Determination of the Deoxyribonucleic Acid Composition and Deoxyribonucleic Acid-Deoxyribonucleic Acid Hybridization of *Fusobacterium fusiforme*, *Fusobacterium polymorphum*, and *Leptotrichia buccalis*: Taxonomic Considerations

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The deoxyribonucleic acids (DNA) of *Fusobacterium polymorphum* ATCC 10953 and *F. fusiforme* ATCC 23726 were submitted to DNA-DNA hybridization procedures. A polynucleotide homology of 78% was demonstrated, indicating that the recent grouping of all oral *Fusobacterium* strains into one species, *F. nucleatum*, should be reevaluated. The polynucleotide homology between *Leptotrichia buccalis* ATCC 19616 and the two strains of *Fusobacterium* tested was less than 30%. The guanine plus cytosine (G+C) values of the DNA of *Fusobacterium* and *Leptotrichia* strains tested were similar and unusually low (24.5 to 25.6 mol%). Since bacteria of the family *Bacteroidaceae* reportedly have G+C values of 41 to 42 mol%, the placing of the genera *Fusobacterium* and *Leptotrichia* into this family is questionable. The similar unusually low G+C values of the DNA of *Fusobacterium* and *Leptotrichia* may indicate a close phylogenetic relationship for these genera.

Considerable confusion exists in the literature concerning oral strains of *Fusobacterium*. Knorr (14, 15) originated the generic name *Fusobacterium* and described three species based on cellular morphology: *F. plauti-vincetii*, *F. polymorphum*, and *F. nucleatum*. However, it became evident that the cellular morphology of the fusobacteria was too inconsistent to be a reliable taxonomic criterion. Several additional classification schemes were subsequently proposed that utilized colonial morphology and serological and biochemical characteristics (8, 24-26). Unfortunately, none of these characteristics was sufficient to allow subclassification of the oral fusobacteria. As a result, in 1941 Boe (1) proposed combining all oral fusobacteria into one species. More recently, a similar conclusion was reached by Werner and co-workers (27) when they also were unable to define consistent phenotypic criteria for the subclassification of these microorganisms. Thus, Moore and Holdeman (21) combined all oral fusobacteria into one species, *F. nucleatum*, the type species of *Fusobacterium*. In addition, the genus *Fusobacterium* was retained in the family *Bacteroidaceae* by Moore and Holdeman (21).

A second genus of oral bacteria, *Leptotrichia*, has often been confused with *Fusobacterium* because of morphological similarities (8, 25, 26). Boe (1) attempted to separate these genera,

but the confusion continued when Hoffman (9) described *L. buccalis* as *F. fusiforme*. Moore and Holdeman (21) recognized the genus *Leptotrichia* as a separate entity and placed it in the family *Bacteroidaceae*. The present study was designed to investigate the taxonomic relationship between strains of *Fusobacterium* and of *L. buccalis* by comparing the guanine plus cytosine (G+C) contents of their deoxyribonucleic acids (DNA) and by using DNA-DNA hybridization. In addition, the placement of the genera *Fusobacterium* and *Leptotrichia* into the family *Bacteroidaceae* was evaluated by comparing the G+C content of their DNA to that reported for members of *Bacteroides*, the type genus of the family *Bacteroidaceae*.

(A preliminary report of this research was presented at the 75th Annual Meeting of the American Society for Microbiology, New York, N.Y., 27 April to 2 May 1975. This paper is based on a portion of a thesis submitted by L. R. P. to the Graduate School, University of Maryland at Baltimore City in partial fulfillment of the requirements for the Ph.D. degree, 1975.)

MATERIALS AND METHODS

Bacterial strains. The strains used in this study were *F. fusiforme* ATCC 23726, *F. polymorphum* ATCC 10953, *L. buccalis* ATCC 19616, and *L. buccalis* ATCC 23471. In Table 1 are listed the metabolic characteristics useful in distinguishing between *Fusobacterium* and *Leptotrichia*.

Cultivation. Cells were grown in a modified

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TABLE 1. *Metabolic characteristics useful in distinguishing between Leptotrichia and Fusobacterium*

Genus	H ₂ S production ^a	Indole production ^b	End product
<i>Leptotrichia</i>	—	—	Lactate
<i>Fusobacterium</i>	+	+	Butyrate

^a Semisolid agar (BBL); 48-h growth.

^b Indole nitrate medium (BBL), 24-h growth, followed by the addition of Kovacs reagent.

tryptone medium consisting of 1% tryptone (Difco), 1% yeast extract (Difco), 0.125% K₂HPO₄, 0.125% MgSO₄·7H₂O, and 0.2% glucose, at pH 7.2 (7). Cultures were grown on a rotary shaker in GasPak anaerobic jars (BBL) at 37°C for 24 h and harvested by centrifugation at 10,000 × *g* for 10 min; the bacterial pellets were stored at -20°C until needed for DNA extraction.

Isotope labeling of DNA. DNA labeling was accomplished by adding 10 µCi of [5-³H]adenine (5 to 15 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and [2-³H]thymidine (6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml to the above-mentioned medium. Cells were grown, harvested, and stored as described above.

Lysis of cells. A modification of the Marmur technique (20) was used for lysis of bacterial cells and DNA purification. Frozen bacterial pellets were suspended in a small volume of saline-EDTA (5 ml/g of frozen pellet weight; 0.15 M NaCl, 0.1 M ethylenediaminetetraacetate [EDTA], pH 8). Lysozyme (3× crystalline; Nutritional Biochemicals Corp., Cleveland, Ohio) was added to a final concentration of 1.5 mg/ml of cell suspension. The suspension was then incubated in a 37°C water bath for 4 to 5 h. At this time, an equal volume of a Pronase-SLS solution (2% sodium lauryl sulfate [SLS] [Fisher Scientific Co., Pittsburgh, Pa.] containing 0.5 mg of nuclease-free Pronase [B grade; Calbiochem, LaJolla, Calif.] per ml) was added, and the suspension was incubated for 16 to 20 h in a 37°C water bath, after which lysis was complete.

Extraction and purification of DNA. The lysate was centrifuged at 8,000 × *g*, and the supernatant was recovered for DNA extraction. The addition of sodium perchlorate to a final concentration of 1 M was followed by a chloroform-isoamyl alcohol protein extraction. Nucleic acids were spooled onto glass rods after the addition of 2 volumes of cold ethyl alcohol. The nucleic acids were suspended in 2 to 5 ml of 1× SSC (0.15 M NaCl, 0.015 M Na₃ citrate, pH 7.0), and heat-treated ribonuclease (80°C for 10 min, 5× crystalline; Nutritional Biochemicals Corp., Cleveland, Ohio) was added to a final concentration of 100 µg/ml. The solution was incubated in a 37°C water bath for 40 min. Nuclease-free Pronase, self-digested for 1 h at 37°C, was added to a final concentration of 100 µg/ml, and incubation was continued for another 45 min. The solution was placed into 5-ml cellulose nitrate centrifuge tubes, and cesium chloride (code 62; Rare Earth Division, Ameri-

can Potash and Chemical Co., W. Chicago, Ill.) was added to a final concentration of 1.251 g/ml. The gradients were centrifuged at 25°C for 48 h at 69,100 × *g*. Visible bands of DNA, detected when tubes were placed over high-intensity light, were recovered through the sides of the tubes. Labeled DNA was dialyzed with 2× SSC (0.3 M NaCl, 0.03 M Na₃ citrate, pH 7.0). Unlabeled DNA was dialyzed with 6× SSC (0.1 M NaCl, 0.09 M Na₃ citrate, pH 7.0). Quantitative measurements of DNA were by the method of Burton (4). The absence of protein contamination was monitored by the procedures of Lowry et al. (17).

Determination of DNA base composition. Thermal denaturation of DNA samples was carried out by the method of Mandel and Marmur (18). Buoyant-density determinations were carried out, using an analytical ultracentrifuge (19), on calf thymus DNA and *Bacterionema matruchotii* WHB9A DNA to determine the accuracy of our thermal denaturation data. The G+C difference between the two methods was less than 2 mol%.

DNA-DNA hybridization. The hybridization procedure used was a slight modification of that described by Legault-Demare et al. (16) and later by DeLey and Tijtgat (5). Unlabeled DNA was denatured to single-stranded form by placing 50 ml of a 6× SSC solution containing 30 µg of DNA per ml into a flask and heating it in a 100°C water bath for 20 min. The flask was transferred to an ice bath, and 100 ml of cold 6× SSC was added quickly. Denaturation was monitored by comparing the absorbance at 260 nm of the undiluted DNA solution before and after heating. DNA was bound to nitrocellulose membrane filters (25 mm, 0.45-µm pore size, type B-6; Schleicher and Schuell, Inc., Germany) by passing it through 5 ml of the denatured DNA solution. Binding efficiency was determined by comparing the absorbance at 260 nm of the DNA solution before and after the passages through the filters. The filters were washed with 6× SSC and dried.

Labeled DNA, dialyzed in 2× SSC, was fragmented by sonic treatment and denatured by the method described above.

At least 5 µg of labeled DNA can be added to a filter with 30 µg of unlabeled DNA (1:6 ratio) without saturating the filter (5). However, in this study a minimum 1:35 ratio was used to insure a wide margin below the saturation level. To vials containing the dried filters was added 0.8 ml of 2× SSC-Me₂SO₄ (1.403 g of NaCl, 0.706 g of Na₃ citrate, 50 ml of distilled water, and 30 ml of N₂-bubbled dimethyl sulfoxide [Me₂SO₄]), after which 0.2 ml of labeled DNA (1 µg) was added and the vials were incubated in a 43°C water bath for 18 h (5, 16). Filters were then washed once in 30% Me₂SO₄-70% 2× SSC, once in 15% Me₂SO₄-85% 2× SSC, and three times in 2× SSC. After the filters were dried, scintillation fluid consisting of 4 g of Omnifluor (New England Nuclear Corp., Boston, Mass.) per liter of toluene was added, followed by scintillation counting. Percent hybridization was determined by dividing the counts per minute for the heterologous hybridization samples by the counts per minute for the homologous hybridization samples.

RESULTS AND DISCUSSION

The G+C contents of the two strains of *Fusobacterium* and two strains of *Leptotrichia* tested, as well as those of other members of the family *Bacteroidaceae*, are shown in Table 2. The fusobacteria tested in this study, as well as those tested by Johnson (13), have similar, very low G+C values (25 to 27 mol%). The *Leptotrichia* strains also have similarly low G+C values. In contrast, the *Bacteroides* species have G+C values between 41 and 42 mol%, considerably higher than those for *Fusobacterium* and *Leptotrichia* strains.

The average binding efficiency of unlabeled DNA to nitrocellulose membrane filters was 92%. Only filters with at least 35 µg of DNA bound were used in these experiments.

The average efficiency of renaturation for these hybridization experiments was 47.3%.

Table 3 lists the DNA-DNA homology of the *Fusobacterium* and *Leptotrichia* strains tested. The DNA of each strain was labeled and tested against the unlabeled DNA of the other strain.

In the present study, *F. fusiforme* ATCC 23726 and *F. polymorphum* ATCC 10953 were found to have a 78% polynucleotide homology. DNA homology studies reported in the literature usually have revealed that strains of the same species have over 80% homology (2, 3, 6). On the other hand, bacteria of different species occasionally have been shown to have high homology levels ranging from 75% to as much as 94% (10, 12, 22, 23). The homology range of 75 to 85% seems to be the borderline for species differentiation. Because of the lack of definite guidelines for species differentiation, the combining of all oral fusobacteria into one species cannot be shown to be untenable on the basis of this study. However, at best, the DNA homology of the strains of *F. polymorphum* and *F.*

TABLE 3. Average percent homology of DNA of selected members of the family *Bacteroidaceae*

Strain	<i>Leptotrichia buccalis</i> ATCC 19616	<i>Fusobacterium fusiforme</i> ATCC 23726
<i>Fusobacterium polymorphum</i> ATCC 10953	29.7	78.1
<i>Fusobacterium fusiforme</i> ATCC 23726	28.6	100

fusiforme tested was at the lower end of the range acceptable for different strains of the same species. These results suggest that the combining of oral fusobacteria into one species should be reevaluated.

The polynucleotide homology between the two strains of *L. buccalis* and the two strains of *Fusobacterium* tested is less than 30%, too low to indicate more than the possibility of closely related genera.

The DNAs of the *Leptotrichia* and *Fusobacterium* strains studied have similar, very low G+C values (24.5 to 25.6 mol%). There is approximately a 16 mol% difference in the G+C content of the DNA of strains of these two genera and those of other members of the family *Bacteroidaceae* reported in the literature. It is unlikely that such large differences are due to variation in laboratory techniques, especially since one of the studies cited (13) reported G+C values for the *Fusobacterium* strains tested within 2% of the values reported here. It is concluded that *Fusobacterium* and *Leptotrichia* are not closely related to the *Bacteroidaceae* species reported and that these two genera probably do not belong in the family *Bacteroidaceae*. Furthermore, the similar, unusually low G+C values for *Fusobacterium* and *Leptotrichia* may indicate a close phylogenetic relationship for these genera.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant 5 TO1 DE00088-13 from the National Institute of Dental Research and by the Drew Endowment Fund of the University of Maryland.

REPRINT REQUESTS

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TABLE 2. G+C content of DNA of selected members of the family *Bacteroidaceae*

Taxon	G+C content (mol%)
<i>Bacteroides fragilis</i>	42 ^a
<i>B. melaninogenicus</i>	41.5 ^b
<i>B. nigrescens</i>	41.5 ^b
<i>Fusobacterium varium</i>	27 ^a
<i>F. mortiforum</i>	27 ^a
<i>F. polymorphum</i> ATCC 10953	25.6 ^c
<i>F. fusiforme</i> ATCC 23726	25.3 ^c
<i>L. buccalis</i> ATCC 19616	24.5 ^c
<i>L. buccalis</i> ATCC 23471	24.8 ^c

^a See reference 13.

^b See reference 11.

^c Data derived from the present investigation.

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