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Heterogeneity among isolates of *Fusobacterium nucleatum* has been recognized for many years. The phenotypic properties of 340 strains considered to be *F. nucleatum* were examined. While these strains were phenotypically similar and fit the description of *F. nucleatum*, they could be differentiated into three groups on the basis of electrophoretic patterns of whole-cell proteins and DNA homology. Strains in groups I and II showed >80% DNA homology within groups and <75% similarity between groups. Strains of group III demonstrated >85% DNA homology to each other and <65% similarity to strains in groups I and II. We propose that *Fusobacterium nucleatum* be divided into the following three subspecies: *Fusobacterium nucleatum* subsp. *nucleatum*, with type strain ATCC 25586; *Fusobacterium nucleatum* subsp. *polymorphum*, with type strain ATCC 10953; and *Fusobacterium nucleatum* subsp. *vincentii*, with type strain ATCC 49256.

Strains of *Fusobacterium nucleatum* have been cultivated from human oral cavities for approximately 100 years. Fusiform organisms were first detected in samples of dental plaque in 1693 by van Leeuwenhoek (3), and later they were detected by Plaut in 1894 and by Vincent in 1896 in samples taken from lesions of acute necrotizing ulcerative gingivitis and Vincent's angina. In 1898, Veillon and Zuber isolated fusiform microorganisms in pure culture, naming them *Bacillus fusiformis*. In 1922, the genus name *Fusobacterium* was proposed by Knorr (17, 18), and the following three species were described: *Fusobacterium polymorphum*, *F. nucleatum*, and *Fusobacterium plauti-vincenti* (a later synonym of *Leptotrichia buccalis*). Between 1930 and 1970, various classification schemes were devised for the differentiation of these species; however, many of the distinguishing features of the three species overlapped, making differentiation difficult. In 1974, *F. polymorphum* and *F. plauti-vincenti* were combined into a single species, *F. nucleatum* (22).

*F. nucleatum* is of considerable interest to oral microbiologists. It is the most frequently detected species in predominant cultivable studies of the microbiota of subgingival plaque, averaging 8 to 10% of the microbiota (5, 6, 20). *F. nucleatum* has been associated with lesions of destructive periodontal disease, but has also been found in high numbers in successfully treated sites and in healthy sites (5, 6, 20). Investigators have noted the heterogeneity of *F. nucleatum* on the basis of cell and colonial morphology (28), protein profiles (1, 21), long-chain fatty acid analysis (1, 15), antigenic determinants detected in both whole cells and cell fractions (9, 13, 16, 19), enzyme electrophoresis (11), and DNA-DNA hybridization (25; Y. Selin and J. L. Johnson, J. Dent. Res. 60:415, 1981). Such findings suggest that distinct subgroups exist within the species *F. nucleatum* and that some subgroups may be associated with disease and others may be associated with health. Studies in our laboratory indicated that there were three patterns that were exhibited by the total cell proteins of strains of *F. nucleatum* when we used sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and a polychromatic silver stain (Gelcode; Pierce Chemical Co., Rockford, Ill.). Therefore, we decided to determine whether the isolates in the SDS-PAGE groups also differed on the basis of DNA-DNA homology.

The data from this study indicated the presence of three different homology groups of *F. nucleatum* that were consistent with the SDS-PAGE groups. Group II contained the type strain of *F. nucleatum*, strain ATCC 25586, and group III contained strain ATCC 10953T (T = type strain). We propose that group III strains be designated *F. nucleatum* subsp. *polymorphum*, since strain ATCC 10953T was originally named *F. polymorphum*. Group II strains are designated *F. nucleatum* subsp. *nucleatum* on the basis of the presence in this group of the current type strain of *F. nucleatum*, strain ATCC 25586. A new subspecies, *F. nucleatum* subsp. *vincentii*, is proposed for the strains of group I, with strain ATCC 49256 as the type strain.

**MATERIALS AND METHODS**

Sources of strains and phenotypic characterization. *F. nucleatum* strains were isolated from subgingival plaque samples of healthy patients (17 isolates), localized juvenile periodontitis patients (3 isolates), and patients with other forms of periodontitis (320 isolates), as previously described (6). The samples were dispersed, diluted, plated onto Trypticase soy blood agar plates (BBL Microbiology Systems, Cockeysville, Md.), and incubated at 37°C in an anaerobic atmosphere containing 80% N₂, 10% CO₂, and 10% H₂ for 5 to 7 days. A total of 50 isolates were picked randomly and were characterized from each sample. The identities of strains presumptively considered to be *F. nucleatum* on the basis of cell and colonial morphology were confirmed by biochemical characterization and analysis of acid end products by gas-liquid chromatography (4, 6). Fresh isolates were compared with the following reference strains obtained from the American Type Culture Collection, Rockville, Md.: *F.
nucleatum ATCC 25586T, ATCC 10953T, and ATCC 23726, Fusobacterium simiae ATCC 33568T (a monkey strain), and Fusobacterium periodonticum ATCC 33693T.

**PAGE of soluble proteins.** (i) Gel preparation. We prepared 10% SDS-discontinuous polyacrylamide gels (thickness, 1.5 mm) by using a model SE600 vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, Calif.), as described by Tanner et al. (30). Stacking gels containing 4% acrylamide (Pierce) were prepared and degassed, and 20-well combs were inserted. The gels were allowed to polymerize overnight before use.

(ii) Bacterial cell preparation, loading, and running of gels. Colonies of *F. nucleatum* were scraped from the surfaces of blood agar plates into 0.2 ml of distilled water. The optical density was adjusted to a MacFarland no. 6 turbidity standard (48 ± 6 µg of protein per 10 µl), and the suspension was sonicated for 1 min with a microultrasonic cell disrupter (Kontes, Vineland, N.J.). Then 0.2 ml of sample loading buffer containing 0.125 M Tris-hydrochloride (pH 6.8), 4% SDS, 20% glycerol, and 10% 2-mercaptopethanol was added. The proteins were then denatured by boiling for 5 min, and 10 µl of 0.01% phenol red (Sigma Chemical Co., St. Louis, Mo.) tracking dye was added. A 10-µl portion of each suspension was applied to a well of the gel, and electrophoresis was conducted at a constant current of 30 mA until the tracking dye reached the bottom of the gel (approximately 4 h).

(iii) Staining. After electrophoresis, the gels were fixed overnight in a solution containing 50% ethanol and 5% acetic acid. The gels were then washed in distilled water for 2 h with one change after the first hour. Gelcode color silver stain (Pierce) was used as described by the manufacturer, with the following modifications. The gels were stained for 20 min in one-half the recommended concentration of silver concentrate solution. They were then washed with double-distilled water for 60 s and developed by adding one-half the recommended concentration of reducer solution for approximately 5 min. The gels were rinsed again (1 min), and the full concentration of stabilizer solution was added to each gel for approximately 1 h. Duplicate gels were stained by using the monochromatic silver staining procedures described by Wray et al. (32) and Morrisey (24).

**DNA-DNA hybridization.** Bacterial cell cultures were grown to log phase in 400 ml of mycoplasma broth (BBL) supplemented with 5 mg of hemin per liter and were harvested by centrifugation. DNA was extracted by using the method of Smith et al. (27), with the addition of pretreatment of the cell suspensions with 200 µg of lysozyme per ml for 30 min at 60°C. DNA preparations were dialyzed overnight in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate [pH 7.0]), after which the optical density at 260 nm was adjusted to 1.8 and the DNA was sheared by using a high-speed recording spectrophotometer (29). Percentages of homology were calculated as described by DeLey et al. (2). Each homology calculation was performed in triplicate by using three separate DNA preparations for each strain to overcome concerns about the possible effects of variables in the extraction process. The variation in the method was ±2.6%, as determined by measuring replicate levels of homology of the strains with themselves.

In an initial study, five cultures from the American Type Culture Collection and seven fresh isolates (two from each group and one ungrouped isolate) were chosen for DNA-DNA hybridization. The DNA isolated from each strain in the matrix was hybridized in triplicate with the DNA of every other isolate and with itself. Representative strains were chosen from the results of this study, based on the recommendation of Hartford and Sneath (12) that a complete data matrix be used to choose strains to represent a cluster or homology group. The strains chosen were strains EM48 (group I), ATCC 25586T (group II), and ATCC 10953T (group III). DNAs were extracted from 137 additional fresh isolates and were tested against each of the three central strains chosen. Fresh isolates were assigned to a homology group based upon the highest percentage of similarity to the three central strains. In addition, the same strains were also grouped by using SDS-PAGE without knowledge of the homology grouping.

**RESULTS AND DISCUSSION**

**Phenotypic features.** The phenotypic features of 340 strains of *F. nucleatum* isolated from subgingival sites of healthy patients and periodontally involved patients, as well as strains from the American Type Culture Collection, were examined and were confirmed as *F. nucleatum* by using conventional phenotypic criteria (14, 23). All of the strains were anaerobic, gram-negative, nonmotile, tapered rods and displayed either an irregular or flecked appearance on the surface of blood agar. Carbohydrate fermentations were negative, with the exception of a few isolates which demonstrated weak acid production (decrease of 0.5 to 1.0 pH unit) from fructose, glucose, and sucrose. Indole was produced by all strains. Acid end products from broth cultures (with or without glucose) included major amounts of butyrate (<10 meq/liter), with lesser amounts of propionate and acetate. Subgroups within the species could not be distinguished on the basis of phenotypic characteristics.

**PAGE protein patterns.** In accord with the findings of other workers (1, 21, 25), there was considerable heterogeneity in the protein profiles detected by the conventional silver stain, making grouping of strains difficult. In our hands, the Gelcode stain was more useful for grouping isolates because of its ability to color the various proteins or other macromolecules. After we examined the Gelcode-stained protein profiles of all of the isolates, it became apparent that distinct marker bands were present in certain strains and absent from others. A number of these marker bands were yellow, possibly denoting the presence of lipoproteins (26). The most obvious banding pattern, and the distinguishing marker of group I, was an intense yellow band at 22,600 ± 500 daltons (Fig. 1). In contrast, group III strains exhibited a series of yellow bands, a doublet at 43,100 and 40,800 daltons and single bands at 32,700 ± 1,000, 27,700 ± 1,200, and 19,700 ± 600 daltons, but no band at 22,600 daltons. Group II strains did not exhibit bands at 32,700, 27,700, or 22,600 daltons. While the marker bands were consistent within each group, heterogeneity was observed in the positions of many of the remaining bands of the groups.

Of 340 fresh isolates, 187 were assigned to group I, 34 were assigned to group II, and 102 were assigned to group III. Strains ATCC 25586T and ATCC 23726 exhibited the banding pattern of group II, and strain ATCC 10953T exhibited the banding pattern of group III. However, there were strains which exhibited different banding patterns and could
FIG. 1. Protein electrophoretic patterns of the three subspecies of *F. nucleatum*. The gel was stained with a polychromatic silver stain. The arrows indicate the yellow bands which discriminate among subspecies. Lanes 1 and 2, group I strains 364 and EM48, respectively; lanes 3 through 5, group II strains FC38, ATCC 25586T, and EL28, respectively; lanes 6 through 8, group III strains JS, ATCC 10953T, and MG, respectively.

not be placed into any of the three groups. These included *F. periodonticum* ATCC 33693T and *F. simiae* ATCC 33568T. The 17 unclassified fresh isolates of *F. nucleatum* might represent additional groups.

**DNA-DNA hybridization.** The levels of DNA-DNA hybridization of DNAs isolated from the same strains on different occasions averaged 102.0 ± 5.8%. A cluster analysis of the complete data matrix of five cultures from the American Type Culture Collection and seven fresh isolates of *F. nucleatum* is shown in Fig. 2. The dendogram shows the percentages of similarity for the different strains. A dashed vertical line was arbitrarily placed at 80% similarity. Three clusters were formed. The three strains exhibiting the PAGE group III profile clustered at >85% similarity and included strain ATCC 10953T. Four strains representing PAGE group II, including strains ATCC 25586T and ATCC 23726, clustered together at >90% similarity. Two strains of PAGE group I clustered at 80% similarity. Three strains that did not fit into any PAGE group did not cluster with the other strains. These included the strains of *F. periodonticum* and *F. simiae* and a fresh isolate of *F. nucleatum*, strain FD39. It should be noted that within-group levels of homology were all >80%, but levels of homology between groups were, with one exception, <80%. The percentages of similarity of *F. simiae* to strains EM48, ATCC 25586T, and ATCC 10953T were 70, 49, and 64%, respectively. The percentages of similarity of *F. periodonticum* to strains EM48, ATCC 25586T, and ATCC 10953T were 60, 52, and 58%, respectively. *F. simiae* and *F. periodonticum* exhibited SDS-PAGE patterns that were distinct from those of the proposed subgroups of *F. nucleatum* and from each other.

There was 100% agreement between PAGE and DNA homology data when the DNAs of 137 fresh isolates were tested against each of the three central strains. Of the 137 isolates, 74 were classified as group I, 26 were classified as group II, and 37 were classified as group III. The mean percentages of similarity and ranges were as follows: group I, 84 and 73 to 98%; group II, 91 and 82 to 106%; and group III, 85 and 78 to 95%.

In this investigation, the difficulty in separating subgroups on the basis of phenotypic traits or SDS-PAGE patterns with conventional silver staining was confirmed. However, the use of SDS-PAGE with a polychromatic silver stain and DNA hybridization techniques allowed a robust subgroup-
ing. Because DNA hybridization is not practical for subgrouping large numbers of fresh isolates, the PAGE method provides a practical means of assigning strains to groups. Perhaps more rapid methods will be devised for distinguishing subgroups, such as hemagglutination (7, 8, 10), enzyme markers (11), or cloned (J. M. DeRienzo, personal communication) or oligonucleotide DNA probes (B. J. Paster and F. E. Dewhirst, personal communication; G. L. F. Smith et al., J. Dent. Res. 68:241, 1989).

Investigators have recognized the heterogeneity among isolates of F. nucleatum but have had difficulty in developing methods to identify subgroups in this species reliably. Variations observed in numerous morphological, biochemical, and serological characteristics and in DNA relatedness have confounded the separation of this species into subgroups. Indeed, the level of relatedness of strains within this species has been reported to be as low as 30% and as high as 100% (25; Selin and Johnson, J. Dent. Res. 60:415, 1981), depending upon the methodology used. In this study, interstrain DNA-DNA hybridization values ranged from 50 to 100%, which was in good agreement with the findings of other workers. DNA homology studies indicate that strains of the same species usually exhibit more than 70% DNA relatedness (32). Based upon the report of Wayne et al. (31) and the fact that the levels of relatedness in the majority of hybridizations in this study and other studies were >70%, it seems tenable to place strains identified conventionally as F. nucleatum into one species. However, the detection of F. nucleatum subgroups in this study with <80% similarity between groups suggests the existence of subspecies. The hybridization data also suggest that F. simiae and F. periodonticum may represent additional subspecies of F. nucleatum. However, more strains of these species should be examined to confirm or refute this suggestion.

Since the DNA homology and SDS-PAGE data suggest that different subspecies of F. nucleatum exist and group II contained the type strain of F. nucleatum, strain ATCC 25586, the name F. nucleatum subsp. nucleatum should be retained for this group. Group III strains were clearly different from the other groups. Since group III contained strain ATCC 10953T, which was originally named F. polymorphum, we propose the name F. nucleatum subsp. polymorphum for members of this group, with strain ATCC 10953T the type strain. The name F. nucleatum subsp. vincentii (vin. cen. ti. i. L. gen. n. vincentii, of Vincent, referring to H. Vincent who studied the organism originally isolated from Vincent’s angina and necrotizing ulcerative gingivitis), is proposed. The type strain of F. nucleatum subsp. vincentii is strain ATCC 49256. The proposed subspecies of F. nucleatum can be differentiated by SDS-PAGE patterns and DNA-DNA hybridization.

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LITERATURE CITED


