Glutamate Dehydrogenase and 2-Oxoglutarate Reductase Electrophoretic Patterns and Deoxyribonucleic Acid-Deoxyribonucleic Acid Hybridization among Human Oral Isolates of *Fusobacterium nucleatum*

SAHEER E. GHARBIA* AND HAROUN N. SHAH

Department of Oral Microbiology, The London Hospital Medical College, London E1 2AD, England

The electrophoretic mobilities of glutamate dehydrogenase and 2-oxoglutarate reductase were compared for three reference strains and 30 human, oral isolates of *Fusobacterium nucleatum*. Both enzymes allowed the same strains to be grouped into three electrophoretic clusters, designated groups Fn-1, Fn-2, and Fn-3. Group Fn-1 contained the type strain of *F. nucleatum*, strain ATCC 25586, and nine clinical isolates. Group Fn-2 comprised 20 strains and appeared to contain the strains of *F. nucleatum* that are isolated most commonly from oral cavities. Strain NCTC 10953 (formerly "*Fusobacterium polymorphum*") was a member of this cluster. Strains of group Fn-3 were rarely isolated; this group contained three isolates and reference strain NCTC 11362, which was listed previously as "*Fusobacterium fusiforme*." The deoxyribonucleic acid (DNA) base compositions of all strains were between 25 and 27 mol% guanine plus cytosine. Under optimal conditions of DNA-DNA hybridization, all of the strains exhibited high levels of DNA homology (73 to 99%) to the three reference DNA probes belonging to groups Fn-1, Fn-2, and Fn-3. However, under stringent DNA hybridization conditions there was evidence of more genetic homogeneity within each group.

The genus *Fusobacterium*, as currently described in *Bergey's Manual of Systematic Bacteriology* (16), has undergone major revisions since it was first described (5, 6, 11, 12, 15). The natural habitat of the type species, *Fusobacterium nucleatum*, appears to be oral cavities (3). This organism is frequently isolated from mucous membranes and from both supra- and subgingival dental plaque (24, 25). Several properties, such as cellular fatty acids (1, 9), peptidoglycan composition (14, 26), and bile sensitivity, distinguish *F. nucleatum* from nonoral species, such as *Fusobacterium varium* and *Fusobacterium mortiferum* (8). However, in spite of the apparent homogeneity among strains belonging to the species *F. nucleatum*, several workers have reported differences in morphology (13), serological properties (7), antibiotic susceptibilities (3), and both soluble protein and polypeptide profiles (1, 2, 13).

In a preliminary study of the properties and distribution of glutamate dehydrogenase (GDH) (4), we recently reported heterogeneity in GDH electrophoretic patterns in *F. nucleatum*. Whereas within other species electrophoretic bands were similar, three human isolates and one animal isolate of *F. nucleatum* possessed GDH bands that migrated differently (4). In this study, therefore, a large number of *F. nucleatum* strains that were isolated from normal and diseased sites in human oral cavities were subjected to a similar GDH electrophoretic analysis. Another important catabolic enzyme, 2-oxoglutarate reductase (OGR), was included to confirm the stability of the groups derived from the study of GDH electrophoretic mobilities. Deoxyribonucleic acid (DNA) base composition and DNA-DNA hybridization were used to assess the taxonomic status of the clusters formed.

MATERIALS AND METHODS

Bacterial strains. The following strains were used: *F. nucleatum* ATCC 25586^T (T = type strain), NCTC 10953 (formerly "*Fusobacterium polymorphum*"), and NCTC

11362 (formerly "*Fusobacterium fusiforme*"), as well as 30 clinical isolates from normal and diseased sites of human oral cavities.

Cultivation and identification. All of the strains were maintained by weekly subculture on 2.5% (vol/vol) blood agar plates in an atmosphere containing 10% (vol/vol) CO₂ and 5% (vol/vol) H₂ in N₂ at 37°C. Each strain was inoculated onto freshly prepared Columbia agar (Oxoid Ltd., London, England) supplemented with 6% horse blood and 0.1% vitamin K and was incubated anaerobically for 48 h. A bacterial suspension corresponding to a McFarland no. 4 density standard was used to inoculate a RapID ANA Kit (API System, La Balme-Les-Grottes, France), and the strips were incubated aerobically at 37°C for 4 h. Strains were identified as described by Murdoch et al. (17). The method used for acid end product analysis for culture supernatants has been described previously (8).

Preparation of cell-free extracts and enzyme electrophoresis. Cells were harvested from 2-day-old blood agar cultures and were suspended in 0.5 ml of 0.1 M tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 8.5). Each cell suspension was shaken with Ballotini beads (grade 12) for 10 min in a Mickle tissue disintegrator at 4°C. The suspension was centrifuged at 37,000 × g for 10 min, and the supernatants were electrophoresed as described previously (20). Electrophoresis was for 90 min at 15 V/cm of gel in 0.04 M barbitone-acetic acid buffer (pH 8.6). GDH was visualized by using a solution of glutamate (20 mg/ml), nicotinamide adenine dinucleotide (5 mg/ml), phenazine methosulfate (0.04 mg/ml), and thiazolyl blue tetrazolium (0.02 mg/ml) in 0.1 M tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 9.0). OGR was visualized by replacing the glutamate with a 2-hydroxyglutarate solution (10 mg/ml).

Isolation, purification, and base composition determination of DNA. DNAs from all strains were prepared as described previously (4). The DNA base composition was estimated from the melting temperature of the DNA in saline citrate (0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0) by

* Corresponding author.

using a model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) and a model 2527 thermal programmer (Gilford) as described previously (21).

Preparation of labeled nucleic acid. All reference DNA preparations were dialyzed in deionized water for 48 h before labeling. For hybridization experiments, 3 µg of each reference DNA was labeled in vitro with deoxy[1',2',5-³H]cytidine 5'-triphosphate by nick translation (10), using a commercial nick translation kit (Amersham Corp., Arlington Heights, Ill.). The reaction was terminated after 40 min, and the DNA was separated from unincorporated nucleotides by gel exclusion chromatography through Sephadex G-50 and dialyzed in 0.42 M NaCl. The DNA was sheared by sonication for 3 min with a 3-mm probe at a power setting of 7 (3 to 4 A for three 10-s bursts; Soniprobe; Dawes Instruments, Ltd., London, England).

DNA-DNA hybridization. DNA homology experiments were performed as described previously (19), with minor modifications. The unlabeled DNAs to be used for all hybridizations with the labeled probe DNAs (from the three reference DNA preparations) were dialyzed in 0.42 M NaCl and sheared as described above to give a fragment size of approximately 500 base pairs. A reaction mixture containing 0.02 µg of the labeled reference DNA and 20 µg of unlabeled DNA was mixed in 200 µl of 0.42 M NaCl, and 200 µl of light paraffin oil was layered on top. The mixture was denatured for 10 min at 100°C, and hybridization was allowed to proceed for 93 h at either 55°C (optimal hybridization) or 75°C (stringent conditions of hybridization). The tubes were then placed in an ice bath for 20 min to stop the reaction. S1 nuclease (100 U/ml; Sigma Chemical Co., Dorset, England) in a buffer containing 0.1 mM ZnSO₄, 0.15 M NaCl, 30 mM sodium acetate (pH 4.5), and 20 µg of sheared and denatured calf thymus DNA (type V; Sigma) per ml was added to all tubes. The mixture was incubated for 20 min at 50°C, precipitated with 10% trichloroacetic acid, and filtered on membrane filters (Schleicher & Schuell, Dassel, Federal Republic of Germany). Cocktail T scintillation fluid (BDH, Essex, England) was added to the filters, which were counted with a model SL30 liquid scintillation counter (Intertechnique, Dover, N.J.). The level of hybridization was determined as a percentage of the value for the homologous reaction. The level of recovery of labeled DNA in the homologous reaction was 82%. A correction was made for self-reassociation of labeled DNA by using calf thymus DNA as a control.

RESULTS AND DISCUSSION

The three reference strains and 30 clinical isolates used in this study were readily identified as *F. nucleatum* by using the RapID ANA system (API System). In addition, the metabolic end products of these organisms corresponded to those reported previously (8). Care was taken to distinguish these bacteria from other oral species, such as *Fusobacterium sulci*, *Fusobacterium alocis*, and *Fusobacterium periodonticum*, by using previously described tests (2, 23). Among the 33 strains of *F. nucleatum* tested, three groups were clearly discernible on the basis of GDH electrophoretic patterns. Furthermore, the compositions of the three GDH clusters formed were supported by the OGR electrophoretic mobility patterns (Fig. 1). The mobility clusters were designated groups Fn-1 (strains which had GDH and OGR mobilities of 0.8 and 1.8 cm, respectively), Fn-2 (isolates which had GDH and OGR mobilities of 1.3 and 2.3 cm, respectively), and Fn-3 (strains which had GDH and OGR mobilities of

FIG. 1. Electrophoretic mobilities of GDH and OGR of *F. nucleatum* strains.

1.8 and 2.0 cm, respectively) (Fig. 1). The variation in GDH and OGR electrophoretic mobilities within groups was minimal (ca. 1 to 2 mm), and the patterns, which were tested on several occasions, were consistent. Group Fn-1 comprised 10 strains and included *F. nucleatum* ATCC 25586^T. Isolates which belonged to this group were readily identified by their distinctive morphology on blood agar plates, typical of that associated with *F. nucleatum*. After overnight growth, colonies were round, convex, and 0.5 to 1.0 mm in diameter and had a greyish white appearance. Group Fn-2 strains appeared to be the most frequently isolated organisms from human oral cavities. This group contained the previously described species "*F. polymorphum*" and consisted of 20 identical strains. After overnight culture, colonies on blood agar plates were characteristically round, convex, 1 to 2 mm in diameter, smooth, glistening, and entire. If cultures were incubated for more than 3 days, they became indistinguishable from group Fn-1 isolates. Group Fn-3 strains were the least commonly isolated strains of *F. nucleatum*. However, the four strains which belonged to this group were recognized by their distinctive colonial morphology; this group contained the previously described organism "*F. fusiforme*" (5, 6) NCTC 11362 (National Collection of Type Cultures, Central Public Health Laboratory, London, England). Colonies on blood agar plates were 1 to 3 mm in diameter and irregular and had a low cone shape with speckled surfaces, and pitting was frequently observed if the colonies were dislodged.

Despite the clear subdivision of *F. nucleatum* strains into three electrophoretic subgroups, DNA base composition determinations and DNA-DNA hybridization experiments indicated that human isolates of *F. nucleatum* belong to the same species. The guanine-plus-cytosine values ranged be-

TABLE 1. DNA base compositions and DNA-DNA hybridization of *F. nucleatum* strains

Strain	Guanine-plus-cytosine content (mol%)	% Hybridization with [³ H]DNA from:					
		Strain ATCC 25586 ^T		Strain NCTC 10953		Strain 010	
		Optimum conditions (55°C)	Stringent conditions (75°C)	Optimum conditions (55°C)	Stringent conditions (75°C)	Optimum conditions (55°C)	Stringent conditions (75°C)
Group Fn-1							
ATCC 25586 ^T	26	100	100	82	79	90	79
1336	25	92	83	86	78	80	69
1337	25	93	85	90	83	79	79
338	26	97	90	88	80	80	71
11	25	98	90	91	89	80	76
13	25	99	89	92	79	80	73
28	27	95	87	89	80	84	69
78	27	96	84	88	79	82	70
D	27	98	90	90	82	86	75
A	27	92	86	92	80	90	80
Mean		96	88	89	81	83	74
Group Fn-2							
NCTC 10953	27	90	82	100	100	81	69
21	26	90	82	97	90	87	77
33	26	93	85	87	80	79	70
38	25	97	88	85	79	90	83
41	27	90	79	83	76	88	78
G	26	93	80	96	81	85	80
1446	25	90	82	87	75	90	79
009	26	90	78	98	90	89	80
337	26	93	77	97	90	94	80
339	26	91	81	95	89	96	83
B109	25	93	80	93	88	90	76
1445	27	97	89	90	87	80	70
1407	25	95	90	82	74	83	69
312	27	97	90	85	73	80	69
304	27	95	87	90	80	79	64
302	26	92	88	89	76	75	60
318	25	94	87	85	70	73	60
720	27	96	88	87	72	80	69
708	25	97	88	86	79	76	65
001	26	95	87	98	94	80	70
Mean		98	84	90	82	84	73
Group Fn-3							
NCTC 11362	26	90	80	82	71	87	80
010	26	92	80	82	70	100	100
45	26	87	79	80	73	90	78
24	27	89	80	89	80	97	90
Mean		89	80	83	73	93	87

tween 25 and 27 mol% (Table 1), well within the limits of a clearly defined species. DNA-DNA hybridization experiments carried out against three reference DNA probes belonging to groups Fn-1, Fn-2, and Fn-3 confirmed the relatedness of these strains. Initially, DNA-DNA reassociation experiments were performed under optimal conditions (55°C). The levels of DNA homology were high with each probe and ranged between 73 and 99% (Table 1). Under more stringent conditions, these values ranged between 60 and 90%. Although we found high levels of DNA homology among all strains under stringent conditions, there was more genetic homogeneity within each of the three groups with their corresponding probes. For example, group Fn-1 strains hybridized with the group Fn-1 probe at levels between 83 and 90%, whereas group Fn-3 strains exhibited between 69 and 80% homology with the same probe (Table 1).

Variations in electrophoretic mobilities occur due to differences in the net changes between enzyme molecules (22). A single amino acid substitution may alter the surface properties of a molecule and affect its electrophoretic mo-

bility. However, several substitutions may also take place without affecting the electrophoretic properties of an enzyme (N. Dance and D. C. Watts, *Biochem. J.* 84p:114, 1962). Differences in electrophoretic migration patterns of enzymes may result from minor genetic alterations and may not reflect taxonomic identity, as in the present study. Often such differences reflect genetic heterogeneity (18, 20, 21) and can be used to identify species which are otherwise difficult to differentiate. Since the electrophoretic patterns of GDH and OGR resulted in three clusters of the same strains, these subdivisions within *F. nucleatum* cannot be overlooked. Further studies involving 16S ribonucleic acid sequencing are now in progress to study the phylogenetic relatedness of these organisms to other members of the *Bacteroidaceae*.

F. nucleatum strains form part of the normal flora of several sites within oral cavities (3). This species is now regarded as an important periodontal pathogen, and its association with acute necrotizing ulcerative gingivitis has long been recognized (28). As yet, no virulence determinants have been clearly identified, nor is it clear whether virulent

and avirulent strains exist. Although most chemotaxonomic data indicate that *F. nucleatum* is a homogeneous species (9, 14, 26), several studies of oral isolates to determine soluble protein or polypeptide patterns have indicated that there is considerable heterogeneity (1, 2, 13). However, the electrophoretic patterns derived were too cumbersome and varied for clustering of isolates (1).

As GDH consistently had a distinctive migrating position within each group, strains could be readily assigned to one of the three clusters by using this enzyme pattern. However, the combined use of GDH and OGR may be helpful. To understand the role of *F. nucleatum* in its natural ecosystem, it may be necessary to recognize the diversity within the species. Tests used so far either have obscured this diversity or have been too complex to assess. The method which we describe provides a simple and reliable technique for studying the ecology of *F. nucleatum*.

ACKNOWLEDGMENT

S.E.G. acknowledges an ORS award for this work.

LITERATURE CITED

- Calhoon, D. A., W. R. Mayberry, and J. Slots. 1983. Cellular fatty and soluble protein profiles of oral fusobacteria. *J. Dent. Res.* **62**:1181-1185.
- Cato, E. P., L. V. Moore, and W. E. C. Moore. 1983. *Fusobacterium alocis* sp. nov. and *Fusobacterium sulci* sp. nov. from the human gingival sulcus. *Int. J. Syst. Bacteriol.* **35**:475-477.
- Finegold, S. M., W. L. George, and M. E. Mulligan. 1985. Disease-a-month, p. 3-69. In N. J. Cotstonas, Jr. (ed.), *Anaerobic infections*, part 1, vol. 31. DM Yearbook Medical Publisher, Chicago.
- Gharbia, S. E., and H. N. Shah. 1988. Characteristics of glutamate dehydrogenase, a new diagnostic marker for the genus *Fusobacterium*. *J. Gen. Microbiol.* **134**:327-332.
- Hine, M. K., and G. P. Berry. 1937. Morphological and cultural studies on the genus *Fusiformis*. *J. Bacteriol.* **34**:517-534.
- Hoffman, H. 1957. Genus *Fusobacterium* (Knorr 1922), p. 436-440. In R. S. Breed, E. G. D. Murray, and N. Smith (ed.), *Bergey's manual of determinative bacteriology*, 7th ed. The Williams & Wilkins Co., Baltimore.
- Hofstad, T. 1979. Serological responses to antigens of *Bacteroidaceae*. *Microbiol. Rev.* **43**:103-115.
- Holdeman, L. V., and W. E. C. Moore. 1972. Anaerobe laboratory manual. Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg.
- Jantzen, E., and T. Hofstad. 1981. Fatty acids of *Fusobacterium* species: taxonomic implications. *J. Gen. Microbiol.* **123**:163-171.
- Kelly, R. B., N. R. Cozzarelli, M. P. Deutscher, I. R. Lehman, and A. Kornberg. 1970. Enzymatic synthesis of deoxyribonucleic acid. XXXII. Replication of complex deoxyribonucleic acid by polymerase at a single strand break. *J. Biol. Chem.* **245**:39-45.
- Knorr, M. 1922. Über die fusospirillane Symbiose, die Gattung *Fusobacterium* (K. B. Lehman) und *Spirillum sputigenum* (Zugleich ein Beitrag Zur Bakteriologie der Mundhöhle). I. Mitteilung: die Epidemiologie der fusospirillaren Symbiose, besonders der Flaut-Vincentischen Angina. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig.* **89**:531-545.
- Knorr, M. 1923. Über die fusospirillane Symbiose, die Gattung *Fusobacterium* (K. B. Lehman) und *Spirillum sputigenum*. (Zugleich ein Beitrag Zur Bakteriologie der Mundhöhle). II. Mitteilung: die Gattung *Fusobacterium*. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig.* **89**:4-22.
- Love, D. N., E. P. Cato, J. L. Johnson, R. F. Jones, and M. Bailey. 1987. Deoxyribonucleic acid hybridization among strains of fusobacteria isolated from soft tissue infections of cats: comparison with human and animal type strains from oral and other sites. *Int. J. Syst. Bacteriol.* **37**:23-26.
- Miyagawa, E., R. Azuma, and T. Suto. 1981. Peptidoglycan composition of Gram-negative obligatory anaerobic rods. *J. Gen. Appl. Microbiol.* **27**:199-208.
- Moore, W. E. C., and L. V. Holdeman. 1974. Genus *Fusobacterium* (Knorr, 1922), p. 404-418. In R. E. Buchanan and N. E. Gibbons (ed.), *Bergey's manual of determinative bacteriology*, 8th ed. The Williams & Wilkins Co., Baltimore.
- Moore, W. E. C., L. V. Holdeman, and W. Kelley. 1984. Genus II. *Fusobacterium* Knorr 1922, 4^{AL}, p. 631-637. In N. R. Krieg (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. The Williams & Wilkins Co., Baltimore.
- Murdoch, D. A., I. J. Mitchelmore, R. A. Nash, J. M. Hardie, and S. Tabaqchali. 1988. Preformed enzyme profiles of reference strains of gram-positive anaerobic cocci. *J. Med. Microbiol.* **27**:65-70.
- Shah, H. N., S. E. Gharbia, T. A. R. Al-Jalili, R. A. Nash, and S. V. Seddon. 1987. Enzymes of diagnostic importance within the *Bacteroidaceae*: use as possible ecological markers. *Microbiol. Health Dis.* **1**:115-121.
- Shah, H. N., T. J. M. van Steenberg, J. M. Hardie, and J. deGraaff. 1982. DNA base composition, DNA-DNA reassociation and isoelectric-focusing of proteins of strains designated *Bacteroides oralis*. *FEMS Microbiol. Lett.* **13**:125-130.
- Shah, H. N., and R. A. D. Williams. 1982. Dehydrogenase patterns in the taxonomy of *Bacteroides*. *J. Gen. Microbiol.* **128**:2955-2965.
- Shah, H. N., R. A. D. Williams, G. H. Bowden, and J. M. Hardie. 1976. Comparison of the biochemical properties of *Bacteroides melaninogenicus* from human dental plaque and other sites. *J. Appl. Bacteriol.* **41**:473-492.
- Shaw, C. R. 1965. Electrophoretic variation in enzymes. *Science* **149**:936-942.
- Slots, J., T. V. Potts, and P. A. Mashimo. 1983. *Fusobacterium periodonticum*, a new species from the human oral cavity. *J. Dent. Res.* **62**:960-963.
- Tanner, A. C. R., C. Haffer, G. T. Bratthall, R. A. Visconti, and S. S. Socransky. 1979. A study of the bacteria associated with advancing periodontitis in man. *J. Clin. Periodontol.* **6**:278-307.
- Van Palenstein Helderman, W. H. 1975. Total viable count and differential count of *Vibrio (Campylobacter) sputorum*, *Fusobacterium nucleatum*, *Bacteroides ocharaceus* and *Veillonella* in the inflamed and non-inflamed human gingival crevice. *J. Periodontal Res.* **10**:294-305.
- Vasstrand, E. N., H. B. Jansen, T. Miron, and T. Hofstad. 1981. Composition of peptidoglycan in *Bacteroidaceae*: determination and distribution of lanthionine. *Infect. Immun.* **36**:114-122.
- Veillon, A., and A. Zuber. 1898. Recherches sur quelques microbes strictement anaerobies et leur role en pathologie. *Arch. Med. Exp. Anat. Pathol.* **10**:517-545.
- Williams, B. L., R. M. Pantalone, and J. C. Sherris. 1976. Subgingival microflora and periodontitis. *J. Periodontal Res.* **11**:1-18.