Identification of *Fusobacterium* species by the electrophoretic migration of glutamate dehydrogenase and 2-oxoglutarate reductase in relation to their DNA base composition and peptidoglycan dibasic amino acids

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Summary. Rapid identification of *Fusobacterium* spp. is hampered by their inability to ferment carbohydrates and the availability of relatively few useful phenotypic characters. In an attempt to identify new diagnostic markers for species, we reported recently the potential utility of glutamate dehydrogenase (GDH) electrophoretic mobilities for distinguishing eight species of *Fusobacterium*. We have extended these observations to include all recognised members of the genus except *F. prausnitzii* and *F. perfoetens*, and our results show that they cluster into three broad electrophoretic groups. Some species, such as *F. periodonticum*, *F. simiae* and *F. necrophorum*, possessed GDH with similar electrophoretic mobilities. However, within such clusters, the electrophoretic migration of 2-oxoglutarate reductase (OGR) distinguished between species. Neither GDH or OGR mobility alone clearly differentiated all species, but their combined use provided unambiguous discrimination of all species except *F. varium* and *F. mortiferum*. The DNA base compositions of all species except *F. naviforme* (ATCC 25832) and *F. sulci*, were within the range 26–34 mol% G + C, suggesting the genus may be homogeneous. However, the peptidoglycan composition divided the genus into two major groups that contained either lanthionine or diaminopimelic acid; *F. mortiferum* peptidoglycan contained both dibasic amino acids.

Introduction

Fusobacterium spp. are now frequently reported in clinical specimens and appear to form a significant component of the bacterial flora in mixed anaerobe infections.¹ In man, examples of such infections include necrotic tonsillitis, oral and dental infections, brain abscesses, empyema, hepatic and intra-abdominal abscesses and endocarditis.¹ Infections in animals vary widely and include liver abscesses in cattle² and foot rot in sheep.² However, the clinical importance of these organisms is perhaps still not fully appreciated as their identification is largely overlooked in most diagnostic laboratories.

Traditional methods of identification, which rely largely on carbohydrate fermentation reactions, are of limited value because *Fusobacterium* spp. are generally non-fermentative. Consequently several alternative approaches have been used. At the genus level, all species are characterised by the production of butyric and acetic acids as major end products of metabolism^{1,2} and their cellular fatty acids comprise predominantly straight-chain and mono-unsaturated fatty acids.³ Polypeptide analysis by sodium dodecylsulphate-polyacrylamide gel electrophoresis has been investigated extensively; however, the electrophoretic patterns derived were too complex and inconsistent within a species for routine identification.⁴ Pyrolysis-mass spectrometry of whole cells of *Fusobacterium* has recently been employed⁵ but, with few exceptions, isolates of most species were assigned in several clusters of the dendrogram.

The electrophoretic mobility of glutamate dehydrogenase (GDH) has been reported in eight species of *Fusobacterium*.⁶ However, application of this method to study other species, such as *F. alocis*, *F. periodonticum*, *F. simiae*, *F. sulci* and *F. ulcerans*, resulted in similar electrophoretic migration of enzymes from several species. Here we report the electrophoretic mobility of another enzyme, 2-oxoglutarate reductase (OGR) and show that the combined use of these enzymes can be applied to identify most species. Furthermore, we examined the possible correlation of these data with the heterogeneity in peptidoglycan composition and we also report the guanine plus cytosine (mol% G + C) content of the DNA of all strains used in this study.

Materials and methods

Bacterial strains

The *Fusobacterium* strains used in this study and their sources are given in table I.

Growth conditions and preparation of cell-free extracts

All strains were maintained by weekly subculture on Blood Agar Base (Oxoid) containing sheep blood (Oxoid) 5% in an atmosphere of CO₂ 10%, H₂ 10%, N₂ 80% at 37°C. Cell-free extracts were prepared as described previously.⁷ Briefly, cells were harvested from 24-h blood-agar cultures and were suspended in 0.5 ml of 100 mM

Table I. Sources of *Fusobacterium* strains

Strain designation	Source	Strain designation	Source
<i>F. nucleatum</i> ATCC 25586 ^T	ATCC	<i>F. periodonticum</i> ATCC 33693 ^T	ATCC
<i>F. alocis</i> ATCC 35896 ^T	ATCC	LH-61	LHMC
VPB CA-49-4	D. Love	LH-62	LHMC
VPB CA-45-7	D. Love	LH-61	
VPB CA-76-3	D. Love	LH-62	
<i>F. gonidiaformans</i> ATCC 25563 ^T	ATCC	<i>F. russii</i> ATCC 25533 ^T	ATCC
VPI 4879	T. Hofstad	VPI 0307	G. Sundqvist
VPI 0482 A	B.I. Duerden	VPB 3327	D. Love
<i>F. mortiferum</i> ATCC 25557 ^T	ATCC	VPB 3393	D. Love
VPI 4249	B.I. Duerden	VPB 3310	D. Love
E1	B.I. Duerden	VPB 3356	D. Love
VPI 4234	B.I. Duerden	<i>F. simiae</i> ATCC 33568 ^T	ATCC
VPI 0473	B.I. Duerden	LH-45	LHMC
XI-88	T. Mitsuoka	<i>F. sulci</i> ATCC 35585 ^T	ATCC
<i>F. naviforme</i> NCTC 25832 ^T	NCTC	LH-45	LHMC
SG-1	LHMC	<i>F. ulcerans</i> NCTC 12111 ^T	B. Adrians
4890-34	S.M. Finegold	NCTC 12112	B. Adrians
<i>F. necrogenes</i> ATCC 25556 ^T	B.I. Duerden	<i>F. varium</i> NCTC 10560 ^T	T. Hofstad
VPI 2368	B.I. Duerden	VPI 0499A	T. Hofstad
VII-29	T. Mitsuoka	VPI 4234	T. Hofstad
XI-90	T. Mitsuoka	6090-26	S.M. Finegold
<i>F. necrophorum</i> ATCC 25286 ^T	ATCC	6297-27	S.M. Finegold
6308	S.M. Finegold	5011-41	S.M. Finegold
85-56B	S.M. Finegold	6668-42	S.M. Finegold
VII-50	T. Mitsuoka		
NCTC 10575	G. Sundqvist		
NCTC 10577	B.I. Duerden		

ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; LHMC, The London Hospital Medical College; T, type strain.

tris(hydroxymethyl)aminomethane HCl 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 supernates were used immediately for electrophoresis.

Electrophoresis and staining of the enzymes

Cell-free extracts were subjected to electrophoresis for 90 min at 15 V/cm on 5.7 × 14-cm Cellogel support strips with 4 mM barbitone-acetic acid buffer (pH 8.6). GDH was visualised by staining with a solution of (/L) glutamate 20 g, nicotinamide adenine dinucleotide 5 g, phenazine methosulphate 4 g, and thiazolyl blue tetrazolium 0.02 g in 100 mM Tris(hydroxymethyl)aminomethane-HCl buffer (pH 9.0). 2-Oxoglutarate reductase was visualised by replacing the glutamate with a solution of 2-hydroxyglutarate 10 g/L.

Isolation, purification and determination of base composition of DNA

DNA was purified by the method of Marmur.⁸ The DNA base composition was estimated from its melting temperature in SSC with a Gilford (model 240) spectrophotometer and a Gilford 2527 thermal programmer as described previously.⁷

Preparation and examination of cell walls

Cell walls were prepared and hydrolysed by the method of Schleifer and Kandler.⁹ The dibasic amino acids of the mucopeptides were analysed qualitatively by descending chromatography on Whatman No. 1 paper with methanol:pyridine:concentrated HCl:water (80:20:2.5:17.5) as running solvent. Amino acids were identified by reference to authentic standards and by their distinctive colours when treated with ninhydrin 0.25% in acetone containing glacial acetic acid 7%.⁷

Results

Representative strains of 13 species of *Fusobacterium* all possessed high levels of GDH and OGR such that only 1 µl of a cell-free extract (*c.* 10⁶–10⁸ cells/ml) was sufficient to detect both enzymes reliably after electrophoresis. Both enzymes required NAD for substrate oxidation but their pH range for activity differed. GDH was stained over a wide pH range (*c.* 7.5–10.5), whereas OGR was visualised within a narrower pH range (*c.* 8.5–9.0). *F. nucleatum*, *F. russii*, *F. gonidiaformans* and *F. ulcerans* had characteristic GDH electrophoretic mobilities, but enzyme analysis clustered the remaining species into three broad groups. *F. periodonticum*, *F. simiae* and *F. necrophorum* had

similar GDH mobilities of 3.0–3.6 cm; the GDH extracted for *F. varium*, *F. mortiferum*, *F. alocis* and the type strain of *F. naviforme* co-electrophoresed at 3.9 cm; and the GDH mobility of *F. alocis* (2.4 cm) was similar but not identical to that of *F. necrogenes* (2.6 cm) (fig. 1). Within these clusters, the electrophoretic mobility of OGR provided a further means of distinguishing species, e.g., *F. alocis* and *F. necrogenes*, which had similar GDH mobilities, were distinguished readily by the migration distances of OGR.

The type strain of *F. naviforme* had electrophoretic mobilities of 4.1 cm for GDH and 5.2 cm for OGR, but the three clinical isolates examined had mobilities of 2 and 2.6 cm for GDH and OGR respectively. Furthermore, the dibasic amino acid of the peptidoglycan of the type strain contained diaminopimelic acid, whereas that of the three clinical strains had lanthionine (fig. 1; table II). Of the species tested, only *F. naviforme* showed heterogeneity in peptidoglycan composition (table II).

The DNA base composition of all species was within the range 25–34 mol% G + C, except for *F. sulci* (39%) and the type strain of *F. naviforme*, ATCC 25832 (49%) (table II). Clinical isolates which were phenotypically very similar to *F. naviforme* had DNA base compositions of 32–33 mol% G + C (table II). The peptidoglycan composition divided the genus into two distinctive groups, those species that contained diaminopimelic acid (*F. naviforme*, *F. sulci*, *F. ulcerans* and *F. varium*) and that also possessed GDH with mobilities of 4–7 cm, and species that contained lanthionine and had GDH mobilities < 4 cm. *F. mortiferum* was atypical in possessing both dibasic amino acids; it clustered with the high-GDH-mobility group. Fig. 1 gives an overview of these inter-relationships.

Discussion

Previously, we reported the importance of glutamate as an energy source for *Fusobacterium* spp. and showed its rapid uptake from a chemically defined medium.¹⁰ The presence of both GDH and OGR, catabolic enzymes of glutamate metabolism, in *Fusobacterium* spp. further supports these physiological findings. The broad pH range of activity for GDH (*c.* 7–10) enables this enzyme to be stained readily in most electrophoretic systems. However, similarity in migration distances of GDH of some species such as *F. alocis* (2.4 cm) and *F. necrogenes* (2.6 cm) limits its use in identifying species. Enzymes which migrate closely can be mixed and electrophoresed together to distinguish between

Fig. 1. Electrophoretic mobilities of GDH and OGR in reference and clinical strains of *Fusobacterium* (○), clinical strains of *F. naviforme* (◐) and the type strain of *F. naviforme* (●), and the distribution of peptidoglycan dibasic amino acids among *Fusobacterium* spp. Species with higher GDH mobilities had diaminopimelic acid whereas those with slower migrating enzymes had peptidoglycans containing meso-lanthionine.

them, but ideally, the application of another enzyme mobility which differs significantly between the species should be employed. In a previous study, we screened for several enzymes involved in nitrogen metabolism of *Fusobacterium* spp. The first enzyme which was present in all species and reliably corroborated the GDH patterns was OGR. Thus, the three GDH electrophoretic patterns of *F. nucleatum*⁶ were recently confirmed by OGR patterns in more than 30 human isolates.¹¹

The GDH electrophoretic mobility of eight species of *Fusobacterium* has been reported.⁶ This study extends these findings to five further species—*F. alocis*, *F. periodonticum*, *F. sulci*, *F. ulcerans* and *F. simiae*—and emphasises the importance of GDH as a diagnostic marker at both the generic and species level. Although its potential value for delineating all *Fusobacterium* spp. has been restricted by common electrophoretic mobilities in some species (fig. 1), a combination of the electrophoretic migration of GDH and OGR provided

diagnostic patterns that can be used to distinguish all *Fusobacterium* spp. except *F. varium* and *F. mortiferum*.

In an earlier study, Love *et al.*¹² reported that although clinical isolates of *F. naviforme* were phenotypically similar to the type strain, there was negligible DNA-DNA homology between the type strain and clinical isolates. In this study, we have shown that their dibasic amino-acid composition and enzyme electrophoretic profiles also show a high degree of discordance (fig. 1). Thus, clinical isolates that resemble phenotypically the type strain of *F. naviforme* (a rodent isolate) differ markedly from the type strain and warrant placement in new species. The name *F. asaccharolyticum* is tentatively proposed.

The genus *Fusobacterium* has been shown to be heterogeneous in peptidoglycan composition.¹³⁻¹⁵ Species such as *F. nucleatum* and *F. necrophorum* possess a directly cross-linked peptidoglycan, based upon the sulphur-containing dibasic amino acid

Table II. DNA base compositions and dibasic amino acids of the peptidoglycan of reference and clinical strains of *Fusobacterium* species

Species	Mol% G + C	Dibasic amino acid
<i>F. nucleatum</i> ATCC 25586	26	meso-lanthionine
<i>F. alocis</i> ATCC 35896	34	"
3 clinical isolates	32-34	"
<i>F. gonidiaformans</i> ATCC 25563	33	"
3 reference strains	31-33	"
<i>F. necrogenes</i> ATCC 25556	28	"
3 clinical strains	27-28.7	"
<i>F. necrophorum</i> ATCC 25286	32	"
5 clinical isolates	32-35	"
<i>F. periodonticum</i> ATCC 33693	28	"
2 clinical isolates	27-28.1	"
<i>F. russii</i> ATCC 25533	26	"
5 clinical isolates	24-26	"
<i>F. simiae</i> ATCC 33568	28	"
1 clinical isolate	28	"
<i>F. sulci</i> ATCC 35585	39	Diaminopimelic acid
1 clinical isolate	40	"
<i>F. ulcerans</i> NCTC 12111	25	"
1 reference strain	25.4	"
<i>F. varium</i> NCTC 10560	26	"
6 clinical strains	25-26	"
<i>F. naviforme</i> ATCC 25832	49	"
2 clinical strains	32-33	meso-lanthionine
<i>F. mortiferum</i> ATCC 25557	25-26.5	meso-lanthionine and diaminopimelic acid
6 clinical isolates		

Details of strains listed as clinical or reference strains are given in table I.

lanthionine.¹⁵ Fig. 2 shows the nature of the linkage. The mucopeptide of several other species, such as *F. varium*, contains diaminopimelic acid as its sole dibasic amino acid.¹³ Interestingly, we observed in the present study that species which belonged to the slower GDH-OGR mobility cluster possessed lanthionine whereas those with faster enzyme mobilities had a peptidoglycan based on diaminopimelic acid (fig. 1). *F. mortiferum*, which

contained both dibasic amino acids, showed more similarity with *F. varium* in electrophoretic patterns. This similarity between the latter species has also been confirmed by 5S-rRNA sequence data¹⁶ and is presently being compared by 16S-rRNA sequence analysis.

The results of the present study indicate that the combined use of GDH and OGR electrophoretic patterns provide a useful and rapid method for identifying most species of *Fusobacterium*. Furthermore, both enzymes appear to be useful for preliminary investigations of the natural diversity of species before undertaking such studies as DNA-DNA hybridisation. With this method, three subgroups of *F. nucleatum* designated Fn-1, Fn-2 and Fn-3 among human oral isolates were resolved and this segregation has been supported by rRNA restriction patterns.¹⁷ Differences between these groups and animal isolates of *F. nucleatum* led to the recent proposal of four subspecies.¹⁸ Recognition of these subspecies has facilitated clearer

Fig. 2. Nature of the cross-linkage of the meso-lanthionine-containing peptidoglycan of some *Fusobacterium* spp.

discrimination among isolates of *F. nucleatum* and highlighted their possible ecological distribution. Thus, *F. nucleatum* subsp. *nucleatum* was shown

recently to be more commonly associated with diseased sites, whereas the remaining subspecies were associated with healthy sites in the mouth.¹⁹

REFERENCES

1. Finegold SM, George WL, Mulligan ME. Anaerobic infections part 1. *Dis Mon XXXI* 1985; **31**:1-77.
2. Langworth BF. *Fusobacterium necrophorum*: its characteristics and role as an animal pathogen. *Bacteriol Rev* 1977; **41**:373-390.
3. Jantzen E, Hofstad T. Fatty acids of *Fusobacterium* species: taxonomic implications. *J Gen Microbiol* 1981; **123**:163-171.
4. Calhoon DA, Mayberry WR, Slots J. Cellular fatty acid and soluble protein profiles of oral fusobacteria. *J Dent Res* 1983; **62**:1181-1185.
5. Magee JT, Hindmarch JM, Bennett KW, Duerden BI, Aries RE. A pyrolysis-mass spectrometry study of fusobacteria. *J Med Microbiol* 1989; **28**:227-236.
6. Gharbia SE, Shah HN. Characteristics of glutamate dehydrogenase, a new diagnostic marker for the genus *Fusobacterium*. *J Gen Microbiol* 1988; **134**:327-332.
7. Shah HN, Williams RAD, Bowden GH, Hardie JM. Comparison of the biochemical properties of *Bacteroides melaninogenicus* from human dental plaque and other sites. *J Appl Bacteriol* 1976; **41**:473-495.
8. Marmur J. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* 1961; **3**:208-218.
9. Schleifer KH, Kandler O. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Revs* 1972; **36**:407-477.
10. Gharbia SE, Shah HN. The uptake of amino acids from a chemically defined medium by *Fusobacterium* species. *Curr Microbiol* 1989; **18**:189-193.
11. Gharbia SE, Shah HN. Glutamate dehydrogenase and 2-oxoglutarate reductase electrophoretic patterns and deoxyribonucleic acid-deoxyribonucleic acid hybridization among human oral isolates of *Fusobacterium nucleatum*. *Int J Syst Bacteriol* 1989; **39**:467-470.
12. Love DN, Cato EP, Johnson JL, Jones RF, Bailey M. Deoxyribonucleic acid hybridization among strains of *Fusobacterium* isolated from soft tissue infections of cats: comparison with human and animal type strains from oral and other sites. *Int J Syst Bacteriol* 1987; **37**:23-26.
13. Kato K, Umemoto T, Fukuhara H, Sagawa H, Kotani S. Variation of dibasic amino acid in the cell wall peptidoglycan of bacteria of the genus *Fusobacterium*. *FEMS Microbiol Lett* 1981; **10**:81-85.
14. Miyagawa E, Azuma R, Suto T. Peptidoglycan composition of gram-negative obligately anaerobic rods. *J Gen Appl Microbiol* 1981; **27**:199-208.
15. Vasstrand EN, Jansen HB, Miron T, Hofstad T. Composition of peptidoglycans in Bacteroidaceae: determination and distribution of lanthionine. *Infect Immun* 1982; **36**:114-122.
16. Van Den Eynde H, De Baere R, Shah HN *et al.* 5S-ribosomal ribonucleic acid in *Bacteroides* and *Fusobacterium*: evolutionary relationships within these genera and among eubacteria in general. *Int J Syst Bacteriol* 1989; **39**:78-84.
17. Lawson PA, Gharbia SE, Shah HN, Clark DR. Recognition of *Fusobacterium nucleatum* subgroups Fn-1, Fn-2 and Fn-3 by ribosomal RNA gene restriction patterns. *FEMS Microbiol Lett* 1989; **53**:41-46.
18. Gharbia SE, Shah HN. Heterogeneity within *Fusobacterium nucleatum*, proposal of four subspecies. *Lett Appl Microbiol* 1990; **10**:105-108.
19. Gharbia SE, Shah HN, Lawson PA, Haapasalo M. The distribution and frequency of *Fusobacterium nucleatum* subspecies in the human oral cavity. *Oral Microbiol Immunol* 1990; **5**, in press.