JOURNAL OF CLINICAL MICROBIOLOGY, May 1980, p. 522-526
0095-1137/80/05-0522/05$02.00/0

Vol. 11, No. 5

Characteristics of *Bacteroides asaccharolyticus* from Dental Plaques of Beagle Dogs

S. A. SYED

Department of Oral Biology, Dental Research Institute, School of Dentistry, The University of Michigan, Ann Arbor, Michigan 48109

Gram-negative, non-saccharolytic, brown- or black-pigment-forming, nonmotile anaerobic coccobacilli, capable of decomposing hydrogen peroxide and identified as *Bacteroides asaccharolyticus* (*B. melaninogenicus* subsp. *asaccharolyticus*), were isolated from the supra- and subgingival plaques of beagle dogs with gingivitis or periodontitis. The organisms remained viable for many hours in an aerobic atmosphere as evidenced by their ability to grow subsequently in an anaerobic environment. They also grew well on agar media that were not reduced before use. Although blood was required for pigmentation of colonies, organisms grew on media that lacked hemin, menadione, blood, or reducing compounds. Increased oxygen tolerance, catalase activity, and different nutritional requirements differentiate these organisms from strains of *B. asaccharolyticus* isolated from humans.

Bacteroides asaccharolyticus (2), formerly called *Bacteroides melaninogenicus* subsp. *asaccharolyticus* (5), is an anaerobic, gram-negative, nonmotile coccobacillus which forms dark brown- or black-pigmented colonies on enriched blood agar media. This organism does not ferment glucose or other carbohydrates and does not produce catalase (4, 5). It has been isolated from the dental plaque and periodontal pockets of periodontitis patients (7, 10, 12), from periodontal plaque of monkeys (11), and from supra- and subgingival plaques of beagle dogs with gingivitis (S. A. Syed, M. Svanberg, and G. Svanberg, J. Periodont. Res., in press), and periodontitis (S. A. Syed, M. Svanberg, and G. Svanberg, Int. Assoc. for Dent. Res. Abstr. no. 553, J. Dent. Res. Vol. 56, special issue A, 1977). Considerable information as to the physiological and biochemical characteristics of this organism (4, 5, 9, 13) and its role as a suspected pathogen in human mixed anaerobic infections (1) and in periodontal disease (12) is available. In the present investigation, 200 isolates of *B. asaccharolyticus* from supra- and subgingival plaques or periodontal pockets of beagle dogs were studied. Characteristics of these strains and their similarities to or differences from human *B. asaccharolyticus* strains are described in this report.

MATERIALS AND METHODS

Sample source and culturing. The plaque samples were collected in a reduced transport fluid (15) from three groups of healthy female dogs, namely, (i) 8 to 9 year olds, kept on daily oral hygiene and with no gingivitis, (ii) 1 to 6 year olds, with minimal or moderate gingivitis, and (iii) 8 to 9 year olds with periodontitis. The samples were dispersed for 20 s by sonication with a Kontes sonifier (S. A. Syed and W. J. Loesche, J. Dent. Res. **57**:982, 1978), serially diluted, and cultured in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.) which had the following gas mixture: 85% N_2, 10% H_2, and 5% CO_2. In all primary isolation experiments, a prereduced enriched Trypticase soy agar (BBL Microbiology Systems) with 3% defibrinated sheep blood (ETSA, Table 1) was used. The inoculated plates were incubated at 37°C for 7 days and examined with a stereomicroscope. Colonies with tan-orange, reddish-brown, or black pigments were observed among the predominant organisms of all the dental plaque specimens.

Identification and characterization. The strains that formed pigmented colonies were tentatively identified as *B. melaninogenicus* on the basis of colony fluorescence on exposure to ultraviolet light (Wood lamp, 365 nm), lack of growth in aerobic or microaerophilic conditions, and Gram reaction. Representative isolates from each plaque specimen were subcultured on ETSA, and their purity was confirmed by stereomicroscopic examination of colonies, microscopic cellular morphology, and Gram staining. Two hundred isolates were subcultured in a prereduced basal esculin-nitrate broth (BEN, 16) and an anaerobic glucose broth (Table 2) and incubated anaerobically at 37°C for 7 to 14 days. The organisms were also streaked on a variety of agar media listed in Table 3 to determine their ability to grow on such media and their growth characteristics. Additional carbohydrates (Table 3) were tested using the BBL Minitek System for anaerobes. The pH of cultures in anaerobic glucose broth was measured with a Beckman combination pH electrode. A pH of 6.5 to 7 was considered negative.

Subcultures of anaerobically grown organisms were made on ETSA plates and incubated in room atmosphere, in a candle jar, and in the anaerobic glove box atmosphere. The plates were examined after 5 to 7 days for growth.

TABLE 1. *Composition of enriched Trypticase soy agar*

Constituents[a]	Amount
Agar (Difco)	4 g
Trypticase soy agar (BBL)	40 g
Yeast extract (Difco)	1 g
Glucose	1 g
Potassium nitrate	0.5 g
Sodium lactate (60%)	1 ml
Sodium succinate	0.5 g
Sodium formate	0.5 g
Sodium fumerate (10% solution)	10 ml
Distilled water	950 ml
Hemin (1.0-mg/ml stock solution)[b]	1 ml
Menadione (0.5-mg/ml stock solution)[c]	2 ml
Sodium carbonate (anhydrous)	0.4 g
Cysteine hydrochloride (Sigma)	0.4 g
Dithiothreitol (Sigma)	0.1 g
Distilled water	20 ml

[a] The first 10 constituents were mixed and autoclaved at 121°C for 15 min. A solution of the last six compounds was sterilized by means of a membrane filter (0.22-μm pore size). The sterile solution and defribrinated sheep blood (30 ml) were added to the autoclaved medium cooled to 50°C. The solution was freshly prepared each time the medium was made.

[b] Prepared by dissolving 0.2 g of hemin (Sigma Chemical Co., St. Louis, Mo.) in 200 ml of 0.1 N KOH solution in 50% ethanol: the solution was stored in a screw-capped bottle in the refrigerator.

[c] Prepared by dissolving 0.05 g of menadione in 100 ml of 50% ethanol in distilled water, filter sterilized, and stored in a brown screw-capped bottle in the refrigerator. Commercial source of menadione, Nutritional Biochemical Corporation, Cleveland, Ohio.

Catalase test. The catalase test was performed on cultures grown on Trypticase soy agar, ETSA without blood, gelatin agar, and Schaedler agar. Immediately after the plates were taken out of the anaerobic glove box, a small amount of growth was transferred to a glass slide or watch-glass, and a drop of 30% hydrogen peroxide (Superoxol, Merck Co.) was added. The results were recorded within a few seconds. The test was repeated with 3 and 15% hydrogen peroxide added to pellets of Trypticase soy broth cultures which were prewashed several times with phosphate buffer (pH 7.4).

Physiological tests and gas chromatographic analyses. Tests for nitrate reduction, esculin hydrolysis, and indole production were performed on BEN-grown cultures by standard procedures (6, 8). The gelatinase test was performed by the gelatin-agar plate technique (14). H_2S production was tested with lead acetate paper strips placed between the screw caps and mouth of the test tubes containing glucose broth cultures. The test was repeated with cultures grown on ETSA by exposing the lead acetate paper strips between the lids and the bottoms of the petri plates. Gas-liquid chromatographic analysis of BEN or glucose broth cultures and uninoculated broth was done by an ether extraction procedure for volatile acids (6) with a Varian gas chromatograph model 2740 equipped with a flame ionization detector and a stainless steel column (6 feet by 0.25 in [ca. 182.9 by 0.64 cm]) packed with 15% FFAP (Varian, Palo Alto, Calif.) on Chromosorb W (acid washed, dimethylchlorosilane treated). The operation conditions were as follows: injection port temperature, 175°C; column temperature, 150°C; detector temperature, 180°C; helium flow, 30 cm^3/min; sensitivity, 10^{-10} A; recorder response, 1 mV full scale; and chart speed, 25 mm/min.

Antibiotic susceptibility tests. Sensitivity of the isolates to tetracycline, penicillin G, and clindamycin was determined with antibiotic disks (Difco Laboratories, Detroit, Mich.) certified for susceptibility testing. Suspensions of the colonies from ETSA plates were prepared in 5 ml of anaerobic glucose broth to a turbidity equal to that of one-half of a McFarland no. 1 standard (0.2 ml of 1% $BaCl_2 \cdot 2H_2O$ in 19.9 ml of 1% H_2SO_4). Prereduced ETSA plates (100 by 15 mm) were streaked evenly over the entire agar surface with sterile Calgiswabs (Inolex Corp., Glennwood, Ill.) that were dipped into the standardized culture suspensions. The plates were then allowed to dry for a few minutes. One disk was placed in each of three quadrants and gently pressed with sterile forceps to insure its contact with the agar surface. The quadrant without a disk was the control. The plates were examined after incubation in the anaerobic glove box for 24, 48, and 72

TABLE 2. *Composition of anaerobic broth with glucose*

Constituents[a]	Amount
Tryptone	10 g
Yeast extract	5 g
Mineral salt solution no. 1[b]	75 ml
Mineral salt solution no. 2[c]	75 ml
Sodium chloride	5 g
Potassium nitrate	0.5 g
Distilled water	800 ml
Dithiothreitol (Sigma)	0.2 g
Hemin (1.0-mg/ml stock solution)[d]	1.0 ml
Menadione (0.5-mg/ml stock solution)[e]	2.0 ml
Sodium carbonate (anhydrous)	0.5 g
Glucose	10 g
Distilled water	50 ml

[a] The first seven constituents were mixed and autoclaved at 121°C for 15 min. A solution of the last six compounds was sterilized by means of a membrane filter (0.22-μm pore size). The sterile solution was added to the autoclaved medium cooled to 50°C, mixed, and dispensed in 5-ml aliquots into 15- by 100-mm sterile screw-capped tubes. The solution was freshly prepared each time the medium was made.

[b] Contains 0.6% K_2HPO_4.

[c] Contains 1.2% NaCl, 1.2% $(NH_4)_2SO_4$, 0.6% KH_2PO_4, and 0.25% $MgSO_4$.

[d] Prepared by dissolving 0.2 g of hemin (Sigma Chemical Co., St. Louis, Mo.) in 200 ml of 0.1 N KOH solution in 50% ethanol. The solution was stored in a screw-capped bottle in the refrigerator.

[e] Prepared by dissolving 0.05 g of menadione in 100 ml of 50% ethanol in distilled water; filter sterilized and stored in a brown screw-capped bottle in the refrigerator. Commercial source of menadione: Nutritional Biochemical Corporation, Cleveland, Ohio.

TABLE 3. *Selected characteristics of B. asaccharolyticus isolates from dental plaques of beagle dogs*

Characteristic	Observation[a]
Oxygen tolerance	+
Pigment synthesis and colony fluorescence in ultraviolet light (365 nm) on:	
Agar media with blood (ETSA, Schaedler agar)	+
Agar media without blood (ETSA, gelatin agar)	−
Catalase	+
Urease	−
Oxidase	−
Hydrogen sulfide produced	+
Requirement for vitamin K or hemin	−
Acid from sugars or other substrates[b]	−
Esculin, starch, or gelatin hydrolyzed	−
Indole produced	+
Nitrate or nitrite reduced	−
n-Butyric acid produced	+
Sensitivity to antibiotics[c]	
Penicillin-G, clindamycin, metronidazole, or tetracycline	+
Resistance to aminoglycosides	+

[a] −, Negative result, +, positive result.

[b] Includes glucose, lactose, sucrose, maltose, mannitol, sorbitol, esculin, starch, xylose, arabinose, raffinose, and inulin.

[c] Penicillin G (2-U disk), clindamycin (2-μg disk), metronidazole (10 μg/ml), and tetracycline (5-μg disk).

h. The diameter of the zone of growth inhibition was recorded as soon as the culture growth was evident on the plates.

Sensitivity of the isolates to other antimicrobial agents was tested on ETSA supplemented with kanamycin (50 μg/ml), neomycin (50 μg/ml), or metronidazole (10 μg/ml). Plates were streaked with a loop with a small inoculum from broth cultures. Plates were checked for growth after incubation for 24 to 72 h in an anaerobic atmosphere.

Serotyping by fluorescent-antibody technique. Fluorescein isothiocyanate-labeled antisera to human and beagle dog plaque isolates of *B. asaccharolyticus* were prepared by the methods of Grenier et al. (3). Representative isolates of *B. asaccharolyticus* from beagle dog dental plaque were tested by a direct fluorescent-antibody technique with eriochrome black as a counterstain (3).

RESULTS

Colonial characteristics, pigment production, and microscopic single-cell morphology of all isolates were examined from growth on ETSA. The colonies of all isolates were similar: 3 to 5 mm in diameter and convex with an entire margin. The pigments of cultures grown on ETSA for 48 to 72 h ranged from light brown, tan, to reddish brown. On further incubation, colonies became dark brown or black. Unlike human *B. asaccharolyticus* strains, the pigmented colonies on ETSA plates kept in the anaerobic glove box remained viable for several weeks. The fluorescence characteristics of colonies from the majority of isolates under ultraviolet light (365 nm) remained stable even after the colonies were entirely pigmented. All isolates were gram-negative coccobacilli which exhibited pleomorphism (Fig. 1). Long rods were rarely observed. Cells were nonmotile as tested by dark-field microscopic examination of freshly grown broth cultures. No surface translocating activity on agar media was observed. The organisms grew well and exhibited catalase activity on a variety of media, including those that did not have blood, reducing agents, hemin, or menadione. However, they did not synthesize pigment on media without blood (Table 3). Occasionally, some isolates exhibited beta hemolysis on ETSA.

All the isolates were capable of growth on the agar medium containing either kanamycin or neomycin. However, they did not grow on agar medium supplemented with metronidazole. The results of antibiotic susceptibility tests determined by disk diffusion technique showed that the growth inhibition zone diameter of the test strains for each of the antibiotics was >20 mm, suggesting that the organisms were sensitive to the antibiotics.

To determine the aerotolerance of the isolates, cultures grown anaerobically on ETSA were ex-

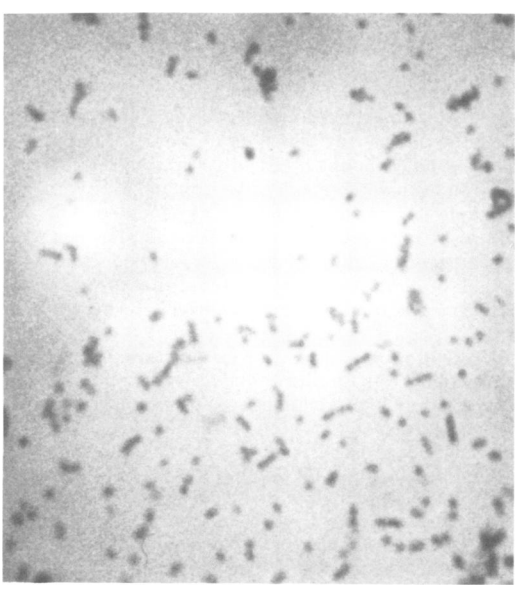

FIG. 1. *Microscopic morphology of B. asaccharolyticus isolated from the subgingival plaque of a beagle dog. Note the coccobacilli of variable size and coccal forms.*

posed to room atmosphere for 2, 4, 6, 8, and 24 h and then streaked on ETSA plates which then were incubated in an anaerobic atmosphere. Excellent growth was produced from colonies exposed to aerobic conditions for up to 8 h. After exposure of colonies for 24 h, only moderate growth resulted when the colonies were streaked on ETSA plates.

The results of serological tests by the direct fluorescent-antibody technique are shown in Table 4. None of the isolates could be typed by the fluorescein isothiocyanate-labeled conjugates prepared with human strains. However, they gave positive tests with canine *B. asaccharolyticus*-fluorescein isothiocyanate-labeled conjugates.

DISCUSSION

The canine strains of *B. asaccharolyticus* were identical in all characteristics tested irrespective of the source of origin, i.e., supragingival plaque, subgingival plaque, or periodontal pockets of beagle dogs. One of the prominent characteristics of these organisms is their ability to produce catalase under anaerobic conditions, a characteristic which, to our knowledge, has not been reported in the literature for pigmented human *Bacteroides* species or for human strains of *B. asaccharolyticus*. The ability of the organisms to survive in the aerobic atmosphere for a relatively long time could be explained in part by their catalase activity. Since the organisms grew well on a variety of growth media with or without blood, reducing agents (dithiothreitol) or other enrichments (hemin or menadione), it would suggest that their nutritional requirements are different from *B. asaccharolyticus* strains isolated from humans. This could also reflect their metabolic efficiency in terms of macromolecule synthesis and their competence for better survival in a complex ecological system. In this regard, these organisms are not as fastidious as are human *B. asaccharolyticus* or *B. melaninogenicus*.

TABLE 4. *Serological reactions of B. asaccharolyticus isolates from beagle dogs by direct fluorescent-antibody (FA) test*

Strain no.	Plaque source	Reactions with undiluted FA conjugates of *B. asaccharolyticus* strains	
		SP1 (human)	D1B1 (beagle dog)
D1B1 (control)	Subgingival	−[a]	4+[b]
O48-2	Supragingival	−	4+
D1-A	Supragingival	−	2+
N1-A	Supragingival	−	2+
N2-A	Supragingival	−	2+
D1-B	Subgingival	−	2+
N1-B	Subgingival	−	2+
SP1 (control)	Subgingival	4+	−

[a] −, Negative reaction.
[b] +, Positive reaction; 4+, strong; 2+, moderate.

Blood was not an essential growth factor for these organisms, but its incorporation in the culture media improved their growth considerably, indicating a stimulatory effect. However, pigment synthesis by these organisms was dependent on the presence of blood in the culture medium. This suggested that the blood was required by the organisms for pigment synthesis, and it served as a source of essential metabolites which could be used by the organisms as precursors of pigment.

The detection of catalase activity in broth cultures or cultures grown on agar media without blood provided further evidence for the synthesis of this enzyme by the canine strains of *B. asaccharolyticus*. Hence, the possibility of pseudo-catalase activity due to pigment, its derivatives, or blood was excluded. The requirement of hemin for synthesis of catalase by these organisms was not determined in this study. Organisms grown on hemin-free growth media exhibited weak catalase activity, suggesting that they would require a minimal amount of hemin for adequate synthesis of this enzyme.

The occurrence of hydrogen peroxide-splitting strains of *B. asaccharolyticus* in the periodontal plaque of monkeys was recently reported by Slots and Hausmann (11). Since the test was performed on colonies grown on Trypticase soy blood agar, the possibility of pseudo-catalase activity exhibited by *B. asaccharolyticus* strains from monkey plaque cannot be ruled out unless proven otherwise by additional studies such as catalase tests on colonies grown on blood-deficient agar media. We have found that *B. melaninogenicus* subsp. *intermedius* and *B. asaccharolyticus* strains isolated on blood agar from human periodontal plaque would decompose hydrogen peroxide, but they would not produce pigment or decompose hydrogen peroxide when grown on agar media without blood (unpublished data). This apparent catalase activity of human oral strains of *B. melaninogenicus* subsp. *intermedius* or *B. asaccharolyticus* appears to be associated with the pigment synthesized by these organisms in the presence of blood. Therefore, hydrogen peroxide-splitting activity of pigmented colonies of these organisms should be interpreted with caution.

Although the majority of the physiological characteristics of *B. asaccharolyticus* strains studied in this investigation are similar to human strains of *B. asaccharolyticus*, they differ in that

they produce catalase, are rather tolerant to atmospheric oxygen, and grow in media lacking menadione, hemin, blood, and reducing compounds. They also are serologically different from the human strains. Although we suspect that these strains may be sufficiently different from the human strains to constitute a different species, we did not determine their guanine plus cytosine moles-percent ratios, their nucleic acid sequence similarity, or their cell wall composition, characteristics that would give a clearer indication of their taxonomic position. Also, the significance of the role of the catalase produced by the canine strains in the microbial ecology of the dog, or in the pathogenicity of this organism in dental infections, is not certain at this time.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants DE02731 and DE03011 from the National Institute of Dental Research.

Janice Stoll and Ruth Roper assisted in the study. Edith Morrison and Gunnar Svanberg kindly provided the plaque samples. Ella Grenier prepared the antisera and performed serological tests. I would also like to acknowledge Martha M. Jones for typing the manuscript.

LITERATURE CITED

1. **Finegold, S. M.** 1977. Anaerobic bacteria in human disease. Academic Press Inc., New York.
2. **Finegold, S. M., and E. M. Barnes.** 1977. Report of the ICBS Taxanomic Subcommittee on Gram Negative Anaerobic Rods. Proposal that the *saccharolytic* and *asaccharolytic* strains at present classified in the species *Bacteroides melaninogenicus* (Oliver and Wherry) be reclassified into two species *Bacteroides melaniogenicus* and *asaccharolyticus*. Int. J. Syst. Bacteriol. **27**:388-391.
3. **Grenier, E. M., W. C. Eveland, and W. J. Loesche.** 1973. Identification of *Streptococcus mutans* in dental plaque by fluorescent antibody technique. Arch. Oral Biol. **18**:707-715.
4. **Harding, G. K. M., V. L. Sutter, S. M. Finegold, and K. S. Bricknell.** 1976. Characteristics of *Bacteroides melaninogenicus*. J. Clin. Microbiol. **4**:354-359.
5. **Holdeman, L. V., and W. E. C. Moore.** 1974. Genus 1. Bacteroides Castellami and Chalmers, p. 385-387, 400-404. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinature bacteriology, 8th ed., The Williams and Wilkins Co., Baltimore, Md.
6. **Holdeman, L. V., and W. E. D. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed., p. 125-128, 134. Virginia Polytechnic Institute and State University, Blacksburg, Va.
7. **Loesche, W. J., R. M. Hockett, and S. A. Syed.** 1972. The predominant cultivable flora of tooth surface plaque removed from institutionalized subjects. Arch. Oral Biol. **17**:1311-1325.
8. **Paik, G., and M. T. Suggs.** 1974. Reagents, stains, and miscellaneous test procedures, p. 930-950. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
9. **Shah, H. N., R. A. D. Williams, G. H. Bowden, and J. M. Hardie.** 1976. Comparison of the biochemical properties of *Bacteroides melaninogenicus* from human dental plaque and other sites. J. Appl. Bacteriol. **41**: 473-492.
10. **Slots, J.** 1977. The predominant cultivable microflora of advanced periodontitis. Scand. J. Dent. Res. **85**:114-121.
11. **Slots, J., and E. Hausmann.** 1978. Longitudinal study of experimentally induced periodontal disease in *Macaca arctoides*: relationship between microflora and alveolar bone loss. Infect. Immun. **23**:260-269.
12. **Socransky, S. S.** 1977. Microbiology of periodontal disease-present status and future considerations. J. Periodontol. **48**:497-504.
13. **Williams, R. A. D., G. H. Bowden, J. M. Hardie, and H. Shah.** 1977. Biochemical properties of *Bacteroides melaninogenicus* subspecies. Int. J. Syst. Bacteriol. **25**: 298-300.
14. **Syed, S. A.** 1976. A new medium for the detection of gelatin-hydrolyzing activity of human dental plaque flora. J. Clin. Microbiol. **3**:200-202.
15. **Syed, S. A., and W. J. Loesche.** 1972. Survival of human dental plaque flora in various transport media. Appl. Microbiol. **24**:638-644.
16. **Syed, S. A., and W. J. Loesche.** 1978. Bacteriology of human experimental gingivitis: effect of plaque age. Infect. Immun. **21**:821-829.