Characteristics of Glutamate Dehydrogenase, a New Diagnostic Marker for the Genus *Fusobacterium*

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Enzymes representative of carbohydrate and nitrogen metabolism were screened for their presence and activity amongst species of the genus *Fusobacterium*. Glutamate dehydrogenase (GDH) was reliably detected in all 25 strains studied. The pH profile of this enzyme and the DNA base composition of selected strains were also determined. DNA base composition of selected strains ranged between 28–32.9 mol% G + C. GDH was active between pH 7.5–11.5 but two pH profiles of activity, with optima at 9.5 and 10.5, were discernible among species. Apart from *Fusobacterium nucleatum*, which had a heterogeneous enzyme pattern, the GDH electrophoretic mobility was constant within a species but in a few cases the enzyme bands overlapped. A combination of the pH profile, the GDH electrophoretic pattern and the DNA base composition provided clear separation of the test organisms into discrete groups; however, a larger number of strains must be examined before the full potential of these tests can be evaluated.

INTRODUCTION

The genus *Fusobacterium*, as defined by Moore *et al.* (1984) in *Bergey's Manual of Systematic Bacteriology*, consists of species of Gram-negative, non-sporing, obligately anaerobic bacteria which characteristically are nonfermentative and produce butyric acid as a major end-product of metabolism. The asaccharolytic nature of these organisms has, however, contributed to the difficulties in routine identification and few tests were reported to be stable and specific for the differentiation of species (Bennett & Duerden, 1985). Recent studies (Love *et al.*, 1987) indicate that the genus *Fusobacterium* is heterogeneous and additional criteria are required to clarify the inter- and intra-generic relationships of these micro-organisms. Attention has turned increasingly in recent years to biochemical and chemical methods for the provision of characters of value in microbial classification (Goodfellow & Minnikin, 1985). Such chemotaxonomic characters for the Bacteroidaceae have led to considerable revision of the genus *Bacteroides* (see reviews by Shah & Collins, 1983; Collins & Shah, 1987). Similar studies on the genus *Fusobacterium* have, however, been very limited. Analysis of their long-chain fatty acids revealed the presence of only straight-chain saturated and mono-unsaturated acids (Jantzen & Hofstad, 1981). Thus, unlike the genus *Bacteroides*, methyl branched acids were not detected, but differences in fatty acid composition were reported among *Fusobacterium* species. Heterogeneity was also observed in peptidoglycan composition. For example, some species such as *F. nucleatum* and *F. necrophorum* possessed a directly cross-linked peptidoglycan, based upon the sulphur-containing dibasic amino acid lanthionine, designated AI δ (Kato *et al.*, 1981; Vasstrand *et al.*, 1979, 1982; Miyagawa *et al.*, 1981). Other species such as *F. varium* and *F. mortiferum* contained diaminopimelic acid as the dibasic amino acid (Miyagawa *et al.*, 1981), that is, the AI γ chemotype of Schleifer & Kandler (1972). Vasstrand *et al.* (1982) reported the

Abbreviation: GDH, glutamate dehydrogenase.

presence of both the above dibasic amino acids in *F. mortiferum*. The majority of species, however, possessed lanthionine as a distinct feature of their peptidoglycan (Vasstrand *et al.*, 1982; Gharbia & Shah, 1986).

In the present study, a wide variety of dehydrogenases – representative of carbohydrate metabolism such as glycolytic and pentose phosphate pathway enzymes and of nitrogen metabolism such as lysine, ornithine, phenylalanine and glutamate dehydrogenase – were screened for their enzyme activities among *Fusobacterium* species. The first enzyme to be reliably detected in all species was glutamate dehydrogenase (GDH) (Shah *et al.*, 1987*b*). This paper describes the electrophoretic mobility and other characteristics of GDH, and compares the electrophoretic patterns obtained with the DNA base composition of species so as to evaluate the use of the former as a potential taxonomic marker.

METHODS

Bacterial strains. The 26 strains of *Fusobacterium* used are listed in Table 1.

Cultivation. All strains were maintained by weekly subculture on 2.5% (v/v) blood agar plates in an atmosphere of 10% (v/v) CO₂ and 5% (v/v) H₂ in N₂ at 37 °C.

Preparation of cell free extracts. Cells were harvested from 3-d-old blood agar cultures and suspended in 0.5 ml 0.1 M-HEPES/NaOH buffer, pH 7.5. The 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 37000 *g* for 15 min; the supernatant, containing 20–30 mg protein ml⁻¹, was used for further studies.

Enzyme activity and pH optimum. The pH optimum for the oxidation of L-glutamate was measured for a representative strain of each species. All assays were done at 25 °C using a Beckman DU-7 or a Gilford model 240 spectrophotometer. Details of the procedure were described previously (Reeves *et al.*, 1971; Shah & Williams, 1982). The buffers used for different pH values were 0.1 M-HEPES, CHES or CAPS (BDH) (adjusted to the appropriate pH with NaOH).

Table 1. *Strains used and their sources*

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Strain no.	Source
<i>Fusobacterium russii</i> VPI 0307	G. Sundqvist
<i>Fusobacterium naviforme</i> SG-1 85-2511, 4890	B. I. Duerden S. M. Finegold
<i>Fusobacterium necrogenes</i> XI-90, VII-29 ATCC 25556*	T. Mitsuoka B. I. Duerden
<i>Fusobacterium varium</i> 5011, 6297 NCTC 10560, * VPI 0499A, VPI 4234	S. M. Finegold T. Hofstad
<i>Fusobacterium mortiferum</i> VPI 4249, VPI 0473, VPI 4123A, E1 XI-88	B. I. Duerden T. Mitsuoka
<i>Fusobacterium necrophorum</i> ATCC 25286* VII-50	B. I. Duerden T. Mitsuoka
<i>Fusobacterium gonidiaformans</i> VPI 4879 VPI 4381, VPI 11360	T. Hofstad B. I. Duerden
<i>Fusobacterium nucleatum</i> 85-28UV, 85-56B, 85-56A B-48	S. M. Finegold B. I. Duerden

* Type strain.

Electrophoresis and staining of enzymes. Cell-free extracts were electrophoresed on 5.7 × 14 cm Cellogel support strips (Reeve Angel Scientific), as described previously (Shah & Williams, 1982). Electrophoresis was done on an LKB Multiphor unit (model 2117) with a LKB Multitemp II thermostatic circulator (model 2219) at 8 °C. Electrophoresis was allowed to proceed for 1 h at 12 V per cm length of gel in 0.04 M-barbitone/acetic acid buffer, pH 8.6. The enzyme was visualized using a solution of glutamate (40 mg ml⁻¹), NAD (10 mg ml⁻¹), phenazine methosulphate (0.04 mg ml⁻¹) and thiazolyl blue tetrazolium (0.02 mg ml⁻¹) in 0.1 M-HEPES/NaOH buffer, pH 9.5.

Isolation, purification and base composition determination of DNA. Cells collected in mid-exponential phase were washed twice with TES buffer (0.05 M-Tris/HCl, 0.005 M-NaCl, 0.005 M-EDTA, pH 8.0) and resuspended in the same buffer containing 50 µg proteinase K (Merck) ml⁻¹ and 0.15 µl diethylpyrocarbonate (BDH) ml⁻¹. Cells were lysed at 37 °C and the DNA was purified by the method of Marmur (1961). The DNA base composition was estimated from its melting temperature in saline citrate (0.15 M-sodium chloride, 0.015 M-sodium citrate, pH 7.0) using a Beckman DU-7 spectrophotometer or a Gilford model 240 spectrophotometer and a Gilford 2527 thermal programmer as described previously (Shah *et al.*, 1976).

RESULTS AND DISCUSSION

Genetic variation can alter the net charge on an enzyme molecule and hence its electrophoretic mobility without altering its catalytic activity. The technique of zone electrophoresis followed by specific staining *in situ* to demonstrate the enzyme bands provides a very sensitive method for the detection of minor structural differences among enzymes (Shaw, 1965). This method has been applied to several genera of Gram-negative anaerobic bacteria (Shah & Williams, 1982) and its correlation with other chemotaxonomic methods, such as lipid analyses, DNA base composition and DNA-DNA hybridization, is good (Baptist *et al.*, 1969; Shah & Collins, 1983). However, identical electrophoretic mobilities can be obtained in spite of gross differences in amino acid sequences in some proteins. Thus, enzyme polymorphism is best studied in parallel with other techniques (Williams & Shah, 1980).

Many studies on enzyme polymorphism have examined esterases: these enzymes hydrolyse an ester bond with low substrate specificity. Dehydrogenases, however, exhibit a high substrate specificity. Thus GDH was selected from a range of other possible enzymes to evaluate its use as a possible diagnostic marker of the genus *Fusobacterium*.

In general, GDH was active between pH 7.5–11.5 but two profiles of activity were discernible among the species (Table 2). Representative pH profiles of both groups are shown in Fig. 1. Thus *F. naviforme*, *F. necrophorum*, *F. nucleatum* and *F. varium* possessed a GDH with a pH optimum near 9.5 whereas *F. gonidiaformans*, *F. mortiferum*, *F. necrogenes* and *F. russii* had maximum enzyme activity at pH 10.5. It is interesting to note that the most frequently reported clinical pathogens of the genus – *F. nucleatum*, *F. naviforme* and *F. necrophorum* – were all in the same pH optimum group (Table 2). The specific activities of GDH differed markedly amongst the species (Table 2). In general, species which possessed enzymes with more alkaline pH optima had higher specific activities (Table 2).

Table 2. GDH specific activity between pH 7.5 and 11.5 of selected strains of *Fusobacterium* species

tr., Enzyme activity < 0.5 µmol (mg protein)⁻¹ min⁻¹.

Species	Strain no.	pH...	GDH activity [µmol (mg protein) ⁻¹ min ⁻¹]				
			7.5	8.5	9.5	10.5	11.5
<i>F. gonidiaformans</i>	VPI 4381		0	0.48	65.9	194.5	138.26
<i>F. necrophorum</i>	VII-50		0.8	2.4	3.2	tr.	0
<i>F. mortiferum</i>	XI-88		11.6	57.87	89	120.86	98.9
<i>F. naviforme</i>	SG-1		4.8	10.45	12.86	0	0
<i>F. necrogenes</i>	ATCC 25556		0	0	6.43	104.5	23.3
<i>F. nucleatum</i>	85-28UV		24.11	32.15	86	4.8	0
<i>F. varium</i>	VPI 0499A		15.0	23	53.15	51	0
<i>F. russii</i>	VPI 0307		0	tr.	66	125.78	60.2

Fig. 1. pH profile of GDH of selected strains of *Fusobacterium*. ---, *F. necrogenes* ATCC 25556; —, *F. nucleatum* 85-28UV.

Fig. 2. GDH electrophoretic mobility of *Fusobacterium* species. The strain number of each species tested is given above the species name in the diagram.

With the exception of *F. nucleatum*, the electrophoretic bands obtained indicated that most species have a characteristic electrophoretic pattern (Fig. 2). Differences in DNA-DNA homology amongst strains of *F. nucleatum* indicated that more than one centre of variation exists within this species (Love *et al.*, 1987). The present GDH patterns as shown by the electrophoretic mobility (Fig. 2) further emphasized the heterogeneity of the species *F. nucleatum*. The only strain of *F. russii* used in the study reproducibly migrated very slowly with a diffuse band but was clearly different from other species (Fig. 2). *F. russii* is therefore particularly distinctive amongst the Bacteroidaceae (Shah & Williams, 1982; Collins & Shah, 1987; Shah *et al.*, 1987a) in being the only species with a near net zero charge at pH 8.6. Further strains must, however, be examined before the reliability of this pattern can be established.

The three strains of *F. gonidiaformans* tested also had diffuse bands that were characteristic of the species (Fig. 2). It was not clear whether these were multiple GDH bands which migrated closely to each other with the main band towards the centre of the staining area. It might be possible, in future studies, to resolve these bands by isoelectric focusing. Strains of *F. naviforme* possessed GDH bands which migrated closely to those of *F. gonidiaformans* but were readily identified by a clearly defined single stained band at 2.1 cm (Fig. 2).

The GDH electrophoretic patterns of *F. necrogenes* and *F. necrophorum* could not be used alone to distinguish between these species (Fig. 2). However, variation in the pH profiles of GDH from both species (Table 2) together with differences in their DNA base composition (Fig. 3, Table 3) permits unequivocal identification of both taxa. Strains of *F. mortiferum* and *F.*

Fig. 3. GDH mobility vs. mol% G + C range of various species of *Fusobacterium*. ⊙, Range of enzyme mobility for each species.

Table 3. DNA base composition of selected strains of *Fusobacterium* species

Species	Strain no.	Mol% G + C
<i>F. nucleatum</i>	ATCC 25586	28.0
<i>F. gonidiaformans</i>	VPI 4879	32.9
<i>F. mortiferum</i>	VPI 4123A	29.0
<i>F. naviforme</i>	SG-1	30.5
<i>F. necrogenes</i>	ATCC 25556	28.0
<i>F. necrophorum</i>	ATCC 25286	32.0
<i>F. russii</i>	VPI 0307	31.0
<i>F. varium</i>	NCTC 10560	29.0

varium had the fastest migrating enzymes of the species tested (Fig. 2). Both taxa were not separable by GDH electrophoretic mobility or DNA base composition but GDH from *F. mortiferum* had a more alkaline pH optimum (Table 2). *F. varium* is, however, atypical of the genus in possessing solely *meso*-diaminopimelic acid as its dibasic peptidoglycan amino acid (Vasstrand *et al.*, 1982). *F. mortiferum*, in common with other members of the genus, possesses lanthionine and, in addition, also contains *meso*-diaminopimelic acid as its peptidoglycan dibasic amino acid (Kato *et al.*, 1981; Vasstrand *et al.*, 1982).

At present, the genus *Fusobacterium* contain species whose DNA base composition ranges between 25–57 mol% G + C (Moore *et al.*, 1984). The mol% G + C of the selected strains used in this study are given in Table 3. The type species, *F. nucleatum*, possesses a DNA base composition of 28 mol% G + C, but different DNA homology subgroups were reported by Love *et al.* (1987). Since it is now generally accepted that a difference of more than 8–10 mol% G + C indicates that species are unrelated at the generic level, several species now grouped within the genus *Fusobacterium* (Moore *et al.*, 1984) are incorrectly classified. In order to resolve these anomalies and facilitate identification of taxa, more reliable biochemical and chemical tests are required. The enzymic characteristics of GDH may provide a useful marker in future studies on the genus *Fusobacterium*.

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