Bacteroides forsythus sp. nov., a Slow-Growing, Fusiform Bacteroides sp. from the Human Oral Cavity

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The characteristics of a group of slow-growing, fusiform, fastidious anaerobes isolated from advanced periodontal lesions in human oral cavities were examined. Our results indicated that 12 fusiform Bacteroides strains belong to a new species in the genus Bacteroides. The name Bacteroides forsythus is proposed for these isolates. The type strain is strain ATCC 43037.

Anaerobic, gram-negative, fusiform organisms which did not resemble previously recognized species were isolated from deep periodontal pockets of humans that showed progressive tissue loss (3, 15, 17). The cells appeared to be long filaments or medium rods with tapered (fusiform) or rounded ends. Some cells had central swellings. Large spheroids were frequently associated with the cells, particularly the cells with filamentous morphology. These strictly anaerobic fusiform Bacteroides strains grew slowly on blood agar plates and grew poorly in all broth media tested and on most agar media tested. Biochemical tests gave inconsistent results and were usually negative. For this reason, the fusiform Bacteroides strains were characterized without using most of the conventional biochemical tests. Deoxyribonucleic acid (DNA) guanine-plus-cytosine (G+C) content, DNA-DNA homology, cell wall ultrastructure, serology, API enzyme substrate tests (Analytab Products, Plainview, N.Y.), and protein profiles of whole sonicated cells obtained by using polyacrylamide gel electrophoresis (PAGE) indicated that these organisms comprise a distinct species in the genus Bacteroides. The name Bacteroides forsythus is proposed for this new species.

Strains and inocula. The strains which we used are listed in Table 1. These organisms were maintained on Trypticase soy agar plates supplemented with 5% sheep blood (TSBA; BBL Microbiology Systems, Cockeysville, Md.) at 35 to 37°C in an atmosphere containing 10% H2, 10% CO2, and 80% N2. The fusiform Bacteroides strains were maintained on TSBA plates; a fusiform Bacteroides strain was streaked on one-half of each plate, and a strain of Fusobacterium nucleatum was streaked on the other half of the plate. The strains were stored in liquid nitrogen by using previously described methods (14). Inocula for tests were obtained by scraping cells from the surfaces of TSBA plates. At least 100 plates of fusiform Bacteroides strains were used to obtain sufficient cells for DNA extraction. Two to four plates were used to provide the inoculum for an enzyme-linked immunosorbent assay and for each of the tests in the API enzyme substrate test series.

DNA-DNA hybridization. Cells of reference strains were grown in 2- to 3-liter broth cultures (mycoplasma broth [BBL] supplemented with 5 mg of hemin per liter) for extraction of DNA. The DNA was extracted by using a method modified (13) from the procedure of Marmur (9).

G+C contents were determined by the thermal denaturation method (1). Levels of DNA-DNA homology were determined by using the initial renaturation rates (2).

API enzyme substrate tests. API ZYM and API An-Ident enzymatic substrate tests were performed in duplicate as recommended by the manufacturer (Analytab Products) (18). Cells from agar plates were suspended in sterile water, inoculated onto the test strips, and incubated in air for 4 h at 35°C.

PAGE (i) Gel preparation. We prepared 9% sodium dode-
FIG. 1. PAGE of whole sonicated cells of *B. forsythus* and oral fusiform species. The upper and lower lines indicate the positions of the superimposed internal standards. *B. forsythus* is characterized by two protein bands at molecular weights of more than 205,000. Lane a, High-molecular-weight standard mixture; lanes 1, 10, and 18, *F. nucleatum* FDC 364; lanes 2 through 9 and 11 through 14, *B. forsythus* strains (lane 2, strain FDC 338{T}= ATCC 43037{T}; lane 3, strain FDC 42; lane 4, strain FDC 148; lane 5, strain FDC 293; lane 6, strain FDC 334; lane 7, strain FDC 436; lane 8, strain FDC 2008; lane 9, strain FDC R145; lane 11, strain FDC BV19; lane 12, strain FDC DL12; lane 13, strain FDC DL38; lane 14, strain FDC BV16); lane 15, *C. gingivalis* ATCC 33624{T}; lane 16, *C. ochracea* FDC 6; lane 17, *C. sputigena* ATCC 33612=.

cyl sulfate discontinuous polyacrylamide gels (thickness, 1.5 mm) with 20 wells in a vertical slab unit (model SE600; 20 by 20 cm) as recommended by Hoefer Scientific Instruments, San Francisco, Calif. The stacking gels contained 4% polyacrylamide. Polyacrylamide gel solutions were degassed for 15 min in a Brewer Jar at a negative pressure of 0.066 Pa (26 to 28 lb/in²). The gels were left overnight before use.

(ii) Loading and running the gels. Cells were suspended in 0.2 ml of water and disrupted by sonication for 1 min with a microultrasonic cell disrupter (Kontes, Vineland, N.J.) at a power setting of 2. A treatment buffer containing 0.125 M tris (hydroxymethyl)aminomethane hydrochloride (pH 6.8), 4% sodium dodecyl sulfate, 20% glycerol, and 10% 2-mercaptoethanol was added, the suspensions were boiled for 5 min, and a phenol red tracking dye was added; 2 to 10 ~1 of each suspension was added to each well. The gels were run at a constant current of 30 mA per gel until the tracking dye was within 1 cm of the end of the gel (about 3 to 5 h).

(iii) Staining. The gels were fixed overnight in 50% methanol–50% distilled water containing 0.1 ml of 37% (wt/wt) formaldehyde per 100 ml of solution. The fixative was removed, 500 ml of a 5-µg/ml dithiothreitol solution was added to each gel, and the gels were agitated for 1.5 h. The dithiothreitol was poured off, 500 ml of silver stain (a 0.1% solution of AgNO₃) was added to each gel, and the preparations were agitated for 1 h. The gels were washed with distilled water for 30 s and then washed twice with 100 ml of developer containing 3% Na₂CO₃ and 0.5 µl of formaldehyde per ml. A 200-ml portion of developer was then added to each gel for 2 min or until the bands were sufficiently stained. Gel staining was stopped by adding 2.3 M citric acid (9.66 g of citric acid in 20 ml of water for each gel) and shaking the preparations for 10 min. The gels were stored wet in plastic wrap in plastic bags.

(iv) Gel analysis. Only gels prepared from the same batch of acrylamide and electrophoresed at the same time in the same tank were compared. The protein bands on the wet gels were scanned by using a model 2202 Ultroscan laser spectrophotometer (LKB, Stockholm, Sweden). Bacterial protein profiles were compared by performing cluster analysis of correlation coefficients as described by Kersters and De Ley (7). The low- and high-molecular-weight standards used were protein bands of *F. nucleatum* FDC 364, which was put on three places on the gel. The selected bands were connected with black lines (Fig. 1 and 2). Preliminary experiments revealed that the fusiform *Bacteroides* strains were characterized by proteins having molecular weights of more than 205,000, the molecular weight of a myosin standard. As previously noted by Weber and Osborn (19), myosin is unsuitable as an internal standard because it yields several electrophoretic bands. The molecular weights of protein bands were estimated by reference to a high-molecular-weight standard mixture (Sigma Chemical Co., St. Louis, Mo.).

Electron microscopy. Cell wall ultrastructure was determined as described previously (16). Cells of fusiform *Bacteroides* strains from 6-day-old colonies on TSBA plates were fixed for 2 h in a mixture containing 5% (vol/vol) glutaraldehyde and 4% (vol/vol) formaldehyde buffered to pH 7.3 with sodium cacodylate (6). Reference strains were harvested...
FIG. 2. PAGE of whole sonicated cells of reference Bacteroides and other oral species. The upper and lower lines indicate the position of the superimposed F. nucleatum internal standards. Lane a, High-molecular-weight standard mixture; Lanes 1, 10, and 18, F. nucleatum FDC 364; lanes 2 and 3, B. fragilis ATCC 25285\textsuperscript{T} and ATCC 23745, respectively; lanes 4 and 5, B. thetaiotaomicron ATCC 29148\textsuperscript{T} and ATCC 12290, respectively; lane 6, B. distasonis ATCC 8503\textsuperscript{T}; lane 7, B. vulgatus ATCC 8482\textsuperscript{T}; lane 8, B. zoogloeiformans ATCC 33285\textsuperscript{T}; lane 9, Bacteroides capillosus ATCC 29799\textsuperscript{T}; lane 11, B. buccae ATCC 33574\textsuperscript{T} and ATCC 33691, respectively; lane 13, B. oris ATCC 33573\textsuperscript{T}; lane 14, B. orafis ATCC 33269\textsuperscript{T}; lane 15, F. gonidiaformans ATCC 25563; lane 16, W. recta ATCC 33238\textsuperscript{T}; lane 17, F. nucleatum FDC 40.

after 3 to 4 days. After a 20-min wash in s-collidine buffer, the cells were postfixed in 2% s-collidine-buffered osmic acid for 90 min. The fixed cells were stored overnight in cold s-collidine buffer, dehydrated in graded ethanol solutions, and embedded in Epon (8). Sections (0.1 μm) were cut, mounted on bare 300-mesh grids, stained with uranyl acetate and lead citrate (11), and examined and photomicrographed with a Philips model EM-300 transmission electron microscope. Negatively stained preparations of whole cells were obtained by applying single drops of cellular material suspended in 2% phosphotungstic acid to carbon-reinforced Formvar-coated grids. The excess liquid was removed by blotting the edge of the grid and lifting the grid to air dry.

Serologic methods. Fusiform Bacteroides strain FDC 338\textsuperscript{T} (T = type strain) harvested from 100 blood agar plates was used to prepare antisera. The cells were suspended in phosphate-buffered saline (0.02 M phosphate, pH 7.5) containing 1 mM ethylenediaminetetraacetate, washed with the same solution and killed with 0.5% neutral Formol-saline (0.5% formaldehyde–0.15 M NaCl [pH 7.0] supersaturated with MgCO\textsubscript{3}) (4). Rabbit antisera were produced by two subcutaneous injections (14 days apart) of 10\textsuperscript{9} Formalin-fixed cells. The globulin fraction was separated from the serum by precipitation with 40% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, conjugated with horseradish peroxidase (10), and stored at −20°C until it was used. The conjugate was tested for antibody activity by using an enzyme-linked immunosorbent assay (5). After preliminary tests with strains belonging to 16 oral species, dilutions that did not exhibit cross-reactions were chosen for species comparisons.

The enzyme-linked immunosorbent assay was performed by using cultures obtained from blood agar plates and suspended in phosphate-buffered saline. Cell suspensions were standardized to an optical density at 580 nm of approx-

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\textsuperscript{a} Determined from the midpoint of thermal denaturation. G + C contents were calculated from the values for reference strains of other Bacteroides species that were 2 mol% higher than previously published values. Therefore, a 2 mol% correction was used for the B. forsythus isolates to determine their G + C contents.

\textsuperscript{b} Mean of at least two determinations obtained by using the initial renaturation rate (2).

Mean of at least two determinations obtained by using the initial renaturation rate (2).
imately 0.3, and 50-μl samples were dispensed into wells of microtiter plates. The plates were centrifuged, and the cells were fixed to the plates with 0.25% glutaraldehyde. The exposed binding sites on the plates were blocked with 1% bovine serum albumin in phosphate-buffered saline supplemented with 0.05% Tween 20 (Fisher Scientific Co., Fairhaven, N.J.). Specific horseradish peroxidase-globulin was then incubated for 30 min with the bacterial strains, and the plates were washed three times with phosphate-buffered saline supplemented with 0.05% Tween 20.

The presence of peroxidase bound to the test strain was determined by adding 0.03% H₂O₂ and N,N,N',N'-tetramethylbenzidine. The N,N,N',N'-tetramethylbenzidine was dissolved in a solution containing 15 mg of glacial acetic acid per ml and diluted 1:50 in 0.05 M sodium citrate buffer [pH 4.2]; 0.1 ml of this solution was added to each well.) After 15 to 20 min at 25°C, NaSCN was added to a final concentration of 0.075 M. Formation of a blue precipitate indicated a positive reaction.

RESULTS AND DISCUSSION

DNAs from 12 fusiform Bacteroides strains showed more than 75% homology with each other, indicating that they belong to a single species (Table 2). These isolates were gram-negative, strictly anaerobic, and rod shaped and had a median G+C content of 46 mol% (Table 2), which is within 2 mol% of the G+C content of the type strain of the genus Bacteroides. The strains were nonmotile. The metabolic products included acetate and succinate (when the organisms were grown in basal medium containing 17 g of Trypticase [BBL] per liter, 3 g of yeast extract [BBL] per liter, 3 g of NaCl per liter, 3 g of KNO₃ per liter, and 3 mg of hemin per liter [15]) or acetate and propionate (when the organisms were grown in mycoplasma broth containing 0.5% glucose, 4 mg of sodium acetate per ml, 10 μg of sodium sulfite per ml, 100 μg of sodium succinate per ml, 0.5 μg of L-cysteine per ml, and 3 mg of hemin per liter). There was little or no DNA-DNA homology between the fusiform Bacteroides strains and the other Bacteroides species tested. The B. forsythus strain showed 0 to 31% homology with Bacteroides fragilis ATCC 25285T, 0 to 28% homology with Bacteroides thetaiotaomicron ATCC 29148T, 0 to 48% homology with Bacteroides distasonis ATCC 8503T, 0 to 20% homology with Bacteroides vulgatus ATCC 8482T, 0 to 30% homology with Bacteroides oris ATCC 33573T, 5 to 14% homology with Bacteroides oralis ATCC 33269T, and 11 to 17% homology with Bacteroides buccae ATCC 33574T. The protein profiles were similar within the new species but different from the protein profiles of reference strains belonging to other species (Fig. 1 through 3). Thus, the fusiform Bacteroides strains appear to belong in the genus Bacteroides, and we propose the new species described below for these strains.

Bacteroides forsythus sp. nov. Bacteroides forsythus (for. sythus. N. L. n. forsythus referring to the Forsyth Dental Center, where the species was first isolated) cells are gram-negative, fusiform with pointed ends (Fig. 4 and 5), and 0.30 to 0.50 μm in diameter (mean ± standard deviation, 0.40 ± 0.05 μm). The cell length varies between 1 and 30 μm (average, 5 μm). Filaments (Fig. 6), cells with central swellings that are up to 3 μm in diameter (Fig. 7), and spheres are frequently observed.

The cells are nonmotile and have no flagella.

Cell wall structure. The outer membrane consists of an...
FIG. 4. *B. forsythus* strain FDC 293. ×32,800. Bar = 1 μm.

FIG. 5. *B. forsythus* strain FDC 334. Ultrastructure of peripheral layers and invagination of cell periphery in the region of septum formation (arrow). ×68,000. Bar = 0.1 μm.
outer layer which is approximately 4 nm thick and is separated by a 2-nm electron-lucent space from an inner layer that is 1 nm thick (Fig. 8 and 9). The inner membrane consists of two electron-dense layers, each approximately 2 nm wide, with a 2-nm intervening electron-lucent layer. The inner and outer membranes are separated by a moderately electron-dense zone that is about 16 nm wide, which appears to lack a distinct peptidoglycan layer. A regularly structured external layer which surrounds the outer membrane consists of subunits, which in some cross-sections resemble adjacent arches that are 10 nm wide and 10 nm high. These subunits are separated from the outer membrane by a 12-nm electron-lucent zone. Electron-dense radial connections appear to extend from the subunits to the outer surface of the outer membrane. Negative staining of tangential sections through the cell periphery indicates that the subunits in the outer-
most layer are packed in an orthogonal array (Fig. 10 and 11).

**Colonies.** Colonies on Trypticase soy blood agar plates after 7 to 10 days are pale pink, speckled, and 1 to 2 mm diameter on primary isolation and when they are growing well (Fig. 12). Colonies of poorly growing cultures are <0.5 mm in diameter.

**Cultural characteristics.** These organisms are strict anaerobes and require CO₂ and H₂ in the atmosphere for growth on agar surfaces (preliminary experiments have revealed no growth on agar under 100% N₂; 90% N₂-10% CO₂; or 90% N₂-10% H₂). To produce visible growth on agar plates, cultures require 4 to 6 days. Complex media are required for growth. Whole blood (e.g., 5% sheep blood) is required for optimal growth on agar. Strains grow on Levinthal agar. Growth on agar plates is stimulated by the presence of *F. nucleatum* (and strains of *Streptococcus sanguis*, *B. fragilis*, *Wolinella recta*, *Bacteroides intermedius*, and *Veillonella parvula* [unpublished data] in a satellite pattern.

Visible growth is produced in mycoplasma broth containing glucose (0.5%), sodium acetate (4 mg/ml), sodium sulfite (10 μg/ml), sodium succinate (100 μg/ml), L-cysteine (0.5 μg/ml), and hemin (3 mg/liter) (unpublished data; S. S. Socransky, personal communication).

Cultures also grow in Trypticase soy or mycoplasma broth supplemented with 8 to 12% gelatin and in biphasic cultures (10 ml of Trypticase soy agar in the solid phase and ≤5 ml of Trypticase soy or mycoplasma broth in the liquid phase), suggesting that agar or gelatin may be a detoxifying agent.

**Biochemical characteristics: API ZYM and API An-Ident reactions.** Strains are positive in both API ZYM and API An-Ident tests for α-fucosidase, β-glucosidase, α-glucuronidase, N-acetyl-β-glucosaminidase, and α-galactosidase activities. In addition, the strains are positive in API ZYM tests for trypsin and β-galactosidase activities and in API An-Ident tests for α-arabinosidase, phosphatase, indoxylacetate, and arginine, alanine, histidine, and phenylalanine.

**FIG. 9.** Fragment of outer membrane (2) and external layer (3), showing the similarity of the unusual external layer and the inner surface of the outer membrane (4). ×272,000. Bar = 0.1 μm.

**FIG. 10.** Tangential section through the external layer of *B. forsythus* ATCC 43037, showing the orthogonal packing of the structural subunits. ×132,000. Bar = 0.1 μm.

**FIG. 11.** Negatively stained preparation of *B. forsythus*, showing the orthogonal arrangement of structural subunits in the external layer. ×132,000. Bar = 0.1 μm.

**FIG. 12.** Colonies of *B. forsythus* strain ATCC 43037. The cells were grown on TSBA that was incubated anaerobically for 10 days without a *F. nucleatum* strain. Bar = 1 mm.
aminopeptidase activities (18). The API ZYM and API An-Ident enzymatic substrate tests are the only biochemical tests in which *B. forsythus* consistently demonstrates clear positive reactions. Strains do not react in the API 20A or API 20E test series. There is no detectable pH decrease in media supplemented with carbohydrates.

**Serologic reactions.** The strains of *B. forsythus* isolated from seven different humans are members of a single serogroup and have no antigenic relationship to *F. nucleatum* FDC 364 or *Capnocytophaga sputigena* ATCC 33612T, which are morphologically similar.

**PAGE of soluble cellular proteins.** *B. forsythus* is characterized by two major protein bands at molecular weights of more than 205,000 (Fig. 1 and 2).

- Isolated from human periodontal pockets.
- G+C content, 46 mol%.
- The type strain is strain FDC 338 (= ATCC 43037).

**Differential characteristics.** A peptidoglycan layer between the outer and inner membranes was readily identified in *B. oris* (Fig. 13), *Bacteroides ovatus*, and *B. thetaiotaomicron*, but was not detected (or was absent) in *Bacteroides zoogofoformans* (Fig. 14), *B. distasonis*, *B. buccae*, and *B. forsythus*. The outer membrane was smooth and tightly adapted to the cell periphery in all species which we examined except *B. buccae*. In addition, *B. zoogofoformans*, *B. distasonis*, *B. buccae*, and *B. forsythus* had an external layer that was peripheral to the outer membrane (Fig. 8 and 14).

Of the other species tested, only *B. distasonis* resembles *B. forsythus* in cell ultrastructure. Both of these species have cell walls with similar inner and outer membranes. However, the highly structured outermost layer of *B. forsythus* is replaced in *B. distasonis* by a uniform moderately electron-dense layer that is approximately 17 nm thick and is in direct contact with the thicker outer layer of the outer membrane. The mean diameter of *B. distasonis* cells is distinctly greater than that of *B. forsythus* cells.

The API ZYM and API An-Ident reactions of species of three genera of oral fusiform organisms differ. By using these tests, *Capnocytophaga* species could be differentiated by their consistent aminopeptidase activity, and *F. nucleatum* strains could be differentiated by their lack of reactivity in
any enzyme substrate test (18). *B. forsythus* was the only species tested which showed trypsin activity. Trypsin activity has also been detected in *Bacteroides gingivalis* (12).

*B. forsythus* ATCC 43047T showed no enzyme-linked immunosorbent assay cross-reactivity with *B. gingivalis* (5 strains), *B. intermedius* (strain FDC 581 and 6 other strains), *B. intermedius* VPI 8944, *Bacteroides melaninogenicus* (2 strains), *Bacteroides denticola* ATCC 10043, *Bacteroides loescheii* ATCC 15930, *C. sputigena* (strain ATCC 33612T and 6 other strains), *Capnocytophaga ochracea* (9 strains), *Capnocytophaga gingivalis* (strain ATCC 33624T and 1 other strain), *W. recta* (strain ATCC 33238T and 2 other strains), *Bacteroides gracilis* (strain ATCC 33236T and 2 other strains), *B. oralis* ATCC 33321, *Eikenella corrodens* (8 strains), *Haemophilus actinomyetemcomitans* (10 strains), or *Haemophilus aphrophilus* (1 strain).

All of the species examined produced different protein profiles (Fig. 1 and 2). As determined by cluster analysis, *B. forsythus* isolates show 88% similarity to each other and less than 55% similarity to other species (Fig. 3). Only *B. forsythus* produced two major protein bands at molecular weights of more than 205,000.

*B. forsythus* is easily differentiated from the fusiform oral gram-negative organisms *F. nucleatum* and *Fusobacterium gonidiformans* (which produce butyric acid) and *Capnocytophaga* species (which ferment carbohydrates) by the presence of swollen cells, the appearance of the colonies, and the absence of growth in glucose broth, the presence of trypsin activity, the electrophoretic pattern of cellular proteins, and serologic reactions.

ACKNOWLEDGMENTS

We thank G. McKinley for providing the API ZYM and API An-Ident plates, S. S. Socransky for help with analysis of polyacrylamide gels, and Carol Des Roches for technical assistance.

This study was supported by Public Health Service grants DE-03488, DE-06085, DE-02623, and DE-04881 from the National Institute of Dental Research.

LITERATURE CITED


