Eubacterium yurii subsp. yurii sp. nov. and Eubacterium yurii subsp. margaretiae subsp. nov.: Test Tube Brush Bacteria from Subgingival Dental Plaque

BARBARA S. MARGARET AND GEORGE N. KRYWOLAP*

Department of Microbiology, University of Maryland School of Dentistry, Baltimore, Maryland 21201

Eighteen strains of anaerobic gram-positive rods exhibiting an unusual three-dimensional cellular arrangement resembling test tube brushes (TTB) were recovered from subgingival dental plaque samples of randomly selected periodontitis patients. The isolates were obtained from subgingival sites exhibiting bone loss of \( \geq 20\% \) and pocket depth of \( \geq 4 \) mm. Based upon biochemical profiles, these organisms belong to the family Propionibacteriaceae and are somewhat similar biochemically to some species of Eubacterium, notably Eubacterium saburreum. Relatedness between TTB bacteria and E. saburreum was assessed by deoxyribonucleic acid base composition and deoxyribonucleic acid renaturation rates. Moles percent guanine plus cytosine were 32 and 33, respectively. Hybridization studies showed 32\% relatedness between E. saburreum and TTB bacteria, indicating that they are related but distinct species. We therefore propose a new species, Eubacterium yurii. Strains of this species characteristically form three-dimensional brushlike aggregates mediated by an amorphous, extracellular material. E. yurii is divided into two subspecies that exhibit 53\% homology: E. yurii subsp. yurii, which is phosphatase positive, and E. yurii subsp. margaretiae, which is phosphatase negative.

Before 1914, descriptions of test tube brush (TTB) bacteria were based solely upon microscopic observations. Davis (2) gave the first account of the cultural characteristics of anaerobic TTB bacteria from “Actinomyces-like” granules. He described structures in which fusiform bacilli were arranged perpendicular to a central stalk. These organisms were named Bacillus fusiformis. Tunnilliff and Jackson (17, 18) reported the isolation of organisms similar to those described by Davis (2). They were difficult to cultivate and were weakly gram positive. Tunnilliff and Jackson concluded that these microorganisms made up a new species within the genus Vibrio and named them Vibrio tonsillicus (18). Rosebury (14) found TTB organisms in 8\% of patients with periodontoclasia and suggested that the bacteria were gram negative and belonged to the genus Bacteroides. In 1976, Listgarten (8) saw TTB forms in situ during his microscopic study of subgingival plaque. He described at least two different types of brushes, coarse and fine, which were composed of gram-negative fusiform or filamentous rods; the brushes typically contained a morphologically mixed population of cells. The relationship, if any, between the organisms to be described here and those of studies by previous investigators remains a matter of unresolved speculation, since no isolates are available for comparison. Spiegel et al. (C. A. Spiegel, G. E. Minah, G. N. Krywolap, and R. K. Nauman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, D8, p. 41) described TTB bacteria that were recovered from subgingival dental plaque. Based on initial biochemical tests, the isolate was reported to be similar to Clostridium malenominatum, even though spores were never demonstrated. Since then we have recovered 17 additional strains of TTB bacteria from 31 sites (showing periodontal disease) and, along with the previously described strain, have characterized them biochemically and genetically. The data from these studies are the basis of this report.

(A preliminary report of this work has been presented [B. S. Margaret, J. W. Hong, and G. N. Krywolap, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, R3 and R4, p. 211] and is based on a portion of a thesis to be submitted by B. S. Margaret to the Graduate School, University of Maryland at Baltimore, in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Subjects for plaque sampling were selected at random from the adult clinic population who agreed to undergo treatment in the postgraduate periodontal clinic at the University of Maryland Dental School. The extent of periodontal disease at sampled sites was characterized by percent bone loss (estimated radiographically) and pocket depth. Subgingival plaque samples were obtained with a sterile no. 23 explorer from the depth of the pocket. The plaque was placed immediately into 1 ml of reduced transport fluid (16), with 0.05\% cysteine hydrochloride and without resazurin, in the inner vial of a Katanga tube (15) fitted with a rubber stopper. Samples were not collected under oxygen-free gas; however, they were promptly transferred to an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) containing an atmosphere of 85\% N\(_2\), 10\% H\(_2\), and 5\% CO\(_2\). Plaque samples were dispersed by sonication with a Kontes micro ultrasonic cell disruptor (Scientific Glassware/Instruments, Vineland, N.J.) with power and tuning each set at 1.5 for 8 to 10 s. Serial 10-fold dilutions of the samples were made in reduced transport fluid and plated in triplicate on fresh prereduced MM10 agar with 2.5\% sheep blood (9). Plates were incubated at 35°C for 6 days.

After incubation, the total colony count was determined, and the plates were examined for typical TTB colonies. Suspect colonies were Gram stained to verify the characteristic brushlike morphology. Colonies then were reisolated on Schaedler blood agar (BBL Microbiology Systems, Cockeysville, Md.) and transferred to Schaedler broth (BBL) to obtain pure cultures and to observe typical growth.

* Corresponding author.
TABLE 1. Biochemical reactions of TTB bacteria

<table>
<thead>
<tr>
<th>No. of strains</th>
<th>Phosphatase</th>
<th>Glucose</th>
<th>Maltose</th>
<th>Sucrose</th>
<th>DNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>w</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>w</td>
<td>−</td>
</tr>
<tr>
<td>1</td>
<td>−</td>
<td>w</td>
<td>w</td>
<td>w</td>
<td>−</td>
</tr>
</tbody>
</table>

* All strains tested were positive for RNase. H₂⁻S, motility, and indole and were negative for amygdalin, cellobiose, dulcitol, esculin (hydrolysis and pH), fructose, gelatin, hirulite, inositol, inulin, lactose, mannitol, melibiose, melezitose, raffinose, salicin, sorbitol, sorbose, starch (pH and hydrolysis), and xylose.

+ w, Weak.

The colony count of black-pigmented Bacteroides spp. was determined to further characterize the sampled sites.

Purified isolates exhibiting typical macroscopic and microscopic morphologies were frozen at −20°C in 10% glycerinated Schaedler broth until needed.

Two methods were used to determine Gram reaction: (i) the Kopeloff modification (5) of the Gram stain, with 1:1 (vol/vol) acetone-ethanol as decolorizer, and (ii) transmission electron microscopy. TTB bacteria were prepared for thin section by the procedure of Kellenberger et al. (6). Cells were grown in PY broth (5) for 48 h, harvested, and fixed with 0.5% glutaraldehyde in Kellenberger (KB) buffer for 30 min in an ice bath, followed by overnight postfixation in 1% OsO₄ at room temperature. The cells were washed in KB buffer, added to 2% Noble agar, and stained with 0.5% uranyl acetate in KB buffer, followed by ethanol dehydration and embedding in Epon 812. Thin sections were cut on an Ultratome (LKB Instruments, Rockville, Md.) and stained with 0.25% lead citrate. Specimens were examined on a Siemens Elmiskop IA microscope at 80 kV (accelerating voltage). A grating replica (E. F. Fullam, Inc., Schenectady, N.Y.) was used to calibrate the microscope.

Substrates were prepared according to standard published formulations (5). Trypticase soy broth (BBL) cultures were used to test for catalase activity. Substrates (5 ml per tube) were inoculated with 2 to 3 drops of a 5-ml, 48-h Schaedler broth culture and incubated for 72 h at 35°C in an anaerobic chamber. Tests were read according to standard procedures (5, 7). Deoxyribonuclease (DNase), ribonuclease (RNase) and phosphatase activities were assayed by incorporating 0.2% salmon deoxyribonuclease acid (DNA), 0.01% Torula yeast RNA, or 0.01% phenolphthalein diphosphate (Sigma Chemical Co., St. Louis, Mo.) into Schaedler agar. Plates were spot inoculated and incubated anaerobically for 48 h. DNase and RNase plates were flooded with 1 N HCl, and clear zones surrounding colonies were interpreted as positive. Phosphatase plates were exposed to fumes of concentrated NH₄OH for 5 min. Colonies exhibiting a bright pink color were interpreted as positive for phosphatase activity.

Volatilizable and nonvolatile fatty acids were analyzed on a model 428 gas chromatograph (Packard Instrument Co., Inc., Rockville, Md.) with a column of 1% H₂PO₄ and SP-1000 100/120-mesh Chromosorb support and model 901 flame ionization detector.

Susceptibility to benzyl penicillin, sodium salt (Sigma), tetracycline hydrochloride (A grade; Calbiochem-Behring, La Jolla, Calif.), erythromycin (Sigma), clindamycin (The Upjohn Co., Kalamazoo, Mich.), and chloramphenicol (Sigma) was determined by the agar dilution method (12) as described previously (7).

Sporulation was checked in the sporulation medium of Duncan and Strong (4) with direct microscopic observation and heat shocking at 80°C for 10 min. C. malenominutum ATCC 25776T was used as a positive control. Optimum temperature and aerotolerance were determined on Schaedler agar.

DNA was isolated from 72-h Schaedler broth cultures. The cells were washed once in phosphate-buffered saline (0.01 M phosphate buffer [pH 7.2] containing 0.15 M NaCl), twice in 0.005 M ethylenediaminetetraacetic acid, and once in distilled water and then suspended in 0.02 M Tris (pH 6.8) to an optical density at 600 nm of 1.5. The cells were lysed by adding 50 μg of mutanolysin (Sigma) per ml, incubating at 37°C for 2 h, and then adding 2.5% sodium dodecyl sulfate and 40 μg of protease K (EM Laboratories, Inc., Elmsford, N.Y.) per ml. Incubation was continued for 1 h to destroy residual DNase activity. The DNA was precipitated with 2 volumes of cold (−20°C) 95% ethanol, scooped and dissolved in 0.1× SSC and adjusted to 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate). The sample was treated with 40 μg of RNase T2 (Sigma) per ml for 2 h at 37°C, followed by an additional 20 μg of protease K per ml for 1 h at 37°C. This preparation was applied to a Sepharose 4B column (1.3 by 28 cm) equilibrated with 1× SSC, with a flow rate of 0.26 ml/min. Absorbance of eluate was monitored at 260 nm, and DNA rich fractions were pooled. A fresh column was used for each sample. The DNA preparations were dialyzed together to ensure uniformity of the SSC concentration: 0.5× SSC was chosen for the determination of base composition; 2× SSC was used for hybridization procedures. Concentrations of recovered DNA were determined by the method of Burton (1), and absence of protein was determined by the procedure of Lowry et al. (10).

DNA base composition (moles percent guanine plus cytosine) of TTB strains and of E. saburreum ATCC 33271T was determined by the thermal melting point method (11) with an automatic recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with thermoprogrammer and stoppered quartz thermocuvettes. DNA from calf thymus (Sigma) was included in each set of analyses as a control. The degree of DNA-DNA binding

<table>
<thead>
<tr>
<th>Organism</th>
<th>CSF</th>
<th>SM21</th>
<th>SM14T</th>
<th>SMN</th>
<th>SM65T</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. saburreum</td>
<td>33</td>
<td>36</td>
<td>27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Averages of duplicate determinations.

* Phosphatase positive.

* Phosphatase negative.
between paired samples was determined from renaturation rates of paired DNA samples and their 1:1 mixture (3, 13).

RESULTS AND DISCUSSION

Biochemical reactions are summarized in Table 1. Volatile fatty acid products were butyrate (>1 meq/100 ml), acetate (>1 meq/100 ml), and propionate (<1 meq/100 ml); no nonvolatile fatty acids were detected. Susceptibility patterns (minimal inhibitory concentrations) for all strains were 0.062 μg (penicillin, erythromycin, tetracycline, or clindamycin) per ml and 0.5 μg (chloramphenicol) per ml.

The moles percent guanine plus cytosine contents of the DNAs of all TTB strains tested and that of E. saburreum were 32 and 33, respectively. DNA hybridization results are summarized in Table 2. TTB strains share 32% sequence homology with E. saburreum and may be separated into two groups exhibiting 53% mean homology. These two homology groups correspond to the phosphatase-positive and phosphatase-negative groups distinguished biochemically (Table 1). Although TTB bacteria exhibit unique morphological characteristics, they appear to belong to the diverse genus Eubacterium. Based on biochemical profile, moles percent guanine plus cytosine contents, and DNA-DNA hybridization studies, we propose placing these organisms into a new species, Eubacterium yurii, possessing the following characteristics.

Eubacterium yurii sp. nov. (yur' i i. masc. noun. Yuri [author]). Obligately anaerobic, nonsporeforming, straight gram-positive rods with slightly rounded ends. Individual cells are motile by means of a single subpolar flagellum (Fig. 1). The cells form three-dimensional brushlike aggregates held together by an amorphous, extracellular substance (Fig. 2 and 3). Cells from 48-h Schaedler broth culture measure approximately 0.5 by 4 μm.

After 48 h of incubation at 37°C on Schaedler blood agar, colonies measure 1 mm in diameter, with delicate, spreading margins. A pale yellow pigment may be evident. Growth on MM10 blood agar is similar, although the colonies are smaller. Colonies do not spread on media without blood.

In Schaedler broth growth is typically as a granular...
sediment, with colonies adhering to the glass; the supernatant fluid remains clear; gas may be formed. Growth is poor in chopped-meat medium.

Vitamin K is not required for growth. The cultures grow well at 34 to 37°C but not at 25 or 40°C. All strains are H₂S, indole, and RNase positive and negative for esculin, gelatin, hippurate, and starch hydrolysis. Catalase is negative; acetyl methylcarbinol is not produced. Most strains are asaccharolytic. DNase, glucose, maltose, sucrose, and phosphatase reactions are variable (Table 1). In peptone-yeast extract-glucose broth, the major product is butyrate, with lesser amounts of acetate and propionate. All strains are susceptible to penicillin, erythromycin, tetracycline, clindamycin, and chloramphenicol.

Habitat: subgingival dental plaque.

The moles percent guanine plus cytosine content is 32 (Tₐ).

Based upon phosphatase and saccharolytic activities and DNA renaturation studies, two subspecies of E. yurii are proposed.

Eubacterium yurii subsp. yurii subsp. nov. (yur' i i. masc. noun. Yuri [author]). Type species. Phosphatase positive; most strains are asaccharolytic; an occasional strain may produce weak acid in sucrose and may be strongly DNase positive. Type strain SM14 is asaccharolytic and DNase negative.

Eubacterium yurii subsp. margaretiae subsp. nov. (mar ga ret' i ae. fem. noun. Margaret [author]). All strains are phosphatase negative; most are asaccharolytic; some may produce weak acid in sucrose, maltose, or glucose. Type strain SM65 is weakly positive for sucrose, maltose, and glucose fermentation.

The single most important characteristic of this species is the ready formation of three-dimensional brushlike aggregates, demonstrable on smears from solid or liquid media, consisting of gram-positive rods embedded in an extracellular material that is roughly ovoid to rectangular and dark when viewed by phase-contrast microscopy. Transmission electron microscopy reveals a more irregularly shaped core. Cultures should be examined for brush formation after 72 h of anaerobic incubation in Schaedler broth at 35 to 37°C, since the aggregates tend to increase significantly in size with culture age. Even young cultures may decolorize readily; cells may appear gram variable or gram negative with gram-positive intracellular beading. After repeated laboratory subcultures, the brush-forming trait may seem to diminish somewhat, although it does not disappear. Brief storage of the culture at -20°C in 10% glycercinated Schaedler broth renews the vigorous expression of this characteristic in the subcultures. Primary isolation is consistently more successful on MM10 sheep blood agar than on Schaedler sheep blood agar; plates should be incubated for 6 days before examination. Subcultures on Schaedler media require only 48 h of incubation for growth to appear. E. yurii is especially sensitive to detergent residue on glassware. Extreme care should be taken to rinse all glassware thoroughly before use.

E. yurii was recovered from shallow (4 to 6 mm) as well as deep (9 to 10 mm) periodontal pockets regardless of the extent of bone loss (20 to 80%). These organisms were not recovered from any of the eight sites from which 10⁷ to 10⁹ colonies of black-pigmented Bacteroides spp. were recovered but were isolated from sites containing lesser numbers of Bacteroides spp. In most samples, E. yurii made up approximately 1% of the cultivable flora.

ACKNOWLEDGMENTS

We thank C. Hawley, S. Hayduk, B. Lescoe, L. Page, and B. Phillips of the Department of Periodontics for clinical characterization of the sample sites and for the collection of dental plaque samples; we also thank R. K. Nauman, Department of Microbiology, University of Maryland Dental School, for his advice on the preparation of electron micrographs. Thanks also to W. Myers, Department of Microbiology, University of Maryland Medical School, for his technical advice in performing the DNA base composition and hybridization experiments.

LITERATURE CITED


Infect. Dis. 14:144–158.