Eubacterium minutum sp. nov., Isolated from Human Periodontal Pockets

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We describe *Eubacterium minutum* sp. nov., which was isolated from human periodontal pockets. This new species was established on the basis of DNA-DNA hybridization data. The guanine-plus-cytosine content of its DNA is 38 to 40 mol%. The results of differential biochemical and enzymatic tests are described. The type strain of this species is strain M-6.

Eubacterium species are being implicated increasingly in chronic periodontal disease (5, 6, 15, 17, 28) and have been found to account for up to 54% of the subgingival plaque microflora (25). Several researchers have recently reported increased antibody titers against these organisms in patients with advanced periodontitis compared with controls (4, 15, 21–24), suggesting that they play an important pathogenic role in this disease. *Eubacterium* strains have also been isolated frequently from carious dentin (1, 3, 6, 8), necrotic pulp (9, 20), and periapical infections (12, 27).

Recently, we isolated several *Eubacterium* strains from periodontal pockets, and these strains could not be classified in any of the previously described species. These strains are asaccharolytic, grow slowly, and are difficult to culture. To determine the taxonomic position of these organisms, we examined their phenotypic and enzymatic profiles, as well as their serologic and genetic characteristics, and compared our results with data for previously described reference species (18, 26) and other undescribed *Eubacterium* species (5, 20).

In this paper we describe the characteristics of three strains of an asaccharolytic *Eubacterium* species. We describe a new species, *Eubacterium minutum*, for these strains on the basis of morphological, biochemical, enzymatic, serologic, and DNA-DNA hybridization data.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1.

Culture conditions. All of the bacterial strains were cultured on brain heart infusion (BHI)-blood agar plates under strictly anaerobic conditions for 7 to 10 days at 37°C in an anaerobic glove box (model AZ-Hard; Hirazawa, Tokyo, Japan) containing 80% N₂, 10% H₂, and 10% CO₂. The bacterial cells were harvested by centrifugation at 10,000 × g for 20 min at 4°C, washed with 10 mM sodium phosphate-buffered saline (pH 7.2), and stored at –20°C until they were used for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting (immunoblotting). The plates, media, buffer solutions, and experimental instruments were stored in an anaerobic box for at least 24 h prior to use. To ensure strictly anaerobic conditions in the box, the reduction of methyl viologen (at –44 mV) was carefully checked whenever experimental procedures were carried out.

Morphological and biochemical characteristics. Cell morphology was determined by examining cells grown on BHI-blood agar plates. Biochemical reactions were determined by the methods described in the *Anaerobe Laboratory Manual* (7). The end products produced in peptone-yeast extract-glucose broth were assayed by gas chromatography, as described previously (10).

API ZYM (Rapid ID 32A) analysis. A Rapid ID 32A kit (API BioMérieux,

Marcy l'Etoile, France) was used as recommended by the manufacturer, except that preparations were incubated under strictly anaerobic conditions.

Preparation of polyclonal antisera. The whole bacterial cells that were to be used as immunogens were suspended in a concentration of 10 mg (wet weight) per 1 ml in sterile saline, and the resulting suspensions (0.1 to 1.0 ml) were injected intravenously into 10-week-old female rabbits twice a week for 3 to 4 weeks. The antisera were stored in small portions at –80°C until they were used.

Analysis of protein profiles by SDS-PAGE. Whole bacterial cells of each of the strains (50 mg) were washed three times in 1 ml of sterile distilled water and suspended in 150 µl of lysis buffer containing 4% (wt/vol) SDS, 40% (vol/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol, and 0.25 M Tris-HCl (pH 6.8). The suspension was boiled and then centrifuged at 12,000 × g for 3 min at 4°C. The resulting supernatant was electrophoresed by the method of Laemmli (13) in a 12.5% polyacrylamide gel by using a model KS-8010 Micro slab gel electrophoresis system (Matsuyoshi Co., Tokyo, Japan). After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 (Nakarai Chemicals, Ltd., Kyoto, Japan).

Western transfer and immunoblotting analysis. After electrophoresis, Western transfer from the gel to a nitrocellulose membrane (pore size, 0.45 µm; Bio-Rad) was performed by using the transfer buffer system described by Burnette (2) in conjunction with the trans blot system (Matsuyoshi Co.) at a constant current of 350 mA for 4 h with cooling. After Western transfer, the nitrocellulose membrane was soaked for 1 h in 20 mM Tris-HCl-buffered saline (pH 7.5) containing 1% (wt/vol) bovine serum albumin (BSA) and then incubated with diluted rabbit antiserum (1:1,000) as the first antibody for 2 h with mild agitation at room temperature. After the nitrocellulose membrane was washed with Tris-HCl-buffered saline containing 0.05% Tween 20, it was soaked in Tris-HCl-buffered saline containing 1% BSA and incubated with goat anti-rabbit immunoglobulin G conjugated with peroxidase (1:1,000) as the second antibody for 2 h, and then the color was developed.

G+C content and DNA-DNA hybridization. Bacterial cells (wet weight, 1.5 g) were resuspended in 5-ml portions of saline-EDTA buffer (0.15 M NaCl, 0.1 M EDTA; pH 8.0) and frozen. The suspensions were subjected to ultrasonication for 10 min after being frozen and thawed three times, and then they were treated with 20 mg of lysozyme (50,000 U/mg of protein) and achromopeptidase (1,000 U/mg) at 37°C for 30 min. After 15 ml of 0.1 M Tris buffer (pH 9.0) containing 1% (wt/vol) SDS was added, each preparation was incubated at 60°C until the

TABLE 1. Bacterial strains and their sources

Taxon	Strain(s)	Source
<i>E. minutum</i>	M-6 ^T , M-7, M-8	Periodontitis
<i>E. brachy</i>	ATCC 33089 ^T	Periodontitis
<i>E. lentum</i>	ATCC 25559 ^T	Rectal abscess
<i>E. nodatum</i>	ATCC 33099 ^T	Periodontitis
<i>E. sapenum</i>	ATCC 49989 ^T	Periodontitis
<i>E. timidum</i>	ATCC 33093 ^T	Periodontitis
<i>Eubacterium</i> group S	S-4, S-6, S-7, S-8, S-22 ^a	Necrotic pulp
	L-24 ^b	Periapical lesion
<i>Eubacterium</i> sp.	D-6 (= D136M-28) ^c	Periodontitis

^a Reference strains S-4, S-6, S-7, S-8, and S-22 were obtained from Sato et al. (20).

^b See reference 12.

^c Reference strain D-6 was obtained from Moore et al. (18).

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TABLE 2. Characteristics that differentiate *E. minutum* and other asaccharolytic *Eubacterium* taxa

Taxon	Fermentation of glucose	End products ^a	Arginine hydrolysis	Nitrate reduction	Esculin hydrolysis ^b	Gelatin liquefaction ^b
<i>E. minutum</i>	—	B or b	—	—	—	—
<i>Eubacterium</i> group S	—	None	+	—	—	—
<i>E. brachy</i>	—	ib, ic, iv, phe-p	—	—	—	—
<i>E. lentum</i>	—	None	+	+	—	—
<i>E. nodatum</i>	—	a, B	+	—	—	—
<i>E. saphenum</i>	—	a, b	—	—	—	—
<i>E. timidum</i>	—	phe-a	—	—	—	—

^a End products in peptone-yeast extract-glucose broth. Abbreviations: a, acetate; b and B, butyrate; ib, isobutyrate; ic, isocaproate; iv, isovalerate; phe-p, phenylpropionate; phe-a, phenylacetate. Uppercase letters indicate that the concentration of the product is ≥ 10 mM, and lowercase letters indicate that the concentration is < 10 mM.

^b Esculin hydrolysis and gelatin liquefaction can be used to differentiate other asaccharolytic *Eubacterium* species, including *Eubacterium combesii* and *Eubacterium dolichum*.

solution became clear. DNAs were isolated from the cell lysates by the method of Marmur (14), with slight modifications. The G+C contents of the DNAs were determined by high-performance liquid chromatography (HPLC) (11).

Levels of DNA-DNA relatedness were determined by the membrane filter method described by Meyer and Schleifer (16). α -³²P-labeled DNA was prepared with a multiprimer DNA labeling system (Amersham, Buckinghamshire, United Kingdom), and the labeled DNA preparation was purified with a Nick column (Pharmacia LKB Biotechnology, Bromma, Sweden); the specific activity ranged from 10^5 to 10^6 dpm/ μ g. Denatured α -³²P-labeled DNA (0.015 μ g) was incubated with unlabeled homologous or heterologous DNA (40 μ g) on a filter (type BA-85; diameter, 23 mm; Schleicher & Schuell, Dassel, Germany) in 1 ml of $6\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-BSA (fraction V; Sigma)-0.02% polyvinylpyrrolidone-0.02% Ficoll 400-0.08% SDS at 60°C overnight. After the filter was washed with $6\times$ SSC and then with $2\times$ SSC and dried, the radioactivity of the hybridized DNA was counted with a liquid scintillation counter. Triplicate tests were performed for each assay, and the results were normalized to 100% for the homologous DNA.

RESULTS AND DISCUSSION

Morphological and biochemical characteristics. The cells of three strains of *E. minutum*, strains M-6^T (T = type strain) M-7, and M-8, were obligately anaerobic, non-spore-forming, nonmotile, gram-positive, short rods; however, the cells in older cultures were gram negative. On BHI-blood agar plates these strains formed colonies that were about 0.3 to 0.5 mm in diameter, circular, convex, entire, and translucent. Growth in broth media was poor but was moderately enhanced by the

addition of 5% bovine serum and 0.3% MgSO₄. The three strains were nonfermentative and produced moderate amounts of butyrate as the sole metabolic end product in peptone-yeast extract medium supplemented with glucose. *E. minutum* resembled *Eubacterium nodatum* and *Eubacterium saphenum* (Table 2), but *E. minutum* did not hydrolyze arginine (in contrast to *E. nodatum*) and did not produce acetate as an end product (in contrast to *E. saphenum*).

API ZYM (Rapid ID 32A) analysis. The enzymatic activities of *E. minutum* and other asaccharolytic *Eubacterium* taxa are summarized in Table 3. The *E. minutum* strains exhibited positive reactions for several aminopeptidases (API code, 0000 0526 01). The previously described species *Eubacterium lentum*, *Eubacterium timidum*, *E. nodatum*, and *E. saphenum* were unreactive in most of the enzymatic tests, while the unclassified taxa *Eubacterium* group S and *Eubacterium* sp. strain D-6 exhibited positive reactions in most of the aminopeptidase tests.

SDS-PAGE protein profile analysis and Western blotting reactions. The protein profile of *E. minutum* (Fig. 1, lane 3) was distinct, having several major high-molecular-weight bands around 150 kDa, when it was compared with the protein profiles of the previously described species *Eubacterium brachy*, *E. timidum*, *E. lentum*, *E. nodatum*, and *E. saphenum* and the unclassified taxa *Eubacterium* group S and *Eubacterium* sp.

TABLE 3. Enzymatic profiles of *E. minutum* and other asaccharolytic *Eubacterium* taxa

Taxon	API ZYM test results ^a													Rapid ID 32A API ZYM code ^b
	Arginine dihydrolase	Nitrogenase	Arginine arylamidase	Proline arylamidase	Leucyl glycine arylamidase	Phenylalanine arylamidase	Leucine arylamidase	Pyroglutamic acid arylamidase	Tyrosine arylamidase	Alanine arylamidase	Glycine arylamidase	Histidine arylamidase	Serine arylamidase	
<i>E. minutum</i>	— ^c	—	+	—	+	—	+	—	—	+	+	+	w	0000 0526 01
<i>E. nodatum</i>	—	—	+	—	—	—	w	—	—	—	—	—	—	0000 0100 00
<i>E. timidum</i>	—	—	—	—	—	—	—	+	—	—	—	—	—	0000 0040 00
<i>E. saphenum</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	0000 0000 00
<i>E. lentum</i>	+	+	+	—	—	—	—	—	—	—	—	—	—	2000 1100 00
<i>Eubacterium</i> group S	w	—	+	+	w	+	+	—	+	+	+	+	w	2000 0337 05
<i>Eubacterium</i> sp. strain D-6	w	+	+	+	+	+	+	—	+	+	+	+	+	2000 0737 05

^a Results obtained with the Rapid ID 32A (API BioMerieux) identification system for anaerobes.

^b Summary of test results.

^c —, negative; +, positive; w, weak.

FIG. 1. Protein profiles on a 12.5% polyacrylamide SDS-PAGE gel. Lane 1, molecular mass markers (molecular masses [in kilodaltons] are indicated on the left); lane 2, *Eubacterium* group S; lane 3, *E. minutum*; lane 4, *E. brachy*; lane 5, *E. lentum*; lane 6, *E. nodatum*; lane 7, *E. saphenum*; lane 8, *E. timidum*.

strain D-6. The oral asaccharolytic *Eubacterium* species had no major bands in common. Western blotting reactions (Table 4) with rabbit antisera revealed that *E. minutum* did not react with antigens from the asaccharolytic species, while antigens from the previously described species were recognized by their respective antisera.

G+C contents. The guanine-plus-cytosine (G+C) contents of the *E. minutum* strains ranged from 38 to 40 mol%, as determined by HPLC (11). As reported previously (19, 25), the G+C content of *Eubacterium limosum*, the type species of the genus *Eubacterium*, is 50 mol%, which is 10 mol% higher than the G+C content of *E. minutum*. Also, the G+C content of *E. minutum* is almost the same as the G+C contents of the asaccharolytic species *E. brachy* (39 mol%) and *E. nodatum* (41 mol%).

DNA-DNA hybridization studies. The levels of reassociation of the DNAs of *E. minutum* M-6^T, M-7, and M-8 with each other were more than 91% (Table 5), while the levels of reassociation with the DNAs of the previously described asaccha-

rolytic taxa *E. saphenum*, *E. nodatum*, *E. timidum*, *E. lentum*, and unclassified *Eubacterium* sp. strain D-6 were 5, 8, 3, 3, and 5%, respectively. These data clearly revealed that the three strains of *E. minutum* were closely related genetically and clearly distinct from the previously described asaccharolytic species. The DNA-DNA hybridization technique used in this study was found to produce accurate results because a labeled *E. timidum* ATCC 33092 DNA probe was 91% homologous to the DNA of *E. timidum* ATCC 33093, the type strain of this species.

It should be noted that the *E. minutum* strains also exhibited a very low level of reassociation (3%) with the DNA of *E. limosum*, the type species of the genus *Eubacterium*, suggesting the possibility that these strains may represent a new genus. However, further studies will be necessary to clarify this.

On the basis of DNA-DNA hybridization data, biochemical and enzymatic characteristics, and the results of SDS-PAGE and Western blotting analyses, we propose a new species, *E. minutum*, for strains M-6^T, M-7, and M-8. The taxonomic description below summarizes the properties of the species.

Description of *Eubacterium minutum* sp. nov. *Eubacterium minutum* (mi.nu'tum. L. adj. *minutum*, minute, small, referring to the minute colonies formed by this organism). Cells are obligately anaerobic, non-spore-forming, nonmotile, gram-positive, short rods. Individual cells are 0.5 by 1.0 to 1.5 μ m and occur singly, in pairs, and in clumps. Cells in actively growing cultures are gram positive, but often cells in older cultures are gram negative. Growth in broth media is poor, and growth is moderately enhanced by the addition of 5% bovine serum and 0.3% MgSO₄.

After 1 week of incubation in an anaerobic glove box, colonies on BHI-blood agar plates are 0.3 to 0.5 mm in diameter, circular, convex, entire, and translucent. After 10 days of incubation, the colonies are about 1 mm in diameter. No hemolysis occurs around colonies on BHI-blood agar plates.

The cells are inert in most biochemical tests. Starch and esculin are not hydrolyzed, nitrate is not reduced, no liquefaction of gelatin occurs, ammonia is not produced from peptone, and arginine, indole, urease, and catalase are not produced. The three strains that have been isolated are nonfermentative and do not utilize adonitol, amygdalin, arabinose, cellobiose, erythritol, esculin, fructose, galactose, glucose, glycogen, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, rhamnose, ribose, salicin, sorbitol, starch, sucrose, trehalose, or xylose.

Moderate amounts of butyrate as the sole metabolic end product are produced in peptone-yeast extract medium supplemented with glucose or peptone-yeast extract-glucose broth. Isolated from human periodontal pockets. The G+C content is 38 to 40 mol%. The type strain of *E. minutum* is strain M-6.

TABLE 4. Western blotting reactions of *E. minutum* and other asaccharolytic *Eubacterium* taxa

Antigen	Reaction with:					
	<i>E. minutum</i>	<i>E. brachy</i>	<i>E. lentum</i>	<i>E. nodatum</i>	<i>E. saphenum</i>	<i>E. timidum</i>
<i>E. minutum</i>	+	—	—	—	—	—
<i>E. brachy</i> ATCC 33089 ^T	—	+	—	—	—	—
<i>E. lentum</i> ATCC 25559 ^T	—	w	+	—	—	—
<i>E. nodatum</i> ATCC 33099 ^T	—	—	—	+	—	—
<i>E. saphenum</i> ATCC 49989 ^T	—	—	—	—	+	—
<i>E. timidum</i> ATCC 33093	—	—	—	—	—	+
<i>Eubacterium</i> group S	—	—	w	—	—	—

^a +, positive; —, negative; w, weakly positive.

TABLE 5. DNA base compositions and levels of DNA relatedness among strains of *E. minutum* and other *Eubacterium* species

Source of competitive DNA	G+C content (mol%) ^a	% Homology with labeled DNA from ^b :		
		Strain M-6 ^T	Strain M-7	Strain M-8
<i>E. minutum</i> strains				
M-6 ^T	38	100		
M-7	40	105	100	
M-8	39	106	102	100
<i>Eubacterium</i> sp. strain D-6	64	6	5	4
<i>E. saphenum</i> ATCC 49989 ^T	45	4	7	3
<i>E. brachy</i> ATCC 33089 ^T	39	3	3	3
<i>E. lentum</i> ATCC 25559 ^T	62	3	3	4
<i>E. nodatum</i> ATCC 33099 ^T	41	7	8	9
<i>E. timidum</i> ATCC 33093 ^T	50	2	4	2
<i>E. limosum</i> ATCC 8486 ^T	50	2	3	2

^a G+C contents were determined by HPLC. The G+C contents of *E. nodatum* and *E. limosum* have been determined to be 38 to 40 and 47 mol%, respectively, by the thermal denaturation method (18).

^b All values were normalized to 100% for the homologous reaction.

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