Investigations of the Taxonomy and Systematics of *Fusobacterium nucleatum* Using Allozyme Electrophoresis

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***Fusobacterium nucleatum* forms part of the resident microbiota in both oral and extraoral sites in humans and animals. It is also involved in infections in such sites. Despite the genetic heterogeneity within the species, it has been divided into five subspecies, the validities of which have been questioned. In the present study, 44 *F. nucleatum* isolates were examined at 21 enzyme loci by using the allozyme electrophoretic technique to establish an accurate genetic framework for taxonomic purposes. Three distinct genetic clusters were identified; one cluster consisted exclusively of extraoral isolates, another cluster consisted predominantly of human oral isolates, and the third cluster consisted of a single human oral isolate. Our results highlight the urgent need for extensive biochemical, immunological, and epidemiological studies to accurately define the systematics of the genus *Fusobacterium* based on the framework derived in this study by using 21 independent genetic characteristics.**

Isolates of *Fusobacterium nucleatum* have been identified in the oral cavities of healthy individuals and a number of extraoral sites, including the gastrointestinal tract (11), where this species constitutes part of the resident microbiota. However, fusobacterial species have also been isolated from a range of oral and extraoral infections (1, 16). The frequent isolation of these organisms from clinical infections suggests a pathogenic role for the genus *Fusobacterium*, but the virulence determinants remain largely unknown (11). Due to the weak fermentation reactions of fusobacterial species (10, 15), carbohydrate utilization tests, which are commonly used for routine diagnosis, are of little use, and thus an alternative identification technique that is rapid, simple, and accurate is required.

DNA-DNA homology experiments have indicated that *F. nucleatum* is particularly homogeneous and that its members exhibit 97.3 to 98.4% sequence similarity under optimal hybridization conditions (13). However, heterogeneity within the species has been observed in cellular and colonial morphologies (5, 6, 10), in whole-cell soluble protein profiles (4), in enzyme electrophoretic patterns (12), in adhesive properties (24), in pyrolysis mass spectrometry analysis data (14), in rRNA gene restriction patterns (12), and in DNA-DNA hybridization data (13). This heterogeneity aids in the identification of members of the species and the division of the strains into taxonomically relevant groups, such as subspecies, but such efforts have not resulted in a widely accepted taxonomy (4, 6, 7). The observed heterogeneity may explain the ability of this species to adapt to a variety of environments, as observed in the diversity of clinical diseases with which it is associated.

Within the species *F. nucleatum*, a number of investigators have identified a range of distinct genetic clusters which have subsequently been designated subspecies, but their results often do not correlate with each other (4, 6, 7). Pyrolysis mass spectrometry (14) has been used to identify two distinct clusters within *F. nucleatum* oral isolates. Dzink et al. (4), using DNA homology techniques and electrophoretic patterns of whole-cell lipoproteins, identified three distinct groups within

this species which were subsequently named *F. nucleatum* subsp. *polymorphum*, *F. nucleatum* subsp. *nucleatum*, and *F. nucleatum* subsp. *vincentii*. However, not all of the isolates tested could be classified as members of one of these subspecies. Gharbia and Shah identified four centers of variation by using cellular and colonial morphologies and the electrophoretic mobilities of the enzymes glutamate dehydrogenase and 2-oxoglutarate reductase (6, 7). DNA-DNA hybridization results obtained under optimal conditions indicated that the isolates in these four centers of variation should be placed in the same species due to their high levels of homology (12), and results obtained under stringent conditions were in agreement with the electrophoretic results (13). Lawson et al. subsequently identified these clusters as *F. nucleatum* subsp. *polymorphum*, *F. nucleatum* subsp. *nucleatum*, *F. nucleatum* subsp. *fusiforme*, and *F. nucleatum* subsp. *animalis* and confirmed this subdivision by differentiating the type strains of the four subspecies by rRNA gene restriction analysis (12). Gharbia et al. suggested that in the human oral cavity, *F. nucleatum* subsp. *nucleatum* is associated with periodontally diseased sites, while *F. nucleatum* subsp. *polymorphum* and *F. nucleatum* subsp. *fusiforme* are associated with healthy subgingival sites (6, 9). Despite this apparent correlation, there seems to be no difference in the putative virulence determinants of these proposed subspecies (8).

In our preliminary taxonomic study of previously assigned *F. nucleatum* subspecies and their designated type strains, we identified massive genetic heterogeneity within the isolates assigned to this species (17). Indeed, the heterogeneity was equivalent to that seen in other bacterial species divisions (18, 22). Furthermore, there was no correlation between any of the observed clusters of isolates and any of the designated type strains. Type strains representing previously designated subspecies often occurred in the same genetic cluster or subspecies. The results of this study highlighted the need for a total revision of the previously designated (genetic) divisions and the methods used for characterization, identification, and taxonomy of *F. nucleatum*.

The aims of the present study were to use previously identified diagnostic loci (17) to estimate the genetic variation within the species *F. nucleatum* by using isolates representing the range of known genetic diversity and to determine whether

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TABLE 1. Origins of the *F. nucleatum* isolates tested

Isolate	Subspecies	Host	Site of isolation or infection	Geographical origin
ATCC 25586 ^T	<i>F. nucleatum</i> subsp. <i>nucleatum</i>	Human	Periodontal disease	United States
Fev-1	<i>F. nucleatum</i> subsp. <i>nucleatum</i>	Human	Periodontal disease	United States
VPI 5040	<i>F. nucleatum</i> subsp. <i>nucleatum</i>	Human	Perirectal	United States
VPI 9240	<i>F. nucleatum</i> subsp. <i>nucleatum</i>	Human	Abdominal	United States
ATCC 10953 ^T	<i>F. nucleatum</i> subsp. <i>polymorphum</i>	Human	Healthy gingiva	United States
D206A4	<i>F. nucleatum</i> subsp. <i>polymorphum</i>	Human	Periodontal disease	United States
F1	<i>F. nucleatum</i> subsp. <i>polymorphum</i>	Human	Healthy gingiva	Berlin, Germany
F3	<i>F. nucleatum</i> subsp. <i>polymorphum</i>	Human	Healthy gingiva	Berlin, Germany
VPI 8818	<i>F. nucleatum</i> subsp. <i>polymorphum</i>	Human	Appendix	United States
NCTC 11326 ^T	<i>F. nucleatum</i> subsp. <i>fusiforme</i>	Human	Healthy gingiva	United States
ATCC 49256 ^T	<i>F. nucleatum</i> subsp. <i>vincentii</i>	Human	Healthy gingiva	United States
D213B3	<i>F. nucleatum</i> subsp. <i>vincentii</i>	Human	Healthy gingiva	United States
D218B17	<i>F. nucleatum</i> subsp. <i>vincentii</i>	Human	Healthy gingiva	United States
VPI 4487R	<i>F. nucleatum</i> subsp. <i>vincentii</i>	Human	Leg	United States
VPI 4487S	<i>F. nucleatum</i> subsp. <i>vincentii</i>	Human	Leg	United States
VPI 6086	<i>F. nucleatum</i> subsp. <i>vincentii</i>	Human	Rectal	United States
VPI 6616D	<i>F. nucleatum</i> subsp. <i>vincentii</i>	Human	Rectal	United States
VPI 8754A	<i>F. nucleatum</i> subsp. <i>vincentii</i>	Human	Scrotal	United States
NCTC 12276 ^T	<i>F. nucleatum</i> subsp. <i>animalis</i>	Monkey	Healthy colon	United States
M 7731	ND ^a	Human	Ear	United Kingdom
M 4322	ND	Human	Abdominal	United Kingdom
M 3832	ND	Human	Dog bite	United Kingdom
M 2109	ND	Human	Pleural	United Kingdom
SG2	ND	Human	Healthy gingiva	Adelaide, Australia
SV2	ND	Human	Healthy gingiva	Adelaide, Australia
G34	ND	Human	Preterm labor	Adelaide, Australia
I34	ND	Human	Preterm labor	Adelaide, Australia
I37	ND	Human	Preterm labor	Adelaide, Australia
10976	ND	Human	Brain	United States
10625	ND	Human	Blood	United States
AIP486-87	ND	Human	Gastric	Paris, France
10772	ND	Human	Pleural	United States
10554	ND	Human	Appendix	United States
10735	ND	Human	Appendix	United States
10213	ND	Human	Foot	United States
10446	ND	Human	Hand	United States
3322	ND	Cat	Periodontal disease	Sydney, Australia
3352	ND	Cat	Periodontal disease	Sydney, Australia
3943	ND	Cat	Healthy gingiva	Sydney, Australia
3949	ND	Cat	Healthy gingiva	Sydney, Australia
KE255	ND	Human	Brain	Perth, Australia
KE393	ND	Human	Vaginal	Perth, Australia
OMZ439	ND	Human	Periodontal disease	Switzerland
F6	ND	Human	Healthy gingiva	Berlin, Germany

^a ND, not determined.

there were any correlations between genetically determined clusters of isolates and other biological parameters, such as site of infection, in order to establish an accurate, taxonomically relevant genetic framework for the species *F. nucleatum* and its proposed subspecies. The null hypothesis under examination was that all isolates identified as *F. nucleatum* by independent methods are genetically homogeneous (i.e., they belong to the same species).

MATERIALS AND METHODS

Microorganisms. Forty-four isolates of *F. nucleatum* (Table 1) were investigated in the present study. The type strains of the five subspecies (4–6) were included, together with 13 isolates previously assigned to one of these subspecies on the basis of homology of the cellular fatty acid profiles and whole-cell protein profiles with the profiles of one of the type strains. The subspecies determination study was performed prior to the present study by L. Holdeman-Moore, Virginia Polytechnic Institute and State University, Blacksburg. The purity of isolates was confirmed during manipulations by using Gram staining, cell morphology, anaerobic growth, and resistance to vancomycin and nalidixic acid. Isolate VPI 4487 (*F. nucleatum* subsp. *vincentii*) divided into smooth and rough colonies during anaerobic growth on blood agar; the two colony types were purified, were des-

ignated S and R, respectively, and were electrophoretically examined as separate isolates (Table 1). All 44 isolates were stored in glycerol broth at -80°C until they were required.

Preparation and electrophoresis of bacterial cell lysates. The test organisms were grown anaerobically for 2 to 3 days at 37°C on blood agar plates, and confluent growth was harvested in ca. 1 ml of 100 mM Tris-HCl buffer (pH 8.0). The cells were then centrifuged, the supernatants were removed, and the resulting cell pellets were stored at -80°C .

Prior to electrophoresis, the pellets were thawed at room temperature and resuspended in an equivalent volume of lysis buffer consisting of 0.1% β -mercaptoethanol and 100 μg of NADP (Sigma Chemical Co.) per ml in 100 ml of H_2O . The resuspended cells were kept on ice and ultrasonically disrupted. The lysed cell suspensions were then centrifuged at $5,000 \times g$ for 10 min at 4°C , and the cell lysates (supernatants) were collected in capillary tubes as 5- μl aliquots and stored at -20°C .

The supernatants were thawed, and approximately 0.5- μl aliquots were electrophoresed on a cellulose acetate (Cellolog; Chemetron, Milan, Italy) support medium at 200 V for 1 to 1.5 h at 4°C .

A total of 21 enzyme loci exhibited sufficient staining intensity, resolution, and separation to allow reliable allozymic scoring. Loci for the following enzymes were examined: acid phosphatase (EC 3.1.3.2), adenylate kinase (EC 2.7.4.3), alkaline phosphatase (EC 3.1.3.1), diaphorase (EC 1.6.2.2), enolase (EC 4.2.1.11), glutamate dehydrogenase (EC 1.4.1.3), aspartate aminotransferase (synonym, glutamate oxaloacetate transaminase) (EC 2.6.1.1), glucose-phos-

phate isomerase (EC 5.3.1.9), alanine aminotransferase (synonym, glutamate pyruvate transaminase) (EC 2.6.1.2), hexokinase (EC 2.7.1.1), lactate dehydrogenase (EC 1.1.1.27), malate dehydrogenase (EC 1.1.1.37), purine nucleoside phosphorylase (EC 2.4.2.1), nucleoside diphosphate kinase (EC 2.7.4.6), 2-oxoglutarate reductase (EC 1.2.4.2), leucine-glycine-glycine peptidase (EC 3.4.11.4), phosphoglycerate mutase (EC 2.7.5.3), phosphoglucomutase (EC 2.7.5.1), pyruvate kinase (EC 2.7.1.40), triose-phosphate isomerase (EC 5.3.1.1), and uridine diphosphate kinase (EC 2.7.4.6). The enzymes were histochemically stained by using the methods described by Richardson et al. (19) and Selander et al. (23).

All 21 enzymes tested in the present study were polymorphic for the isolates tested. The electrophoretic band patterns were allozymically scored in order of increasing anodal migration (Table 2) and were interpreted by using fixed differences as the genetic distance measure (see reference 17 for a detailed discussion). Each isolate was compared individually to each of the other isolates to obtain a matrix of percentages of fixed genetic difference between the isolates tested. The results are presented visually as a phenogram in Fig. 1, which was constructed by using the unweighted pair group method of analysis (19).

RESULTS

Table 2 shows the allelic profiles of 44 *F. nucleatum* isolates at 21 enzyme loci. Figure 1 is the phenogram derived from the allelic profiles and shows the fixed genetic differences between isolates. Three distinct genetic groups of isolates were identified, and the fixed differences between groups ranged from 82.5% of the loci (group I compared with group II) to 90% of the loci (groups I and II compared with group III). The within-group variations were approximately equivalent in groups I and II, with the fixed differences ranging from 0 to 79.4%. Group III was defined by a single isolate, SG2.

Genetic group I. The isolates which defined group I were 3322, Fev-1, ATCC 10953^T (T = type strain), ATCC 49256^T, 10446, 10772, M 4322, M 3832, NCTC 11326^T, D213B3, D218B17, M 7731, 10625, F6, D206A4, VPI 8818, F1, F3, 10554, M 2109, 3943, 3352, 3949, VPI 8754A, ATCC 25586^T, 10213, OMZ439, and SV2. Isolate SV2 differed from all of the other isolates in group I at 79.4% of the 21 enzyme loci examined and formed a unique genetic cluster. The remaining isolates in group I could be divided into six clusters that exhibited approximately equal levels of within-cluster genetic variation. Each of these six clusters exhibited fixed genetic differences with the other clusters which ranged from 54.9 to 79.7% of the 21 enzyme loci examined.

The first cluster contained isolates VPI 8754A, ATCC 25586^T, 10213, and OMZ439. The genetic variation within this cluster was much greater than the variation observed in the other five clusters, ranging from 31 to 69% of the loci. It is interesting that this was the genetic cluster which contained ATCC 25586, the type strain of *F. nucleatum* and *F. nucleatum* subsp. *nucleatum*. The second cluster contained isolates 3943, 3352, and 3949. The levels of genetic heterogeneity within this cluster ranged from 24 to 36% of the loci. The third cluster contained isolates 3322, Fev-1, ATCC 10953^T, ATCC 49256^T, 10446, and 10772, and the genetic variation within this cluster ranged from 28 to 48.8% of the loci. The fourth cluster contained isolates M 4322, M 3832, NCTC 11326, D213B3, D218B17, and M 7731; the within-cluster variation for this cluster ranged from 24 to 42.5% of the loci examined. The fifth cluster contained isolates 10625, F6, D206A4, VPI 8818, F1, and F3; within this cluster, the genetic variation ranged from 0 to 49% of the loci. The sixth cluster contained isolates 10554 and M 2109, which differed from each other at 41% of the loci.

Genetic group II. The second group contained isolates KE393, KE255, G34, I34, I37, VPI 6086, AIP486-87, 10735, VPI 6616D, VPI 5040, VPI 9240, VPI 4487R, VPI 4487S, NCTC 12276^T, and 10976. Within group II, there were five distinct genetic clusters of isolates which differed from each other at 53.4 to 78.7% of the loci examined; these values were comparable to the levels of genetic variation between clusters

in group I. The first group II cluster contained isolates KE393, KE255, G34, I34, and I37, and the within-cluster variation ranged from 7 to 34.3% of the loci. The second cluster contained isolates VPI 6086, AIP486-87, 10735, and VPI 6616D, and the within-cluster genetic variation of this cluster ranged from 29 to 43.7% of the loci. The third group II cluster contained a single isolate, VPI 5040. The fourth cluster contained isolates VPI 9240, VPI 4487R, and VPI 4487S, which exhibited fixed differences ranging from 24 to 42% of the loci. Isolates NCTC 12276^T and 10976 belonged to the fifth cluster of group II and differed from each other at 50% of the loci.

Genetic group III. A single isolate, SG2, defined the third genetic group. This organism differed from all of the other isolates tested at an average of 90% of the 21 enzyme loci studied.

DISCUSSION

For the 44 *F. nucleatum* isolates examined in the present study, fixed differences were detected at 0 to 90% of the 21 previously established enzyme loci examined (17). Despite the fact that isolate SG2 was biologically classified as a member of the species *F. nucleatum* by independent investigators (Institute of Medical and Veterinary Science, Adelaide, South Australia, Australia), who used standard diagnostic techniques, it formed a unique genetic group. The remaining 43 isolates of *F. nucleatum* were divided into two major groups which differed from each other at an average of 82.5% of the 21 enzyme loci examined. This value is equivalent to or higher than the genetic distance measures that define some other prokaryotic and bacterial species.

Genetic group I. Isolate SV2 exhibited a high degree of genetic variation compared to the other isolates in group I. This isolate may represent the extent of genetic heterogeneity in this group, or it may be the only representative of an extensive genetic cluster that could be defined with additional clinical isolates.

Four isolates derived from cat oral cavities were members of group I, but there was an average fixed difference of 54.3% between isolate 3322, which was obtained from a periodontally diseased site, and the other three isolates. The remaining cat-derived isolates formed a genetically distinct cat-specific cluster. These results imply that isolate 3352, which clustered with two isolates obtained from healthy cat oral cavities, may have been isolated from an opportunistic infection, while isolate 3322, which was genetically distinct, may represent a pathogenic, cat-specific genetic cluster. Alternatively, isolate 3322 may have been obtained from an infection due to human contamination, which would explain why it was genetically more closely related to a number of human oral isolates than to the three other cat oral isolates. M 3832 is an isolate that was obtained from a dog bite wound abscess. This isolate clustered with a number of human oral isolates, which implies that the infecting organism may have been transmitted from the dog's owner to the dog. Alternatively, *F. nucleatum* isolates derived from dogs may be more closely genetically related to isolates found in the oral cavities of human than to isolates found in the oral cavities of cats. Isolate NCTC 12276 has been designated the type strain of *F. nucleatum* subsp. *animalis* and therefore was used as a representative of all of the isolates derived from animals. However, the results of the present study indicate that this isolate differed from the other animal isolates at an average of 82.5% of the loci; therefore, its use as an animal type strain is inappropriate. NCTC 12276^T could perhaps be used as a type strain for the animal extraoral isolates in group II, but an alternative animal oral type strain must be chosen for group I.

TABLE 2. Allelic profiles at 21 enzyme loci for 19 *F. nucleatum* isolates previously classified as members of subspecies and 25 *F. nucleatum* isolates whose subspecies status has not been determined

Isolate	Subspecies	Enzyme loci ^a																				
		ACP	AK	AP	DIA	ENOL	GDH	GOT	GPI	GPT	HK	LDH	MDH	NDPK	NP	OGR	Pep-B	PGAM	PGM	PK	TPI	UMPK
ATCC 25586 ^T	<i>F. nucleatum</i> subsp. <i>nucleatum</i>	d	c	b		b	b	d	b	e	a	b	C	c	g	g	b	f	a	a	h	b
Fev-1	<i>F. nucleatum</i> subsp. <i>nucleatum</i>	b	g	c	d	c	c	g	c	c	c	d	C	d	b	d	b	d	c	c	h	b
VPI 5040	<i>F. nucleatum</i> subsp. <i>nucleatum</i>	a	a		c	d	d	a	b	c		b		a	c	b		c	b		d	a
VPI 9240	<i>F. nucleatum</i> subsp. <i>nucleatum</i>	e	b			c	b		b	a	b	c	B	b	c	a	d	b	b		b	
ATCC 10953 ^T	<i>F. nucleatum</i> subsp. <i>polymorphum</i>	b	g	c	c	a	c		c	c	c		A	e	b	d	b	f	b	c	h	b
D206A4	<i>F. nucleatum</i> subsp. <i>polymorphum</i>	b	g	b	c	d	c	f	f	c	d	d	A	e	a	d	b	e	b	c	h	b
F1	<i>F. nucleatum</i> subsp. <i>polymorphum</i>	b	e	b	c	b	c	h	e	c	d	c	A	d	b	d	c	f	d	c	h	b
F3	<i>F. nucleatum</i> subsp. <i>polymorphum</i>	b	e	b	c	b	c	h	e	c	d	c	A	d	b	d	c	f	d		h	b
VPI 8818	<i>F. nucleatum</i> subsp. <i>polymorphum</i>	b	f	d	e	d	c	e	c	c	d	c	A	d	b	d	c	f	c	c	l	b
NCTC 11326 ^T	<i>F. nucleatum</i> subsp. <i>fusiforme</i>	b	g	c	e	d	b	f	b	c	c	d	A	d	b	d	a	e	b	d	h	b
ATCC 49256 ^T	<i>F. nucleatum</i> subsp. <i>vincentii</i>	b	g	b	b	b	c	g	d	d	c	d	C	d	b	d	b	e	c	c	g	
D213B3	<i>F. nucleatum</i> subsp. <i>vincentii</i>	b	f	b	c	d	b	f	b	d	c	a	C	d	b	d	a	e	b	c	g	b
D218B17	<i>F. nucleatum</i> subsp. <i>vincentii</i>	b	f	b	b		b	d	b	c	c	a	A	d	b	d	a	b	b	c	g	
VPI 4487R	<i>F. nucleatum</i> subsp. <i>vincentii</i>	f	b	b		d	b	b	b	a	d	c	B	b	b	a	a	b	a		a	
VPI 4487S	<i>F. nucleatum</i> subsp. <i>vincentii</i>	f	b	b		c	c	l	b		d	c	B	b	b	a	a	b	b		a	
VPI 6086	<i>F. nucleatum</i> subsp. <i>vincentii</i>	a	a		c	c	e		b	b	c	d	B	a		b		b	a	a	d	a
VPI 6616D	<i>F. nucleatum</i> subsp. <i>vincentii</i>	a	b		c	d	a	b	b	b	c	b	B	b	b	b		c	b	a	c	a
VPI 8754A	<i>F. nucleatum</i> subsp. <i>vincentii</i>		c	b	b	c	b			d		d		b		e	b	e	c		f	b
NCTC 12276 ^T	<i>F. nucleatum</i> subsp. <i>animalis</i>	b	a	c		b	f	h	c		c	c	C	a		b	d	a	b		b	
M 7731	ND ^b	b	g	b	b	c	b	d	b	c	c	d	A	d	b	d	a	d	a	c	g	b
M 4322	ND	b	g	b	c	d	b	g	f	c	d	c		d	b	d	a	e	c	c	g	b
M 3832	ND	b	g	b	b	d	b	f	c	b	d	b	C	d	b	d	a	f	c	c	g	b
M 2109	ND	a	g	b	e	b	d	g	c	d	d		C	f	c	d	b	e	c	c	l	
SG2	ND		l		c	e	f	k	g		c				f		e	b	f		k	a
SV2	ND	a	h		c	d	g	j	h	e	d	a	C		e		b	f	f		j	b
G34	ND		b	c		d	b		c	b	a	a	B	b	d	b	a	c	b	a	d	a
I34	ND		b	c		d	b	c	a	b	a	a		b	d	b	a	f	b	a	d	a
I37	ND		b	c		c	b	c	a	b	a			b	d	b		f	b		d	a
10976	ND	f	c	b		b	b	h	c	a	d	c	C	c	d	a	d	a	b		a	
10625	ND	c	d	b	c	d	d	g	d	c	b		A	e	b	d	c	e	b	c	l	b
AIP486-87	ND	a	b		c	d	b	b	b	b	a	d	B	b	d	b		b	a	a	e	a
10772	ND	b	g	b	d	b	d	g	d	c	d	b		f	b	c	b	d	c	c	d	b
10554	ND	c	e		c	b	e		c	d	d	a	C	d	b	d	b	e	c	c	d	a
10735	ND	a	b		c	d	e		d	b	a	e	B		e	b	a	d	a	a	e	a
10213	ND	d	c		e	b	b		c	e	a		C	c		f	a	e	b	a	h	b
10446	ND	b	g	b	d	c	c		e	c	d	b	B		b	d	b	c	c	c	d	b
3322	ND	b	e	b	a	e	c	g	d	b	e	a	C	d	b	d	b	d	c	c	h	b
3352	ND	c	f		c	d	f		d	c	e	a	C	d	b	d	c	c	d		h	a
3943	ND	c	e	a		e	f	g	d	c	e	a	A	d	b	d	c	c	e		h	a
3949	ND	c	f			c	e		d	c	b		A	d	b	d	c	f		c	h	
KE255	ND	b	b	c		c	b	b	c	b	c	b		b	d	b	a	c	b		h	
KE393	ND	b	b	c		c	b	b	c	c	a	b		b	d	b	a	c	b		h	a
OMZ439	ND	d	c	b	e	d	c	i	b	d	b	c	A		h		c	c	a	c	g	b
F6	ND	b	g	b	c	d	c	f	b	c	d	c	A	e	b	d	c	g	b	b	h	b

^a Abbreviations: ACP, acid phosphatase; AK, adenylate kinase; AP, alkaline phosphatase; DIA, diaphorase; ENOL, enolase; GDH, glutamate dehydrogenase; GOT, aspartate aminotransferase (synonym, glutamate oxaloacetate transaminase); GPI, glucose-phosphate isomerase; GPT, alanine aminotransferase (synonym, glutamate pyruvate transaminase); HK, hexokinase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; NDPK, nucleoside diphosphate kinase; NP, purine nucleoside phosphorylase; OGR, 2-oxoglutarate reductase; Pep-B, peptidase leucine-glycine-glycine; PGAM, phosphoglycerate mutase; PGM, phosphoglucomutase; PK, pyruvate kinase; TPI, triose-phosphate isomerase; UMPK, uridine diphosphate kinase. Where resolution was not adequate for allozymic interpretation, banding patterns were scored phenotypically in order of increasing anodal migration and are designated as such in uppercase letters.

^b ND, not determined.

FIG. 1. Phenogram depicting the genetic relationships among 44 isolates of *F. nucleatum* based on an allozyme electrophoresis analysis in which 21 enzyme loci were used.

According to our results, isolates Fev-1, ATCC 10953^T, ATCC 49256^T, 10446, and 10772 all belong to a single genetic cluster. However, according to previous classifications (4–6), the first three of these isolates belong to *F. nucleatum* subsp. *nucleatum*, *F. nucleatum* subsp. *polymorphum*, and *F. nucleatum* subsp. *vincentii*, respectively, and strains ATCC 10953 and ATCC 49256 are the type strains of their respective subspecies.

The other isolates previously identified as members of these subspecies (Table 1) that are not located in this cluster appear to have been incorrectly identified; i.e., the type strains of these subspecies were genetically more closely related to each other than to the isolates that have been classified as members of the subspecies. The published relationships among *F. nucleatum* subsp. *nucleatum*, *F. nucleatum* subsp. *polymorphum*, and *F.*

nucleatum subsp. *vincentii* are confusing. For example, Gharbia and Shah (5, 6) were not able to detect *F. nucleatum* subsp. *vincentii*, as identified by Dzink (4), and claimed that this taxon was identical to their *F. nucleatum* subsp. *fusiforme*. Our results indicate that *F. nucleatum* subsp. *polymorphum* and *F. nucleatum* subsp. *vincentii* are closely related genetically since the type strains of these subspecies were members of a single cluster.

A number of genetic clusters in group I consisted of isolates obtained from a range of sites of infection, suggesting that the isolates are opportunistic colonizers. However, genetic clusters which are diagnostically relevant and which consisted of a number of related isolates were identified. For example, isolates NCTC 11326^T, D213B3, and D218B17 were all obtained from healthy gingival sulci, indicating that their cluster may be a nonpathogenic cluster.

Isolates F1 and F3 have previously been identified as genetically homogeneous clones on the basis of their identical ribosomal DNA restriction patterns (3) and homologous restriction enzyme analysis results obtained by using probes for an outer membrane protein gene and a random segment of DNA (2). Our results support this genetic homology; indeed, these isolates were the only two isolates identical at all of the enzyme loci. This finding illustrates the accuracy and utility of allozyme electrophoresis in epidemiological and taxonomic investigations. It is interesting that although this cluster contained isolates belonging to *F. nucleatum* subsp. *polymorphum*, the type strain of this subspecies, ATCC 10953, was located in a different genetic cluster.

Genetic group II. Group II contained five clusters of isolates and a single type strain, NCTC 12276 (*F. nucleatum* subsp. *animalis*; derived from the colon of a Macaque monkey). Isolates G34, I34, and I37 were obtained from three preterm labor patients. These isolates belonged to a single genetic cluster and exhibited minimal heterogeneity, indicating that they are closely related and possibly derived from a common infecting source. Isolate KE255, from a brain abscess, and isolate KE393, from a vaginal abscess, were both obtained from the same hospital and were in the same genetic cluster as isolates G34, I34, and I37. The four vaginal isolates could be distinguished from isolate KE255 on the basis of hexokinase enzyme mobility. The sex and age of the patient from whom isolate KE255 was obtained are unknown, but if this isolate was from a woman, not only would this genetic subdivision be a vaginal isolate-specific cluster, but it would be a female-specific *F. nucleatum* cluster.

One genetic cluster in group II consisted exclusively of isolates of gastrointestinal origin. The only gastrointestinal isolate not located in this cluster, VPI 5040 from a rectal abscess, was in a unique cluster whose average fixed difference with the previous cluster was 59%. Perhaps the isolates obtained from gastrointestinal tract infections form a genetically related cluster but a cluster with higher-than-expected heterogeneity.

Isolates VPI 4487R and VPI 4487S are colony variants of a single clone which, interestingly, exhibited fixed genetic differences at 24% of the 21 loci examined, indicating that these organisms were not genetically identical.

Within group II, it appears that there are two types of clusters, namely, those that probably originated in the gastrointestinal tract and those that are female specific and originated in the vaginal tract. This observation implies that there are groups of isolates within the gastrointestinal tract that are genetically distinct from strains isolated from the vaginal tract, and these extraoral isolates form a distinct genetic group when they are compared to isolates of oral origin. Confirmation of the taxonomic relevance of the clusters identified in group II

would require further information about the patients from whom the isolates were obtained and simultaneous testing of *F. nucleatum* isolates in individual patients from the site of infection, the oral cavity, the gastrointestinal tract, and, in the case of female patients, the genital tract.

Genetic group III. Genetic group III contained a single isolate, SG2, which exhibited fixed differences with the 43 other isolates tested at 90% of the enzyme loci examined. A possible explanation for this, given the inaccuracy of previously used *F. nucleatum* typing methods, is that this isolate was incorrectly assigned to this species. Alternatively, this isolate may represent the extremity of the genetic heterogeneity of the species.

Comparison with other biological methods of identification.

The results of allozyme electrophoresis do not reflect the results obtained previously by methods such as fatty acid analysis, biochemical profile analysis, and analysis of the molar proportions of guanine and cytosine in the DNAs of *F. nucleatum* isolates (Fig. 2). For this reason, isolates SG2, SV2, ATCC 25586 (the type strain of *F. nucleatum* and *F. nucleatum* subsp. *nucleatum*), and NCTC 11326 (the type strain of *F. nucleatum* subsp. *vincentii*), which represent the extent of diversity within the 44 *F. nucleatum* isolates used in the present study, were sent to an independent diagnostic laboratory for reidentification. On the basis of G+C content and lipase activity, these strains were identified as members of a single species in the genus *Fusobacterium*. However, the results of a series of carbohydrate utilization tests identified isolate SG2 as the only "accurately identified" *F. nucleatum* isolate and isolate SV2 as a member of the closely related species *Fusobacterium necrophorum*. Clearly, this cannot be the case, as outlined in the present study (Fig. 1), where isolate SG2 is in group III, which suggests that alternative and reliable methods are required to genetically characterize the genus *Fusobacterium* and its species and subspecies. Our current and previous results (17) demonstrate that the use of allozyme electrophoresis for 21 enzyme loci provides a reliable method for genetic identification, characterization, and taxonomic classification of isolates of *F. nucleatum*.

Taxonomic implications of the present study. It is important to note that the separation of *F. nucleatum* isolates into distinct genetic groups in this study was not influenced by whether fixed differences or Nei's *D* values were used as measures of genetic distance. The observed levels of genetic variation were equivalent to or higher than the levels detected in previous studies in which prokaryotic and bacterial species were defined (20–22). This study was not designed to provide a nomenclature or a taxonomic revision of the genus *Fusobacterium*. Such studies would require the use of additional type strains representing other fusobacterial species and closely related genera and a comparison of our results with results obtained by using other typing techniques with the same set of isolates. Nevertheless, the present study provides a sound genetic framework which can be used as the basis for an extensive study in which a range of taxonomically relevant techniques are used.

Our results highlight the problems associated with the use of inaccurately identified and characterized type strains, not only for taxonomic studies, but also for the subsequent identification, classification, and characterization of clinical isolates. An extensive epidemiological study must now be undertaken to unequivocally redefine the taxonomic divisions in the genus *Fusobacterium*. Type strains of other fusobacterial species and closely related genera must be included in such a study. Appropriate type strains can then be assigned to represent the taxonomic divisions. It is evident that the currently assigned type strains do not represent any of the distinct genetic clusters

FIG. 2. Phenogram highlighting the genetic relationships among 13 isolates of *F. nucleatum* previously placed in subspecies (thick grey lines) and their relationships to the type strains of five identified subspecies (thick black lines), based on an allozyme electrophoresis analysis in which 21 enzyme loci were used. Abbreviations: (N), *F. nucleatum* subsp. *nucleatum*; (P), *F. nucleatum* subsp. *polymorphum*; (V), *F. nucleatum* subsp. *vincentii*; (F), *F. nucleatum* subsp. *fusiforme*; (A), *F. nucleatum* subsp. *animalis*.

of isolates identified in this study and that the currently identified species *F. nucleatum* consists of at least three distinct species (i.e., genetic groups I, II, and III).

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