Genetic Relationships among *Eubacterium* Species

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The genetic relationships among *Eubacterium* species were assessed by using DNAs from American Type Culture Collection type strains of 10 species and two subspecies of the genus *Eubacterium*, i.e., *Eubacterium aerofaciens*, *E. alactolyticum*, *E. brachy*, *E. lentum*, *E. limosum*, *E. nodatum*, *E. rectale*, *E. saburreum*, *E. timidum*, *E. yurii* subsp. *yurii*, and *E. yurii* subsp. *margaretiae*. The DNA base compositions (determined by high-performance liquid chromatography) of these species varied widely, from 38 to 62 mol% G+C. Seven *Eubacterium* species showed significant differences (nearly 10%) in G+C content compared with *E. limosum*, the type species of the genus. DNA-DNA hybridization (by the membrane filter method) showed that two subspecies, *E. yurii* subsp. *yurii* and *E. yurii* subsp. *margaretiae*, and two strains of *E. timidum* exhibited high levels of DNA relatedness. However, the DNA reassociations among the 10 *Eubacterium* species studied were 1 to 16%. None of the species examined shared a high level of DNA reassociation with the type species of the genus *Eubacterium*. The protein profile patterns (determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) of whole bacterial cells from these *Eubacterium* species were distinct, and no major peptide bands were shared among the 10 *Eubacterium* species. Therefore, the *Eubacterium* species we tested must be considered genetically distinct from each other.

Numerous bacterial strains of non-spore-forming, gram-positive, obligately anaerobic rods belonging to the genus *Eubacterium* have been isolated from human oral cavities (7), including those from dental plaque (3, 6, 16, 30), periodontal lesions (18, 20, 29, 31), infected dental pulp (6, 27), and carious dentine (1, 2, 5). Recognized among them in the past decade are many new species of *Eubacterium* (e.g., *Eubacterium brachy*, *E. nodatum*, *E. timidum*, *E. yurii*, and *E. saphenum*). Some of these species are associated with moderate and severe periodontitis and are less frequent in the supragingival sites or subgingival sites of healthy persons (13, 19, 32, 34, 35).

The genus *Eubacterium* includes more than 40 species exhibiting diverse morphologies and shows biochemical, physiological, and serological heterogeneity (4, 11, 21, 22, 25, 28, 33). We have found that the oral *Eubacterium* species are clearly distinguished by serological reactions (24), and this immunological heterogeneity suggests that they are a genetically heterogeneous group of microorganisms. There is no comprehensive study of relationships among *Eubacterium* species by DNA reassociation, although the limited data on the G+C content of DNA suggests heterogeneity and the need for research.

Ten species and two subspecies of the genus *Eubacterium*, including the type species and type strains, were selected for the present study. Variations in G+C content and DNA-DNA hybridization among these species were determined to assess the extent of heterogeneity among these *Eubacterium* species.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. aerofaciens* ATCC 25986^T (T = type strain), *E. alactolyticum* ATCC 23263^T, *E. brachy* ATCC 33089^T, *E. lentum* ATCC 25559^T, *E. limosum* ATCC 8486^T, *E. nodatum* ATCC 33099^T, *E. rectale* ATCC 33656^T, *E. saburreum* ATCC 33271^T, *E. timidum* ATCC 33092, *E. timidum* ATCC 33093^T, *E. yurii* subsp. *yurii* ATCC

43714^T, and *E. yurii* subsp. *margaretiae* ATCC 43715^T were used as representatives of these species. *Actinomyces viscosus* ATCC 19246 and *Prevotella nigrescens* NCTC 9336 were also used for comparisons of DNA-DNA homology. All bacterial strains were cultured in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with bovine serum, hemin, and vitamin K₁. Cultures were incubated at 37°C for 3 days in an anaerobic chamber (Hirasawa Works, Tokyo, Japan) containing an atmosphere of 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 used for DNA extraction.

Cell lysis and DNA isolation. Bacterial cells (wet weight, 1 g) were resuspended in 5 ml 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) at 37°C for 30 min. After 15 ml of 0.1 M Tris buffer (pH 9.0) containing 1% (wt/vol) sodium dodecyl sulfate (SDS) was added, incubation at 60°C was continued until the solution became clear. DNA was isolated from the cell lysates by the method of Marmur (15) with slight modifications. Briefly, the cell lysate was thoroughly extracted with 0.5 volume of water-saturated liquid phenol with shaking for 30 min at room temperature. The phases were separated by centrifugation, and the aqueous phase was saved. DNA was then precipitated in an ice bath by addition of 2 volumes of 95% ethanol. The precipitate was collected by centrifugation at 4°C and washed with 70, 80, and 90% ethanol, in that order. After drying, the precipitate was dissolved in 0.1× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate, pH 7.0) at 4°C. RNase A (Boehringer GmbH, Mannheim, Germany; previously heated at 80°C for 10 min to deactivate DNase) was then added to a final concentration of 50 µg/ml, and the solution was incubated for 1 h at 37°C. After 50 µl of proteinase K (10 mg/ml; Boehringer) was added, the DNA preparation was incubated at 37°C for 1 h. After that, extraction with equal volumes of a mixture of phenol and chloroform (1:1; previously saturated with water) and ethanol precipitation were repeated

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TABLE 1. DNA base composition and levels of DNA-DNA homology among *Eubacterium* species

Bacterial source of unlabeled DNA	G+C content (mol%)	% Homology with labeled bacterial DNA ^b from strain:													
		25986 ^T	23263 ^T	33089 ^T	25559 ^T	8486 ^T	33099 ^T	33656 ^T	33271 ^T	33092	33093 ^T	43714 ^T	43715 ^T	19246	9336
<i>E. aerofaciens</i> ATCC 25986 ^T	51	100	10	2	3	3	2	2	2	4	2	3	2	2	2
<i>E. alactolyticum</i> ATCC 23263 ^T	61		100	2	8	8	8	7	1	8	5	1	4	3	2
<i>E. brachy</i> ATCC 33089 ^T	39			100	1	2	6	3	16	12	9	6	6	5	1
<i>E. lentum</i> ATCC 25559 ^T	62				100	6	1	1	1	8	4	3	12	5	4
<i>E. limosum</i> ATCC 8486 ^T	50 (47) ^c					100	4	4	5	4	5	3	2	3	3
<i>E. nodatum</i> ATCC 33099 ^T	41 (36–38)						100	3	7	6	8	5	3	4	1
<i>E. rectale</i> ATCC 33656 ^T	38 (30)							100	19	2	4	3	2	1	1
<i>E. saburreum</i> ATCC 33271 ^T	41 (33)								100	4	4	5	2	4	1
<i>E. timidum</i> ATCC 33092	50									100	91	4	4	3	1
<i>E. timidum</i> ATCC 33093 ^T	50										100	4	4	3	1
<i>E. yurii</i> subsp. <i>yurii</i> ATCC 43714 ^T	40 (32)											100	60	3	1
<i>E. yurii</i> subsp. <i>margaretia</i> ATCC 43715 ^T	40 (32)												100	3	1
<i>Actinomyces viscosus</i> ATCC 19246 ^d	66 (59–69)													100	1
<i>Prevotella nigrescens</i> NCTC 9336 ^d	49 (41–44)														100

^a G+C content was measured by HPLC.^b All values were normalized to 100% for the homologous strain.^c The data in parentheses are from reference 14 or 21 and were obtained by the thermal melting method.^d This bacterial strain was used for comparison.

to achieve the desired level of purity. The DNA concentrations of these preparations were determined spectrophotometrically by determining the A_{260} . The purity of each DNA preparation was assessed by measuring the UV A_{260} to A_{280} ratio.

G+C content of DNA. The G+C content of DNA was estimated by high-performance liquid chromatography (HPLC) (10). Each DNA preparation was dissolved in distilled water (1 mg/ml), and then the solution was heated at 100°C for 5 min. After rapid cooling in an ice bath, 10 µl of the denatured DNA solution was mixed with 10 µl of a nuclease P1 (Yamasa Shoyu Co., Chiba, Japan) solution (2 U/ml of 40 mM sodium acetate buffer containing 0.2 mM ZnCl₂) and the mixture was incubated at 50°C for 1 h. The hydrolysate and a standard solution containing equal molar concentrations of four deoxyribonucleotide monophosphates (dAMP, dCMP, dGMP, and dTMP) were subjected separately to HPLC (Hitachi L-6200) with a 3013-N column (4 by 150 mm). The column was eluted with a mobile phase containing 30 mM KCl, 10 mM KH₂PO₄, and 10% CH₃CN (pH 3.5). The deoxyribonucleotide monophosphates were detected with a Hitachi L-4200 apparatus at 267 nm.

DNA-DNA hybridization. The extent of DNA-DNA reassociation was determined by the membrane filter method described by Meyer and Schleifer (17). Briefly, 35 µg of denatured, unlabeled target DNA was loaded onto a filter membrane (BA-85; diameter, 23 mm; Schleicher & Schuell, Dassel, Germany) and the membrane was baked at 80°C for 3 h. The DNA for the probe was labeled with the Multiprime DNA-labeling system (Amersham, Amersham, United Kingdom) with [α -³²P]dCTP, and the labeled DNA preparation was purified with a NICK column (Pharmacia LKB Biotechnology, Bromma, Sweden) and ethanol precipitation. The radioactivities of the DNA probes ranged from 10⁵ to 10⁶ cpm/µg. After preincubation at 60°C for 5 h, the unlabeled DNA on the membrane was hybridized with 20 ng of denatured ³²P-labeled DNA in 1 ml of 6× SSC containing 0.02% (wt/vol) bovine serum albumin (fraction V), 0.02% (wt/vol) polyvinyl pyrrolidone, 0.02% (wt/vol) Ficoll 400, and 0.08% (wt/vol) SDS at 60°C for 18 h in a Hybri-Bag (Cosmo-Bio, Tokyo, Japan). After hybridization, the filter membrane was removed from the hybridization fluid and washed with 6× SSC (three times) and

2× SSC (two times) and then dried. The amount of filter-fixed DNA was estimated by measuring the radioactivity of the hybridized DNA on the filter in a liquid scintillation counter in 10 ml of toluene scintillant containing 33% (vol/vol) Triton X-100, 0.27% (wt/vol) 2,5-diphenyloxazole, and 0.007% (wt/vol) 1,4-bis[2-(5-phenyloxazolyl)]benzene. Triplicate tests were run for each assay, and the results were normalized to 100% for the homologous DNA-DNA hybridization.

SDS-PAGE analysis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (12), with the Micro Slab KS-8010 gel electrophoresis system (Maysol, Tokyo, Japan). Whole bacterial cells of each of the strains were heated in solubilizing buffer (0.125 M Tris buffer [pH 6.8] containing 4% [wt/vol] SDS, 20% [vol/vol] glycerol, and 5% [vol/vol] 2-mercaptoethanol) for 10 min at 100°C. After centrifugation at 10,000 × *g* for 15 min, the protein concentrations of the supernatants were determined by the protein assay kit (Bio-Rad Laboratories, Richmond, Calif.). The supernatant (10 µg of each) from each bacterial strain was applied to an SDS-polyacrylamide linear gradient (10 to 20%) gel. Electrophoresis was conducted at a constant 20 mA at room temperature. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250.

RESULTS AND DISCUSSION

In the present study, DNAs were isolated from the type strains of 10 species belonging to the genus *Eubacterium* and reference strains. All DNA preparations were considered sufficiently pure when the A_{260}/A_{280} ratios were greater than 1.8. As listed in Table 1, the G+C contents of the DNAs from the *Eubacterium* species ranged widely, from 38 to 62 mol%, and the species were separated into three groups based on G+C content. The groupings were *E. aerofaciens*, *E. limosum*, and *E. timidum* at nearly 50 mol% (middle-content group); *E. alactolyticum* and *E. lentum* at roughly 60 mol% (high-content group); and *E. brachy*, *E. nodatum*, *E. rectale*, *E. saburreum*, and *E. yurii* at almost 40 mol% (low-content group). All of the species in the high- and low-content groups had G+C contents significantly different (nearly 10%) from that of *E. limosum*, which is the type species of the genus *Eubacterium*. These

results indicated that of the 10 *Eubacterium* species investigated in this study, 7 species, including two subspecies, were likely to belong to genera separate from *Eubacterium*.

The G+C contents of several *Eubacterium* species have been previously reported (14, 21), and all of these values were estimated by the thermal melting method. In the present study, G+C contents were measured by the HPLC method. To confirm the validity of the method, DNA was extracted from *Escherichia coli* K-12 and the G+C content of the DNA (in moles percent) was preliminarily quantified by HPLC. The DNA of *E. coli* B (purchased from Funakoshi Chemicals, Tokyo, Japan) was also analyzed by this method. The G+C contents of *E. coli* K-12 and B were correctly determined to be 50.7 and 51.4 mol%, respectively. The G+C contents of the DNAs of *E. yurii* subsp. *yurii* and *E. yurii* subsp. *margaretiae* and two strains of *E. timidum* corresponded to each other (40 and 50 mol%, respectively; Table 1). These results demonstrated that the HPLC method used in this study was valid for these determinations.

Quantitative DNA-DNA hybridization studies with the membrane filter technique (Table 1) showed that the labeled DNA probe from *E. timidum* ATCC 33092 was reasonably (91%) homologous to the DNA of *E. timidum* ATCC 33093, which is the type strain of this species. Also, 60% DNA reassociation was observed between *E. yurii* subsp. *yurii* and *E. yurii* subsp. *margaretiae*, which agreed with the results of Margaret and Krywolap (13, 14). These observations support the validity of the data on the species studied.

The reassociation percentages among the DNAs of the different *Eubacterium* species, as well as the DNAs of *A. viscosus* and *P. nigrescens*, which served as the out group for comparisons, were less than 20% in all cases and less than 10% in most cases. Even for *Eubacterium* species in the same G+C content group, the levels of mutual DNA homology were very low values (Table 1).

Johnson (8) has proposed that groups of bacteria with intragroup DNA homology of 80 to 90% and intergroup homology between 60 and 70% could be considered different subspecies within a species. While there has been no general agreement regarding the phylogenetic definition of a genus (36), Johnson has also stated that the minimum DNA-DNA homology level among members of a genus should be 20% (9). These criteria, taken with the results of DNA-DNA hybridizations and estimations of G+C content in the present study, suggest that some of *Eubacterium* species examined may need reassignment to different genera. A background of support for these findings is given by recent studies of 16S rRNA sequences showing that *E. bifforme*, *E. limosum*, *E. tenue*, and *E. alactolyticum* are grouped phylogenetically with distinct clusters of *Clostridium* and *Acetobacterium* species and that the genus *Eubacterium* may be incoherent (26, 37).

When SDS-soluble proteins were analyzed by SDS-PAGE analysis, it was shown that the protein profile patterns of these *Eubacterium* species were quite distinct from each other (Fig. 1). We were unable to detect major bands shared by all of the species examined in the present study. The different protein profiles of ultrasonic extracts from oral *Eubacterium* species, which were estimated by SDS-PAGE with a constant gel concentration (7.5%), were reported previously (23). However, in the present study, whole bacterial cell extracts were used for SDS-PAGE with a gradient gel (10 to 20%). That demonstrated clearly the great heterogeneity of structural proteins among these *Eubacterium* species.

Species of the genus *Eubacterium* are fundamentally different from the other anaerobic non-spore-forming, gram-positive rods, according to the major metabolic products. The

FIG. 1. SDS-PAGE (10 to 20% gradient) profiles of SDS-soluble proteins of bacterial cells from *Eubacterium* species. All samples were prepared as described in Materials and Methods. Lanes: 1, *E. aerofaciens* ATCC 25986^T; 2, *E. alactolyticum* ATCC 23263^T; 3, *E. brachy* ATCC 33089^T; 4, *E. lentum* ATCC 25559^T; 5, *E. limosum* ATCC 8486^T; 6, *E. nodatum* ATCC 33099^T; 7, *E. rectale* ATCC 33656^T; 8, *E. saburreum* ATCC 33271^T; 9, *E. timidum* ATCC 33093^T; 10, *E. yurii* subsp. *yurii* ATCC 43714^T; 11, *E. yurii* subsp. *margaretiae* ATCC 43715^T; 12, protein molecular size standards.

classification is based on assignment of species to the genus *Eubacterium* by a process of elimination from other anaerobic genera (21), which is likely to result in assembly of species which are phylogenetically distant from each other.

The present study demonstrates remarkable genetic heterogeneity among the 10 *Eubacterium* species studied. Although a more complete investigation is necessary before substantial taxonomic decision are made, these results may assist identification of these microorganisms and suggest a basis for reclassification of the genus *Eubacterium*.

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