa, Department of Biomedical Science, Faculty of Agriculture, University of Tokyo, Tokyo, Japan, for helpful discussions and their help in the DNA homology study. We are also grateful to B. L. Pierce for help in preparation of the manuscript and to T. Ozaki for typing.

LITERATURE CITED

1. Haapasalo, M., K. Lounatmaa, H. Ranta, H. Shah, and K. Ranta. 1985. Ultrastructure of *Bacteroides capillus*, *B. buccae*, *B. pentosaceus*, *B. oris*, *B. oralis*, *B. veroralis*, and peptose sugar-fermenting *Bacteroides* sp. from humans with periapical osteitis: occurrence of external proteinaceous cell wall layer. *Int. J. Syst. Bacteriol.* **35**:65-72.
2. Holbrook, W. P., and B. I. Duerden. 1974. A comparison of some characteristics of reference strains of *Bacteroides oralis* with *Bacteroides melaninogenicus*. *Arch. Oral Biol.* **19**: 1231-1235.
3. Holdeman, L. V., E. P. Cato, and W. E. C. Moore. 1977. Anaerobe laboratory manual, 4th ed. Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg.
4. Holdeman, L. V., and J. L. Johnson. 1982. Description of *Bacteroides loeschei* sp. nov. and emendation of description of *Bacteroides melaninogenicus* (Oliver and Wherry) Roy and Kelly 1939 and *Bacteroides denticola* Shah and Collins 1981. *Int. J. Syst. Bacteriol.* **32**:399-409.
5. Holdeman, L. V., R. W. Kelley, and W. E. C. Moore. 1984. Anaerobic gram-negative straight, curved and helical rods. Family 1. *Bacteroidaceae*, p. 602-662. In N. R. Krieg and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*. The Williams & Wilkins Co., Baltimore.
6. Holdeman, L. V., W. E. C. Moore, P. J. Churn, and J. L. Johnson. 1982. *Bacteroides oris* and *Bacteroides buccae*: new species from human periodontitis and other human infections. *Int. J. Syst. Bacteriol.* **32**:125-131.
7. Johnson, J. L. 1981. Genetic characterization, 450-472. In P. Gerhardt (ed.), *Manual of methods for general microbiology*. American Society for Microbiology, Washington D. C.
8. Johnson, J. L., and L. V. Holdeman. 1985. *Bacteroides capillus* Kornman and Holt and *Bacteroides pentosaceus* Shah and Collins, later synonyms of *Bacteroides* Holdeman et al. *Int. J. Syst. Bacteriol.* **35**:114.
9. Kornman, K. S., and S. C. Holt. 1981. Physiological and ultrastructural characterization of a new *Bacteroides* species (*Bacteroides capillus*) isolated from severe localized periodontitis. *J. Periodontal Res.* **16**:542-555.
10. Listgarten, M. A., and C.-H. Lai. 1979. Comparative ultrastructure of *Bacteroides melaninogenicus* species. *J. Periodontal Res.* **14**:332-340.
11. Loesche, W. J., S. S. Socransky, and R. J. Gibbons. 1964. *Bacteroides oralis*, proposed new species isolated from the oral cavity of man. *J. Bacteriol.* **88**:1329-1337.
12. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* **3**:208-218.
13. Moore, W. E. C., D. E. Hash, L. V. Holdeman, and E. P. Cato. 1980. Polyacrylamide slab gel electrophoresis of soluble proteins for studies of bacterial floras. *Appl. Environ. Microbiol.* **39**:900-907.
14. Nakamura, T. 1969. Study on anaerobic heparinase-producing microorganisms in human oral cavity. *Juzen Igakkai Zasshi* **78**:509-530. (In Japanese.)
15. Nakamura, T., Y. Suganaka, and I. Takazoe. 1976. Heparinase activity in lesion of periodontal diseases. *Bull. Tokyo Dent. Coll.* **17**:147-155.
16. Nakamura, T., Y. Suganaka, I. Takazoe, and K. Okuda. 1976. Biological activity of endotoxin from oral heparinase-producing *Bacteroides*. *Matsumoto Shigaku* **2**:12-19. (In Japanese.)
17. Schildkraut, C. L., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. *J. Mol. Biol.* **4**:430-443.
18. Shah, H. N., and M. D. Collins. 1981. *Bacteroides buccalis*, sp. nov., *Bacteroides denticola*, sp. nov., *Bacteroides pentosaceus*, sp. nov., new species of the genus *Bacteroides* from the oral cavity. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe C* **2**:235-241.
19. Slots, J. 1977. The predominant cultivable microflora of advanced periodontitis. *Scand. J. Dent. Res.* **5**:235-241.
20. Takazoe, I., and T. Nakamura. 1971. Experimental mixed infection by human gingival crevice material. *Bull. Tokyo Dent. Coll.* **12**:85-93.
21. Taniguchi, H., S. Fujimura, K. Takeuchi, and T. Nakamura. 1983. Purification and characterization of mucopolysaccharidase from an oral strain of *Bacteroides* sp. *Appl. Environ. Microbiol.* **46**:1252-1257.
22. Tanner, A. C. R., C. Haffer, G. T. Bratthall, R. A. Visconti, and S. S. Socransky. 1979. A study of bacteria associated with advanced periodontitis in man. *J. Clin. Periodontol.* **6**:278-307.
23. Watabe, J., Y. Benno, and T. Mitsuoka. 1983. Taxonomic study of *Bacteroides oralis* and related organisms and proposal of *Bacteroides veroralis* sp. nov. *Int. J. Syst. Bacteriol.* **33**:57-64.
24. Yamamoto, A., and I. Takazoe. 1976. Efficient fractionation of collagenolytic sample from *Bacteroides melaninogenicus*. *Bull. Tokyo Dent. Coll.* **17**:123-130.