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Further Characterization of *Bacteroides endodontalis*, an Asaccharolytic Black-Pigmented *Bacteroides* Species from the Oral S Cavity

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In this study, the isolation, characterization, and identification of *Bacteroides endodontalis* is described. It was found that this asaccharolytic black-pigmented *Bacteroides* species is associated with infected dental root canals and oral submucous abscesses. *B. endodontalis* could be differentiated from *B. gingivalis* by a negative direct hemagglutination test and the absence of trypsin and *N*-acetyl-β-glucosamidase. *B. endodontalis* could be differentiated from *B. asaccharolyticus* by the absence of α-fucosidase, its inability to grow in an atmosphere of 95% N_2–5% H_2, and a growth requirement for menadione. Immune serum raised against *B. endodontalis* strain HG 370^T agglutinated only *B. endodontalis* cells. Precautions for the isolation of *B. endodontalis* are discussed.

The role of gram-negative obligate anaerobic bacteria in the etiology and pathogenesis of periodontal diseases is well recognized today. Within this group of microorganisms, black-pigmented *Bacteroides* bacteria play an important role (13, 17, 24). This is true not only for periodontal diseases but also for infections of dental pulp tissues (5), periodontitis apicalis (18), and orofacial abscesses (1, 7). The significance of black-pigmented *Bacteroides* species in these infections has been reviewed recently (15).

In the last decade, the classification of black-pigmented *Bacteroides* species has been much improved. The important group of asaccharolytic species are now classified into the following three species: *B. asaccharolyticus* (4), *B. gingivalis* (3), and *B. endodontalis* (21). *B. asaccharolyticus* is usually isolated from nonoral sites, whereas *B. gingivalis* is mainly isolated from periodontal pockets. Recently, we described a third asaccharolytic black-pigmented *Bacteroides* species, *B. endodontalis*, which was isolated from infected dental root canals with severe periapical destruction (21). On the basis of DNA homology studies, guanine and cytosine content, and polyacrylamide gel electrophoresis patterns, the strains studied were found to be different from the two known species. Recently, nine more strains of *B. endodontalis* were isolated, most of them in this laboratory. The purpose of this study is to give further information about *B. endodontalis* concerning biochemical properties, sites of isolation, and tests for discrimination between *B. endodontalis* and the two other asaccharolytic black-pigmented *Bacteroides* species.

MATERIALS AND METHODS

Isolation and cultivation of black-pigmented *Bacteroides* bacteria. Strains used in this study and their sources are mentioned in Table 1. Samples from dental root canals were collected with a nitrogen-flushed syringe as described by Newman and co-workers (11). These samples were transported in prereduced, anaerobically sterilized 0.25-strength Ringers solution. Exudates from submucous periapical abscesses were collected with sterile cotton-wool swabs and transported in charcoal transport medium (Microdiagnostics, Puurs, Belgium). Specimens were processed in the laboratory within 10 min. Samples were plated on horse blood agar (Oxoid no. 2) supplemented with 0.05% hemin and 0.01% menadione. Incubation took place in anaerobic jars under an atmosphere of 80% N_2–10% CO_2–10% H_2. Plates were examined after 5, 7, and 14 days of incubation. Brown- and black-pigmented colonies were subcultured on horse blood agar plates.

Identification of black-pigmented *Bacteroides* bacteria. The fermentation of carbohydrates of the isolates was tested in BM broth (12) containing 1% glucose and the Minitek anaerobic system (BBL Microbiology Systems, Cockeysville, Md.). Catalase production was tested by the slide test. Hemagglutination was carried out by the slide test as described by Slots and Genco (16). Growth in air and in air with 10% CO_2 was investigated for all strains on horse blood agar plates at 37°C.

To investigate some biochemical properties of the three asaccharolytic black-pigmented *Bacteroides* species, growth requirements for hemin and menadione were tested by growth on BM agar plates (12) supplemented with hemin (0.05%) or menadione (0.01%). Plates with both components were used to check for unlimited growth. No blood was added to any of these BM agar plates. Plates were inoculated with a loopful of cells and incubated anaerobically for 7 days as described above. A strain was registered as hemin or menadione dependent when no growth was visible or when only a very limited number of small (<1 mm) colonies developed. If growth occurred, bacterial cells were subinoculated onto the same kind of plate to exclude the influence of hemin or vitamin K storage in the cells.

Isolation of DNA and determination of DNA base composition and DNA homology. Unlabeled DNA was isolated from cells lysed mechanically with a Vibrogen cell mill (Buhler, Tubingen, Federal Republic of Germany) as described previously (19). Tritiated DNA was isolated from Sarkosyl-lysed cells by means of CsCl density gradient centrifugation (22). DNA base composition was determined by the buoyant

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TABLE 1. Strains of black-pigmented *Bacteroides* species used in this study

Collection no.	Origin	Source
B. endodontalis		
HG 181 (H 11a–e)	Dental root canal	G. Sundqvist[a]
HG 182 (BN 11a–f)	Dental root canal	G. Sundqvist
HG 370^T (ATCC 35406)	Dental root canal	Own isolate
HG 400	Oral submucous abscess	Own isolate
HG 410	Dental root canal	Own isolate
HG 412 (TN 42c–l)	Dental root canal	G. Sundqvist
HG 413	Oral submucous abscess	Own isolate
HG 414	Oral submucous abscess	Own isolate
HG 415	Oral submucous abscess	Own isolate
HG 420	Oral submucous abscess	Own isolate
HG 421	Oral submucous abscess	Own isolate
HG 422	Oral submucous abscess	Own isolate
B. gingivalis		
HG 66 (W 83)	Clinical specimen	H. N. Shah[b]
HG 76 (W 50)	Clinical specimen	S. S. Socransky[c]
HG 91 (381)	Periodontal pocket	S. S. Socransky
HG 94 (376)	Periodontal pocket	S. S. Socransky
HG 184	Periodontal pocket	Own isolate
HG 185	Periodontal pocket	Own isolate
HG 372^T (ATCC 33277)	Periodontal pocket	D. Mayrand[d]
HG 416 (BE-c)	Dental root canal	G. Sundqvist
B. asaccharolyticus		
HG 71 (NCTC 9337)	Infected hemorrhoids	H. N. Shah
HG 78 (B 536)	Feces	H. N. Shah
HG 79 (4199)	Feces	H. N. Shah
HG 107^T (ATCC 25260)	Empyema	H. N. Shah
HG 111 (ATCC 27067)	Leg wound	ATCC[e]
HG 124 (B 537)	Leg lesion	H. N. Shah
HG 364 (BM 4)	Dental plaque	D. Mayrand
HG 411 (KC)	Oral submucous abscess	G. Sundqvist

[a] University of Umeå, Umeå, Sweden.
[b] London Hospital Medical College, London, England.
[c] Forsyth Dental Center, Boston, Mass.
[d] Ecole de Médecine Dentaire, Université Laval, Quebec, Canada.
[e] American Type Culture Collection, Rockville, Md.

density method, and the amount of DNA-DNA hybridization was determined by the S1 nuclease method essentially as described previously (22).

Enzyme profiles. To compare the enzyme patterns of *B. gingivalis*, *B. asaccharolyticus*, and *B. endodontalis*, API ZYM (Analytab Products, Plainview, N.Y.) tests were carried out. The API ZYM strips were inoculated, incubated, and read as recommended by the manufacturers. The assays were performed under aerobic conditions. Strains used for this study were grown anaerobically for 4 to 6 days on horse blood agar plates. The inocula were prepared by suspending bacterial cells in sterile 0.85% NaCl in destilled water to a final optical density at 690 nm of 10 to 12 (1×10^{10} to 1.2×10^{10} viable cells). Care was taken not to use cultures which were pigmented, because the pigment obstructs proper reading of the enzyme reactions. All strains were tested twice in separate experiments.

Growth in various gas mixtures. Bacterial strains were inoculated on horse blood agar plates (Oxoid no. 2) supplemented with 0.05% hemin–0.01% menadione and incubated in anaerobic jars at 37°C under atmospheres of (i) 80% N_2–10% H_2–10% CO_2, (ii) 85% N_2–10% H_2–5% CO_2, or (iii) 95% N_2–5% H_2. After 6 days of incubation, growth was recorded with respect to the colony size, i.e., no visible growth, growth of a few colonies at the inoculated area with a size of less than 1 mm, confluent growth of individual colonies but with smaller size as compared with 10% CO_2, and unlimited confluent growth with colonies of 2 to 3 mm.

Antiserum. Whole cells of *B. endodontalis* strain HG 370^T were used for antiserum production. The immunizing strain was grown in BM medium (12) supplemented with 0.05% hemin–0.01% menadione at 37°C for 5 days in an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.) under an atmosphere of 80% N_2–10% H_2–10% CO_2.

The cells were killed with 2% formaldehyde (vol/vol) for 1 h at room temperature and washed three times in sterile phosphate-buffered saline. The cell concentration used for immunization was 1.5×10^{10}/ml. From this cell suspension, an increasing amount of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0 ml was injected intravenously into a male rabbit (2.0 kg) every other day. Preimmune serum was collected before the immunization. At week 1 after the last injection, a booster of 2.0 ml was given intravenously. After 7 days, the animal was bled by cardiac puncture. Serum was separated from the blood cells by centrifugation for 20 min at 3,300 × *g* and stored at −80°C.

Undiluted immune serum, as well as the undiluted preimmune serum, was tested for slide test agglutination of bacterial cells grown on 5% horse blood agar plates. Two to three colonies were suspended in 40 μl of phosphate-buffered saline and examined for autoagglutination, after which 40 μl of undiluted serum was added. The slide was tilted for 2 min before reading the reaction with a binocular (magnification, ×10). All test strains were examined three times.

RESULTS

Besides the three *B. endodontalis* strains described in a previous paper (21), nine more strains were isolated from dental root canals or submucous periapical abscesses. The *B. endodontalis* strains were always isolated from mixed

TABLE 2. Final pH in BM broth, guanine-plus-cytosine contents, and relative levels of hybridization of DNAs from *B. endodontalis* strains with reference DNA from *B. endodontalis* HG 181

Collection no.	Final pH in BM	G+C content (mol%)[a]	% Homology with labeled DNA from HG 181
HG 181	6.9	49.9	100
HG 182	7.0	50.1	93
HG 370	7.1	49.4	92
HG 400	7.3	50.1	83
HG 410	7.3	50.7	93
HG 412	7.2	50.7	85
HG 413	7.2	49.7	92
HG 414	7.1	50.6	81
HG 415	7.2	50.8	74
HG 420	7.0	49.6	89
HG 421	6.7	49.3	87
HG 422	7.2	49.7	84

[a] G+C, Guanine plus cytosine.

cultures which consisted of obligate and facultative anaerobic streptococci, *Fusobacterium* species, and black-pigmented *Bacteroides* species, i.e., *B. intermedius*. In the initial cultures, *B. endodontalis* grew slowly and pigmented only after 7 days or more. After subculturing, *B. endodontalis* often grew faster.

The final pH in BM broth, the guanine-plus-cytosine content of the DNA, and the DNA hybridization of 12 strains are summarized in Table 2. The percentages of the guanine-plus-cytosine content varied between 49.8 and 51.5, with a mean of 50.4. The hybridization of the 12 strains with labeled DNA of HG 181 showed homology which varied between 74 and 93%. With these results, strains were positively identified as *B. endodontalis*. All *B. endodontalis* strains did not ferment glucose, were catalase negative, and showed no hemagglutination of sheep erythrocytes. No growth was observed under aerobic conditions or in air with 5% CO_2. The experiments in which hemin or menadione was omitted from the media showed no growth limitation of the *B. asacccharolyticus* strains when one of the two components was lacking. All *B. gingivalis* strains, on the contrary, needed hemin for growth and did not grow after subinoculation on media without hemin. This hemin dependence was often not observed during the first cultivation. The menadione requirement of *B. gingivalis* varied. HG 66 was menadione independent, whereas strains HG 91, HG 94, and HG 76 were menadione dependent. *B. endodontalis* strains were not dependent on hemin for growth but needed menadione. No growth or only the growth of a few colonies was observed when menadione was depleted from the medium. This dependence was observed during the first cultivation. *B. endodontalis* strains showed no hemolytic activity on blood agar after 7 days of anaerobic incubation. However, after anaerobic incubation, all strains were hemolytic after 24 or 48 h under aerobic conditions at room temperature. An exception is strain HG 370^T which had a slight hemolytic activity under anaerobic conditions.

On horse blood agar medium, *B. endodontalis* and *B. asaccharolyticus* formed low convex, circular colonies with an entire edge and were 1 to 2 mm in diameter after 5 days of incubation. Small brown particles were often observed in both the colonies and the agar under growing colonies of all *B. endodontalis* and some *B. asaccharolyticus* strains. Colony morphology of *B. endodontalis* resembled that of *B. asaccharolyticus* but was quite distinct from *B. gingivalis* colonies which were dome-shaped and were not strongly pigmented in the agar. *B. endodontalis* colonies did not stick to the agar and were easily removed. Growth in BM medium (12) was slow, with a maximal optical density of 0.6 to 0.8 after 7 days. The addition of newborn calf serum (1 to 3%) enhanced the growth rate but not the final density.

The enzyme profiles of the three asaccharolytic black-pigmented *Bacteroides* species as tested with the API ZYM technique are shown in Table 3. Alkaline phosphatase, acid phosphatase, butyrate esterase, caprylate esterase, and phosphoamidase enzyme profiles were similar. However, only *B. gingivalis* was found to produce trypsin like activity and *N*-acetyl-β-glucosamidase activity. An enzyme activity specific for *B. asaccharolyticus* was found for α-fucosidase. All strains hydrolyzed 5 to 10 nmol of 2-naphthyl-α-L-fucopyranoside. Other investigators found no (14) or variable (6) α-fucosidase activity for *B. asaccharolyticus*, although some strains were the same as those we tested. A possible explanation for these differences could be the use of different kinds of culture media. To determine whether these media could be the cause of the different outcomes, we tested all *B. asaccharolyticus* strains with cells grown on *Brucella* agar with 7% human blood (6) and Trypticase soy agar (BBL Microbiology Systems) with 10% sheep blood and supplemented with 5 μg of hemin per ml and 0.2 μg of menadione per ml (14). We found all strains grown on these media to have a α-fucosidase activity. Ten to 20 nmol of 2-naphthyl-α-L-fucopyranoside substrate was hydrolyzed. No enzyme activity specific for *B. endodontalis* was found.

All strains tested grew well after 6 days of incubation in atmospheres with both 10 and 5% CO_2. All strains of *B. endodontalis* failed to grow in an atmosphere of 95% N_2–5% H_2 or produced only a faint growth on the inoculated area without individual colonies visible. All *B. asaccharolyticus* strains tested, however, grew in 95% N_2–5% H_2, although growth was reduced compared with growth in both other atmospheres.

All *B. endodontalis* strains tested were agglutinated by the antiserum raised against strain HG 370^T, whereas no strain agglutinated in phosphate-buffered saline or preimmune serum. Some strains reacted more rapidly than others. HG 370, HG 181, and HG 415 agglutinated within 20 s, whereas the other strains reacted within 1 to 2 min. None of the *B. gingivalis* strains tested were agglutinated by the antiserum, preimmune serum, or phosphate-buffered saline. Two

TABLE 3. Enzymatic characterization of asaccharolytic black-pigmented *Bacteroides* species by the API ZYM system

Organism	Total no. of strains	Enzyme activity of[a]: Alkaline phosphatase	Butyrate esterase	Caprylate esterase-lipase	Myristate lipase	Leucine amino-peptidase	Valine amino-peptidase	Cystine amino-peptidase	Trypsin	Chymotrypsin	Acid phosphatase	Phosphoamidase	α-Galactosidase	β-Galactosidase	β-Glucuronidase	α-Glucosidase	B-Glucosidase	*N*-Acetyl-β-glucosamidase	α-Mannosidase	α-Fucosidase
B. endodontalis	12	++	+	+	−	−	−	−	−	−	++	++[b]	−	−	−	−	−	−	−	−
B. gingivalis	8	++	+(3)	+	−	−	−	−	++	−	++	++	−	−	−	−	−	++	−	−
B. asaccharolyticus	6	++	+	+	−	−(3)	+	−	−	−	++	++	−	−	−	−	−	−	−	+

[a] Symbols: ++, strong reaction; (API ZYM test reading of 3 to 5 according to manufacturer's manual); +, moderate reaction (API ZYM test reading of 1 to 2 according to manufacturer's manual); and −, no reaction. Data in parentheses show number of positive strains.

[b] Negative for strain HG 412.

TABLE 4. Identification characteristics of asaccharolytic black-pigmented *Bacteroides* species

Organism	G+C content (mol%)	Hemagglutination	Phenylacetic acid	Cell-free protease	Menadione requirement	α-Fucosidase	CO_2 requirement	Agglutination with *B. endodontalis* antiserum
B. gingivalis	49	+	+	+	Variable	−	Variable	−
B. asaccharolyticus	54	−	−	−	−	+	−	−
B. endodontalis	50	−	−	−	+	−	+	+

strains of *B. asaccharolyticus* (HG 111 and HG 124) were autoagglutinable. The other strains tested did not agglutinate with the antiserum or the preimmune serum.

DISCUSSION

Based on the guanine-plus-cytosine contents and on the DNA hybridizations with labeled DNA of HG 181, the nine strains studied were classified as *B. endodontalis*. These results agree with previous ones in which only strains HG 370, HG 181, and HG 182 were studied and proposed as a new species, i.e., *B. endodontalis* (21).

Not only morphologically but also physiologically, *B. endodontalis* seems to be more related to *B. asaccharolyticus* than to *B. gingivalis*. This accounts for the lack of hemagglutination, proteolytic activity, and production of phenylacetic acid as well as the colony morphology.

Formerly, *B. endodontalis* could be identified by means of DNA studies, serology, and polyacrylamide gel electrophoresis patterns. In this study, some characteristic differences between the three asaccharolytic black-pigmented *Bacteroides* species were found. The specific menadione requirement of *B. endodontalis* as well as the enzyme profiles and the specific antiserum against *B. endodontalis* HG 370^T can be used for discrimination between the three asaccharolytic black-pigmented *Bacteroides* species. Differentiation between *B. endodontalis* and *B. gingivalis* is distinct by the positive hemagglutination reaction of *B. gingivalis*. Another difference between *B. asaccharolyticus* and *B. endodontalis* is the menadione requirement of the latter species.

The API ZYM technique has proven to be a useful test for the identification of the asaccharolytic black-pigmented *Bacteroides* species. The trypsin-like activity of *B. gingivalis* has been reported earlier (8, 14). In our study, all strains of *B. asaccharolyticus* tested were found to have an α-fucosidase activity. Different kinds of media did not influence this activity. These results do not explain the differences in activity found in this study and those from Hofstad (6) and Slots (14). So far, we have no explanation for this discrepancy.

We found that α-fucosidase activity is a reliable property of *B. asaccharolyticus* and can well serve as a differentiating test between *B. endodontalis* and *B. asaccharolyticus*. Growth in 95% N_2–5% H_2 proved to be a valuable test for the discrimination of *B. endodontalis* and *B. asaccharolyticus*. This test is easy to perform if a gas cylinder containing N_2 and H_2 is available. Although results with these tests were consistent in our experiments, it is advisable to perform all tests described for the identification of *B. endodontalis*. When there is doubt, a determination of the percentage of guanine-plus-cytosine content can be very helpful. Tests for rapid identification of the three asaccharolytic black-pigmented *Bacteroides* species are summarized in Table 4.

With the specific antiserum against HG 370^T, we confirmed that *B. endodontalis* is antigenetically distinct from the two other asaccharolytic black-pigmented *Bacteroides* species (21). The specific antiserum proved to be a useful tool for the rapid identification of *B. endodontalis*. Earlier, *B. gingivalis* was found to be serologically distinct from *B. asaccharolyticus* (10).

Up to now, *B. endodontalis* has been isolated from infected dental root canals with periapical destruction and submucous abscesses. These abscesses were probably an aggravation of a periapical osteitis. *B. endodontalis* seems to be involved in endodontic infections which can intensify and thus result in serious inflammation, loss of alveolar bone, or both. In all cases, *B. endodontalis* was isolated as part of a mixed microbiological flora in which four to six other bacterial species were involved. From experimental mixed infections with oral bacteria, it is known that black-pigmented *Bacteroides* species play an obligate role (9, 19). Up to now, it was unknown whether *B. endodontalis* plays a similar essential role in mixed infections. It seems that the three asaccharolytic black-pigmented *Bacteroides* species are isolated from different sites of the human body. *B. asaccharolyticus* is commonly associated with nonoral infections, *B. gingivalis* is associated with the various forms of periodontitis, and *B. endodontalis* is associated with endodontic infections, although there are some exceptions (21, 22). It is not known whether *B. endodontalis* is part of the oral cavity or whether this species has a nonoral ecological niche.

The *B. endodontalis* strains seem to be very sensitive to oxygen. Media had to be freshly prepared to get satisfactory growth. In an earlier study, two strains of black-pigmented *Bacteroides* (HG181 and HG182), which are now classified as *B. endodontalis*, were found to be most oxygen sensitive when compared with other *Bacteroides* strains (2). This oxygen sensitivity may be one of the reasons that *B. endodontalis* was not often isolated previously. Another reason may be that this species grows much slower and produces pigment only after 7 days of incubation. Furthermore, several researchers in oral microbiology use selective media for the isolation of black-pigmented *Bacteroides* species. Often, a combination of kanamycin and vancomycin is used in these selective media. However, vancomycin is inhibitory to all asaccharolytic black-pigmented *Bacteroides* species (23). Among the black-pigmented *Bacteroides* species, *B. asaccharolyticus* and *B. endodontalis* appeared to be the most sensitive. If vancomycin is used as a selective agent in isolation media, *B. endodontalis* will probably not be recovered from clinical samples.

Not much is known about the virulence of *B. endodontalis*. Only two strains of this species have been studied in earlier virulence experiments (18, 20). However, additional studies are currently under investigation. On the basis of the

results presented in this paper, it seems that *B. endodontalis* is involved in endodontic infections and the possible subsequent submucosal abscesses. We have also described some identification properties for discriminating between the asaccharolytic black-pigmented *Bacteroides* species.

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