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

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A new species, *Eubacterium saphenus* sp. nov., established on the basis of the results of DNA-DNA hybridization, was proposed for strains isolated from human periodontal pockets. Differential characteristics are given.

Asaccharolytic strains of *Eubacterium*, which were obligately anaerobic, non-spore-forming, nonmotile, gram-positive short rods and produced moderate amounts of acetate and butyrate as end products, were isolated from human periodontal pockets and comprised 14% of a total of 422 isolates from patients with advanced periodontitis (10). According to criteria described in the Virginia Polytechnic Institute manual (2) and *Bergey's* manual (9), they most resembled *E. nodatum* of the established species of *Eubacterium* (9) but did not hydrolyze arginine (10), unlike *E. nodatum*. They also resembled *Eubacterium* strain D-6 (1) but did not produce phenylacetate as one of the end products (10), unlike *Eubacterium* strain D-6 (1). Thus, the isolates were tentatively classified as *Eubacterium* U group strains (10).

A DNA-DNA hybridization technique was adopted to evaluate the genetic relationship of the U group strains to *E. nodatum*, *Eubacterium* strain D-6, and some other asaccharolytic *Eubacterium* species.

MATERIALS AND METHODS

Bacterial strains. *E. saphenus* U 133-14, U 146-24, U 164-47^T (T = type strain), U 175-34, and U 183-27 were isolated from periodontal pockets of different patients. The isolation procedure used has been described in detail previously (10). *E. nodatum* ATCC 33099^T, *E. brachy* ATCC 33089^T, *E. lentum* ATCC 25559^T, *E. timidum* ATCC 33093^T,

and *E. limosum* (type species) ATCC 8486^T were obtained from the American Type Culture Collection. *Eubacterium* strain D-6 (D136M-28) was obtained from L. V. H. Moore. The bacterial cells were maintained on brain heart infusion (BHI)-blood agar plates (2) under strictly anaerobic conditions in an anaerobic glove box (model AZ-Hard; Hirasawa, Tokyo, Japan) containing 80% N₂, 10% H₂, and 10% CO₂ and stocked in a refrigerator at 4°C in the anaerobic glove box or in a freezer at -80°C. The plates, media, buffer solutions, and experimental instruments were stored in the anaerobic box for at least 24 h prior to use. To ensure strictly anaerobic conditions in the box, the reduction of methyl viologen (at -446 mV) was carefully checked whenever the experimental procedures were carried out.

Biochemical tests. Biochemical reactions were tested according to the methods described in the Virginia Polytechnic Institute manual (2). End products produced in peptone-yeast extract-glucose broth (PYG) were assayed by gas chromatography, as described previously (3, 4).

DNA-DNA hybridization. DNA hybridization experiments with *E. saphenus*, *E. nodatum*, and *Eubacterium* strain D-6 were carried out, since phenotypical and morphological characteristics of *E. saphenus* were similar to those of *E. nodatum* and *Eubacterium* strain D-6. The level of DNA relatedness among *E. saphenus*, *E. brachy*, *E. lentum*, and *E. timidum* was also determined, because these asaccharolytic *Eubacterium* species are frequently isolated from the human oral cavity. *E. combesii* and *E. dolichum* were

TABLE 1. Levels of relatedness among strains of *E. saphenus* and six other *Eubacterium* spp.

Competitive DNA from:	G+C content ^a (mol%)	% Relatedness with labeled DNA from <i>E. saphenus</i> strain				
		U 133-14	U 146-24	U 164-47 ^T	U 175-34	U 183-27
<i>E. saphenus</i>						
U 133-14	46	100				
U 146-24	48	96	100			
U 164-47 ^T	45	91	93	100		
U 175-34	44	>100	>100	>100	100	
U 183-27	45	>100	>100	>100	83	100
<i>E. brachy</i> ATCC 33089 ^T	39	9	3	3	4	9
<i>E. lentum</i> ATCC 25559 ^T	62	2	1	1	1	3
<i>E. limosum</i> ATCC 8486 ^T	50	3	1	1	1	3
<i>E. nodatum</i> ATCC 33099 ^T	41	2	1	1	1	1
<i>E. timidum</i> ATCC 33093 ^T	50	4	2	1	8	6
<i>Eubacterium</i> strain D-6 D136M-28	64	2	1	1	1	1

^a G+C content was measured by high-performance liquid chromatography. G+C values for *E. nodatum* and *E. limosum* had been reported as 38 to 40 and 47 mol%, respectively, by the thermal denaturation method (9).

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TABLE 2. Differential characteristics of *E. saphenus* and four other asaccharolytic *Eubacterium* species

Characteristic	<i>E. saphenus</i>	<i>E. brachy</i>	<i>E. lentum</i>	<i>E. nodatum</i>	<i>E. timidum</i>
Fermentation of carbohydrates	—	—	—	—	—
Product(s) ^a (PYG cultures)	a, b	ib, iv, ic, phe-p	ND ^b	a, B	phe-a
Arginine hydrolysis	—	—	+	+	—
Nitrate reduction	—	—	+	—	—

^a a, acetate; b, butyrate; ib, isobutyrate; iv, isovalerate; ic, isocaproate; phe-a, phenylacetate; phe-p, phenylpropionate. Capital letters represent an amount of product equal to or greater than 10 mM; small letters represent an amount of product less than 10 mM.

^b ND, not determined.

excluded from the DNA hybridization experiment because the characteristics of both species (9) are different from those of *E. saphenus*. The bacterial cells were grown in BHI broth supplemented with 2% bovine serum and harvested by centrifugation. The cell pellet was resuspended in 5 ml of saline-EDTA buffer (0.15 M NaCl, 0.1 M EDTA [pH 8.0]) and frozen. After three cycles of thawing and freezing, the suspension was subjected to ultrasonic treatment for 10 min and then incubated with 20 mg of lysozyme at 37°C for 30 min. DNA was extracted by the method of Marmur (7).

DNA-DNA relatedness (%) was determined by the membrane filter method described by Meyer and Schleifer (8). ³²P-labeled DNA was prepared with the Multiprime DNA labeling system (Amersham, Buckinghamshire, United Kingdom), and the amount of radioactivity ranged from 10⁶ to 10⁷ cpm/μg. Denatured ³²P-labeled DNA (0.015 to 0.02 μg) was incubated with unlabeled homologous or heterologous DNA (35 μg) on a filter (type BA-85; diameter, 23 mm; Schleicher & Schuell, Dassel, Germany) in 1 ml of 0.09 M sodium citrate-0.9 M saline solution (6× SSC) containing 0.02% bovine serum albumin (fraction V; Sigma), 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400, and 0.08% sodium dodecyl sulfate (SDS), at 60°C for 18 h. After the filter was washed with 6× SSC and then 2× SSC and dried, the radioactivity of the hybridized DNA on the filter was counted with a liquid scintillation counter. Triplicate tests were run for each assay, and the results were normalized to 100% for the homologous DNAs.

G+C contents. The G+C content of DNA was measured by high-performance liquid chromatography (5).

SDS-PAGE. Whole bacterial cells (200 mg [wet weight]) were resuspended in 20 ml of phosphate-buffered saline and ultrasonicated in a cooling system until more than 95% of the cells were disrupted, as determined by phase-contrast microscopy. The extract was centrifuged at 12,000 × g for 30 min at 4°C, and the supernatant was dialyzed against distilled water and lyophilized. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (6) in 7.5% polyacrylamide gel (1 mm thick), by using the Micro Slab Gel Electrophoresis System KS-8010 (Marysol Co., Tokyo, Japan). The lyophilized sample (50 μg) was solubilized in 0.125 M Tris-HCl buffer, pH 6.8, containing 4% SDS, 20% glycerol, and 5% 2-mercaptoethanol for 10 min at 100°C.

Electrophoresis was conducted at a constant current of 10 mA at room temperature. After electrophoresis, the gels were stained by Coomassie brilliant blue R-250 (Nakarai Chemicals, Ltd., Kyoto, Japan).

RESULTS AND DISCUSSION

DNA-DNA hybridization experiments revealed that five strains of the U group were highly related genetically but

clearly distinct from *E. nodatum*, *Eubacterium* strain D-6, and other asaccharolytic *Eubacterium* species studied (Table 1). SDS-PAGE patterns of the cell extract of the U group strains were also distinct from those of the other species studied (data not shown). These results indicate that the U group strains should be assigned to a new species. Because the organisms had been hidden in the bacterial flora of periodontal pockets and their isolation had been difficult, we propose to name them *E. saphenus*. Phenotypic characteristics useful for differentiating *E. saphenus* from some other asaccharolytic *Eubacterium* species are shown in Table 2.

Description of *Eubacterium saphenus* sp. nov. *Eubacterium saphenus* (sa.phe.'nus. L. adj. hidden; referring to the fact that the organisms had been hidden in a bacterial flora).

The description below is based on a study of the five strains listed in Materials and Methods.

Cells are obligately anaerobic, non-spore-forming, nonmotile, gram-positive short rods (0.5 by 1.0 to 1.2 μm). Cells occur singly, in pairs, and sometimes in chains (Fig. 1). Cells from older cultures often stain gram negative. The growth is poor in broth media and moderately enhanced in the presence of 5% bovine serum, 0.2% lysine, or 0.2% arginine. Strictly anaerobic conditions are required for growth.

After incubation for 7 days in an anaerobic glove box (model AZ-Hard) containing 80% nitrogen with 10% H₂ and 10% CO₂, colonies on BHI-blood agar plates are 0.3 to 0.5 mm in diameter, circular, convex, and translucent. After 14 days, they are about 1.0 mm in diameter. No hemolysis is produced around colonies on BHI-blood agar plates.

Strains are nonfermentative and do not utilize adonitol, amygdalin, arabinose, cellobiose, erythritol, esculin, fructose, galactose, glucose, glycogen, inositol, lactose, maltose, mannose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, starch, sucrose, trehalose, and xylose. Moderate amounts (ca. 5 mM each) of butyrate and acetate are produced in peptone-yeast extract or PYG. They are inert in most of the usual biochemical tests: esculin and starch are not hydrolyzed, nitrate is not

FIG. 1. Sketch of Gram-stained cells from BHI broth cultures (supplemented with 2% bovine serum) of *E. saphenus* ATCC 49989^T.

reduced, no liquefaction of gelatin occurs, ammonia is not produced from arginine, indole is not produced, and catalase and urease are not produced. Ammonia production from arginine may be useful for differentiating this species from *E. nodatum* (Table 2).

Isolated from human periodontal pockets.

The G+C content is 44 to 48 mol%.

The type strain is *E. saphenus* ATCC 49989.

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