Eubacterium exiguum sp. nov., Isolated from Human Oral Lesions

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Eubacterium exiguum sp. nov. is the name proposed for organisms formerly described as *Eubacterium* group S strains and similar bacteria isolated from various types of oral lesions. This new species was established on the basis of the results of DNA-DNA hybridization experiments and DNA base composition determinations (G+C contents, 60 to 64 mol%). The results of an API ZYM analysis, Western blotting (immunoblotting) reactions, and phenotypic tests are also given. The type strain of *E. exiguum* is strain S-7.

Eubacterium spp. are nonsporing, nonmotile, gram-positive, strictly anaerobic bacilli which produce various combinations of butyric, lactic, acetic, and formic acids or no major acids (18, 24). *Eubacterium* spp., particularly asaccharolytic species, are frequently isolated from advanced periodontal pockets (5, 6, 15, 17, 28, 32), dental pulp (9, 22), carious dentin (1, 3, 8), acute dento-alveolar abscesses (31), and periapical infections (12). Several researchers have recently reported increased systemic antibody titers to these organisms compared with healthy controls (4, 23, 25–27), suggesting that they play potent pathogenic roles in oral infectious diseases.

There are six previously described oral asaccharolytic *Eubacterium* species, namely, *Eubacterium timidum*, *Eubacterium brachy*, *Eubacterium nodatum*, *Eubacterium lentum*, and the recently proposed species *Eubacterium sapheum* (29) and *Eubacterium minutum* (21). These species have proven to be difficult to culture and unreactive in conventional biochemical tests. However, by using better anaerobic techniques, we isolated several *Eubacterium* strains which could not be classified in any of the previously established species. These clinical isolates were found to be the predominant organisms among the asaccharolytic *Eubacterium* species in human necrotic pulp samples and were tentatively classified as members of *Eubacterium* group S (22). The phenotypic characteristics of *Eubacterium* group S resembled those of *E. lentum* when *Eubacterium* group S was compared with the previously established asaccharolytic *Eubacterium* species and unclassified *Eubacterium* group D-6 (17). They also resembled the characteristics of another unclassified group, *Eubacterium* cluster 2, which was described previously by Wade et al. (31, 33), although its characteristics were not given in detail.

In this paper we describe the characteristics of *Eubacterium* group S and other unclassified strains as determined by DNA-DNA hybridization, a DNA base composition analysis, a Western blotting (immunoblotting) analysis, a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis, and an API ZYM analysis and propose a new taxonomic position for *Eubacterium* group S.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study and their sources are listed in Table 1. The five *Eubacterium* group S strains (strains S-7^T [T = type strain], S-4, S-6, S-8, and S-22) were isolated from human necrotic pulp samples

(22). The type strains of previously established asaccharolytic *Eubacterium* species and other unclassified *Eubacterium* strains, such as L-24 (12), 108 and 142 (31, 33), and D136M-28 (17), were also included in this study.

Culture conditions. All of the strains were cultured on brain heart infusion (BHI)-blood agar plates under strictly anaerobic conditions for 7 to 10 days in an anaerobic glove box (model AZ-Hard; Hirasawa, Tokyo, Japan) containing 80% N₂, 10% H₂, and 10% CO₂. The bacterial cells were harvested by centrifugation, washed with 10 mM sodium phosphate-buffered saline (pH 7.2), and stored at –20°C until they were used for SDS-PAGE and Western blotting. To ensure that strictly anaerobic conditions were maintained in the glove box, the reduction of methyl viologen (at –446 mV) was carefully checked whenever experimental procedures were carried out.

Morphological and biochemical characteristics. Cellular 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 determined by gas chromatography, as described previously (10).

API ZYM (Rapid ID 32A) analysis. A Rapid ID 32A kit (API BioMerieux, Marcy l'Etoile, France) was used as recommended by the manufacturer, except that preparations were incubated under strictly anaerobic conditions.

SDS-PAGE of cellular proteins. Cellular proteins were extracted by using the SDS-PAGE procedure described by Laemmli (13). Approximately 50 mg (wet weight) of whole cells was suspended in 1 ml of distilled water. Samples were boiled 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 protein concentrations of the supernatants were determined with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.). About 7 to 10 µg of protein per lane was subjected to electrophoresis on a 12.5% polyacrylamide gel by using a model KS-8010 Micro slab gel electrophoresis system (Marsol Co., Tokyo, Japan). The proteins were stained with Coomassie brilliant blue R-250 (Nakarai Chemicals, Ltd., Kyoto, Japan).

Western immunoblotting analysis. Proteins were transferred from SDS-PAGE gels to nitrocellulose membrane filters (pore size, 0.45 µm; Bio-Rad) by using the transfer buffer system described by Burnette (2) in conjunction with the Trans blot system (Marsol) at a constant current of 350 mA for 4 h with cooling. The membrane filters were processed as described previously (21). Briefly, an immune rabbit antiserum (1:1,000) was used as the first antibody, and then the preparation was incubated with goat anti-rabbit immunoglobulin G conjugated with peroxidase (1:1,000) as the second antibody; finally, the color was developed.

DNA isolation and purification. DNA was isolated and purified by a modification of the procedure of Marmur (14), as described previously (21).

DNA base composition. The G+C content of DNA was determined by high-performance liquid chromatography (HPLC), as described previously (11).

DNA-DNA hybridization. DNA-DNA hybridization was performed by the membrane filter method as described previously (16). Briefly, reference DNA was labeled by using a multiprimer DNA labeling kit (Amersham, Buckinghamshire, United Kingdom) and [α -³²P]dCTP and then purified with a Nick column (Pharmacia LKB Biotechnology, Bromma, Sweden). About 40 µg of unlabeled single-stranded DNA immobilized on each nitrocellulose membrane filter and 0.015 µg of labeled reference DNA were reassociated in a solution containing 0.08% SDS, 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400, 0.02% bovine serum albumin (fraction V; Sigma), and 1 ml of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After incubation overnight at 60°C, the filters were washed and dried. The radioactivity was measured with a liquid scintillation counter. Triplicate tests were performed for each assay, and the results were normalized to 100% for the homologous DNA.

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TABLE 1. Bacterial strains and their sources

Taxon	Strain(s)	Source	Reference
<i>Eubacterium</i> group S	S-4, S-6, S-7 ^{Ta} , S-8, and S-22	Necrotic pulp	
Unclassified <i>Eubacterium</i> strains	L-24	Periapical lesion	12
	D136M-28	VPI ^b	17
	108 and 142	Acute dental infections	31
<i>E. minutum</i>	ATCC 700079 ^T , M-7, and ATCC 700080 ^T	Periodontitis	21
<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. saphenum</i>	ATCC 49989 ^T	Periodontitis	
<i>E. timidum</i>	ATCC 33093 ^T	Periodontitis	
	ATCC 33092	Periodontitis	

^a S-7^T, *E. exiguum* S-7^T has been assigned the strain designation ATCC 700122^T.

^b VPI, Virginia Polytechnic Institute and State University, Blacksburg.

RESULTS AND DISCUSSION

Morphological characteristics. The five *Eubacterium* group S strains, strains S-7^T, S-4, S-6, S-8, and S-22, were nonsporing, nonmotile, strictly anaerobic, short, rod-shaped organisms. Growing cells of these bacteria were gram positive, but sometimes cells in older cultures were gram negative. Growth in broth media was poor with or without carbohydrates and was moderately enhanced by the presence of 5% bovine serum, 0.2% lysine, or 0.2% arginine. Like other oral asaccharolytic *Eubacterium* strains, these strains formed typical tiny colonies on BHI-blood agar plates; the colonies were about 0.3 to 0.5 mm in diameter, circular, convex, and translucent. Even after prolonged incubation, the colonies were less than 1 mm in diameter.

Phenotypic characteristics. The biochemical characteristics of *Eubacterium* group S and the reference *Eubacterium* species used are shown in Table 2. The five *Eubacterium* group S strains were nonfermentative, arginine hydrolysis positive, and nonreactive in most of the usual biochemical tests, and no metabolic end product from peptone-yeast extract-glucose medium was detected by gas chromatography. Thus, phenotypically, *Eubacterium* group S most closely resembled *E. lentum* among the previously established *Eubacterium* species and the unclassified organism *Eubacterium* sp. strain D136M-28 (17), but the *Eubacterium* group S strains did not reduce nitrate, unlike *E. lentum* and *Eubacterium* sp. strain D136M-28. The *Eubacterium* group S strains also resembled the unclassified organisms *Eubacterium* sp. strains 108 and 142, which produce

lactic acid and succinic acid as end products (33), but in this study no lactic acid or succinic acid was detected as a metabolic end product.

SDS-PAGE protein profile analysis. The protein profiles of *Eubacterium* group S strains and asaccharolytic reference strains are shown in Fig. 1. The *Eubacterium* group S strains produced typical protein profiles, which had several major bands at low molecular weights (20,000 to 45,000) and were quite similar to the protein profiles of the unclassified organisms *Eubacterium* sp. strains D136M-28, 108, and 142. The profiles of the oral asaccharolytic species had no major bands in common, which suggested that the protein components of these organisms are very heterogeneous.

API ZYM (Rapid ID 32A) analysis. The enzymatic activities of the *Eubacterium* group S strains and reference strains are summarized in Table 3. The *Eubacterium* group S strains and the unclassified organisms *Eubacterium* sp. strains 108 and 142 strains had an enzymatic profile that was quite distinct (API code, 2000 0337 05). These organisms were positive for most aminopeptidase activities, including arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, and serine arylamidase activities.

Most of the previously described oral asaccharolytic *Eubacterium* species (namely, *E. brachy*, *E. nodatum*, *E. lentum*, and *E. saphenum*) were not reactive in most of the aminopeptidase tests. The recently proposed new species *E. minutum* (API code, 0000 0526 01) was positive in most of the aminopeptidase

TABLE 2. Characteristics of *Eubacterium* group S, unclassified *Eubacterium* strains, and other oral asaccharolytic *Eubacterium* species

Taxon	Fermentation of glucose	End products ^a	Arginine hydrolysis	Nitrate reduction
<i>Eubacterium</i> group S ^b	—	—	+	—
Unclassified <i>Eubacterium</i> strains				
L-24	—	—	+	—
108	—	—	+	—
142	—	—	+	—
D136M-28	—	—	+	+
<i>E. minutum</i>	—	B or b	—	—
<i>E. brachy</i>	—	ib, ic, iv, phe-p	—	—
<i>E. lentum</i>	—	—	+	+
<i>E. nodatum</i>	—	a, B	+	—
<i>E. saphenum</i>	—	a, b	—	—
<i>E. timidum</i>	—	phe-a	—	—

^a a, acetate; b and B, butyrate; ib, isobutyrate; ic, isocaproate; iv, isovalerate; phe-a, phenylacetate; phe-p, phenylpropionate. Uppercase letters indicate that the concentration is 10 mM or more, and lowercase letters indicate that the concentration is less than 10 mM.

^b *Eubacterium* group S strains S-7^T, S-4, S-6, S-8, and S-22

FIG. 1. Protein profiles on a 12.5% polyacrylamide SDS-PAGE gel. Lanes 1 and 9, molecular mass markers (molecular masses [in kilodaltons] are indicated on the left); lane 2, *Eubacterium* group S; lane 3, *E. saphenum*; lane 4, *E. minutum*; lane 5, *E. nodatum*; lane 6, *E. lentum*; lane 7, *E. timidum*; lane 8, *E. brachy*.

tests, but this organism was quite distinct from *Eubacterium* group S.

Western blotting analysis. Western blotting reactions (Table 4) with rabbit antisera revealed that antigens of *Eubacterium* group S strains did not react with antisera against the reference oral asaccharolytic species, while antigens of the previously described species were recognized by their respective antisera.

Our data clearly showed that oral asaccharolytic *Eubacterium* species, including *Eubacterium* group S, could be distinguished serologically, which indicates that there is immunological heterogeneity within the genus *Eubacterium*. These data are consistent with the previously published data of Nakazawa and Hoshino (19).

G+C contents. The G+C contents of *Eubacterium* group S strains and reference species, as determined by HPLC, are listed in Table 5. The *Eubacterium* group S strains and *Eubacterium* sp. strains L-24, 108, 142, and D136M-28 had high G+C contents, ranging from 60 to 64 mol%. Most of the previously established *Eubacterium* species had G+C contents of 38 to 50 mol%, values which are significantly lower than the G+C contents of the *Eubacterium* group S strains. Because of their high G+C contents, the *Eubacterium* group S strains can be placed in the high G+C-content group described previously by Nakazawa and Hoshino (20). Of the previously established asaccharolytic *Eubacterium* species, only *E. lentum* had a high G+C content (62 mol%) which was similar to the G+C contents of the *Eubacterium* group S strains.

DNA-DNA hybridization analysis. The DNA-DNA hybridization experiments performed by the membrane filter method (Table 5) showed that the *Eubacterium* group S strains were genetically related because of their very high levels of DNA reassociation. These experiments also revealed high levels of reassociation between the DNAs of the *Eubacterium* group S strains and the DNAs of *Eubacterium* sp. strains 108 (92%), 142 (98%), and D136M-28 (87%). The hybridization analysis revealed that *Eubacterium* group S strains and strains 108, 142, and D136M-28 exhibit very low levels of DNA homology with the previously established asaccharolytic *Eubacterium* species (levels of homology, 1 to 20%). These data clearly indicate that the *Eubacterium* group S strains are completely distinct from the previously established *Eubacterium* species. Consequently, we propose that *Eubacterium* group S should be named *Eubacterium exiguum* sp. nov. Three genetically related, previ-

TABLE 3. Enzymatic profiles of *Eubacterium* group S, unclassified *Eubacterium* strains, and other oral asaccharolytic *Eubacterium* strains

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 arylamidase	Tyrosine arylamidase	Alanine arylamidase	Glycine arylamidase	Histidine arylamidase	Serine arylamidase	
<i>Eubacterium</i> group S ^c	w ^d	—	+	+	w	+	+	—	+	+	+	+	w	2000 0337 05
Unclassified <i>Eubacterium</i> strains														
L-24	w	—	+	+	w	+	+	—	+	+	+	+	+	2000 0337 05
D136M-28	w	—	+	+	+	+	+	—	+	+	+	+	+	2000 0737 05
108 and 142	w	—	+	+	w	+	+	—	+	+	+	+	+	2000 0337 05
<i>E. timidum</i> ATCC 33093 ^T	—	—	—	—	—	—	—	+	—	—	—	—	—	0000 0040 00
<i>E. minutum</i> ATCC 700079 ^T	—	—	+	—	+	—	+	—	—	+	+	+	w	0000 0526 01
<i>E. nodatum</i> ATCC 33099 ^T	—	—	+	—	—	—	w	—	—	—	—	—	—	0000 0100 00
<i>E. saphenum</i> ATCC 49989 ^T	—	—	—	—	—	—	—	—	—	—	—	—	—	0000 0000 00
<i>E. lentum</i> ATCC 25559 ^T	+	+	+	—	—	—	—	—	—	—	—	—	—	2000 1100 00
<i>E. brachy</i> ATCC 33089 ^T	—	—	—	—	—	—	+	—	—	—	—	—	—	0000 4200 00

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

^b Summary of test results.

^c *Eubacterium* group S strains S-7^T, S-4, S-6, S-8, and S-22.

^d w, weak; +, positive; —, negative.

TABLE 4. Western blotting reactions of *Eubacterium* group S, unclassified *Eubacterium* strains, and other oral *Eubacterium* strains

Antigen	Reaction with antiserum against:					
	<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>Eubacterium</i> group S ^a	— ^b	—	w	—	—	—
Unclassified <i>Eubacterium</i> strains						
L-24	—	—	w	—	—	—
108 and 142	—	—	w	—	—	—
D136M-28	—	—	—	—	—	—
<i>E. minutum</i> ATCC 700079 ^T	+	—	—	—	—	—
<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 ^T	—	—	—	—	—	+

^a *Eubacterium* group S strains S-7^T, S-4, S-6, S-8, and S-22.^b —, negative; +, positive; w, weakly positive.

ously unclassified strains, strains D136M-28, 108, and 142, should also be placed in this species.

Description of *Eubacterium exiguum* sp. nov. *Eubacterium exiguum* (ex. i.gu'um. L. adj. *exiguus*, scanty, small, referring to the scanty or poor growth of this organism). Cells are short gram-positive rods that are obligately anaerobic, nonmotile, and nonsporing. Individual cells are 0.5 by 1.0 μ m, and the cells occur singly or in clumps. Sometimes cells from older cultures stain gram negative. On BHI-blood agar plates, the cells form minute colonies that are less than 1 mm in diameter, circular, convex, and translucent even after prolonged incubation in an anaerobic glove box. Growth in broth media is poor in the presence or in the absence of carbohydrates and is moderately enhanced in the presence of 5% bovine serum, 0.2% lysine, or 0.2% arginine. No hemolysis occurs around colonies on BHI-blood agar plates.

The cells are inert in most biochemical tests. Starch and esculin are not hydrolyzed, and nitrate is not reduced. No liquefaction of gelatin occurs. Indole, urease, and catalase negative. Ammonia is produced from arginine. All strains are nonfermentative and do not utilize adonitol, amygdalin, arabi-

nose, cellobiose, erythritol, esculin, fructose, galactose, glucose, glycogen, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, rhamnose, ribose, salicin, sorbitol, starch, sucrose, trehalose, or xylose.

No detectable metabolic end products are produced in peptone-yeast extract medium supplemented with glucose or peptone-yeast extract-glucose broth.

Isolated from human necrotic pulp samples, periapical infections, and acute dento-alveolar abscesses.

The G+C content is 60 to 64 mol%. The type strain is strain S-7.

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TABLE 5. DNA base compositions and levels of DNA relatedness among *Eubacterium* group S strains, unclassified *Eubacterium* strains, and other asaccharolytic *Eubacterium* strains

Source of DNA	G+C content (mol%) ^a	% Homology with labeled DNA from ^b :							
		S-7 ^T	S-8	S-4	L-24	108	142	D136M-28	ATCC 33093 ^T
<i>Eubacterium</i> group S strains									
S-7 ^T	60	100							
S-8	63	>100	100						
S-4	61	>100	>100	100					
Unclassified <i>Eubacterium</i> strains									
L-24	61	>100	>100	>100	100				
108	62	>100	97	92	>100	100			
142	61	>100	98	99	>100	>100	100		
D136M-28	64 ^c	87	95	100	>100	>100	99	100	
<i>E. lentum</i> ATCC 25559 ^T	62 ^c	10	13	10	4	20	13	10	4
<i>E. timidum</i> ATCC 33093 ^T	50 ^c	2	1	3	2	5	3	3	100
<i>E. timidum</i> ATCC 33092	50 ^c	5	1	6	6	5	12	3	96
<i>E. minutum</i> ATCC 700079 ^T	38-40 ^c	6	5	6	4	13	12	6	2
<i>E. saphenum</i> ATCC 49989 ^T	45 ^c	3	7	6	5	3	11	2	4
<i>E. nodatum</i> ATCC 33099 ^T	41 ^c	1	1	1	2	2	2	2	8
<i>E. brachy</i> ATCC 33089 ^T	39 ^c	3	7	3	5	4	6	2	9
<i>E. limosum</i> ATCC 8486 ^T	50 ^c	2	3	4	3	6	6	2	5

^a G+C contents were determined by HPLC.^b All values were normalized to 100% for the homologous reactions.^c Data from references 20, 21, and 29.

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