Glucose-6-Phosphate Dehydrogenase and Malate Dehydrogenase Enzyme Electrophoretic Patterns Amongst Strains of *Bacteroides fragilis*

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Fifty-two strains of *Bacteroides fragilis* were examined for their enzyme electrophoretic patterns of glucose-6-phosphate dehydrogenase (G6PDH) and malate dehydrogenase (MDH). All strains tested possessed high levels of both enzymes but the G6PDH reduced NADP whereas MDH was NAD-dependent. Twenty-seven strains produced single bands of both G6PDH and MDH. In all cases G6PDH migrated faster than MDH. Strains clustered by a single linkage algorithm were recovered in eight clusters at the 77% similarity level. The remaining 25 strains produced multiple bands of one or both enzymes. These were recovered in six clusters at the 72% similarity level using the same algorithm. The results of this study revealed considerable heterogeneity of enzyme patterns within *B. fragilis*.

INTRODUCTION

Bacteroides fragilis is the most common species of the '*B. fragilis* group' of species associated with infections in man. This species, however, represents only about 0.5% of the bowel microflora and is substantially outnumbered by *B. distasonis*, *B. vulgatus* and *B. thetaiotaomicron* (Moore, 1977). The clinical importance of *B. fragilis* is well documented (Gorbach & Bartlett, 1974; Onderdonk *et al.*, 1977; Kasper *et al.*, 1983; Elhag *et al.*, 1986). Early studies on more than 300 strains of *B. fragilis* indicated that there was a continuum of variants within the species (Holdeman & Moore, 1972). Subsequent serological studies (Elhag *et al.*, 1977; Elhag & Tabaqchali, 1978*a, b*) and chemotaxonomic analysis (Shah & Collins, 1980; Shah & Williams, 1982) of *B. fragilis* further emphasized this heterogeneity. Preliminary studies in this laboratory using SDS-PAGE analysis of polypeptides and lipopolysaccharide electrophoretic patterns in silver-stained gels (unpublished data) also indicate considerable heterogeneity of *B. fragilis*.

The value of enzyme electrophoretic patterns to investigate the suspected heterogeneity within the genus *Bacteroides* has been reported previously (Shah *et al.*, 1976; Williams & Shah, 1980; Shah & Williams, 1982). In particular the patterns of malate dehydrogenase (MDH), glutamate dehydrogenase (GDH), glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) have helped to clarify several chemotaxonomic problems within the pigmented and non-pigmented bacteroides (Shah & Collins, 1983). In the present paper, we report the use of MDH and G6PDH electrophoretic patterns to probe the heterogeneity of strains of *B. fragilis* isolated from a wide variety of clinical sites.

METHODS

Bacterial strains. All 52 strains of *B. fragilis* used in this study were of human origin. Details of the test strains and their sources are given in Table 1.

Cultivation and identification of strains. All strains were maintained by weekly subculture on 5% (v/v) blood agar

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Abbreviations: MDH, malate dehydrogenase; GDH, glutamate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase.

Table 1. *Strains and their sources*Reference strains: *B. fragilis* NCTC 9343, NCTC 10584, NCTC 8560 and NCTC 10581

<i>Bacteroides</i> strain		Site of isolation	Clinical condition
Group	Strain		
A	11c, 19, 20, 32, 43b, 69	Faeces	Normal human subjects
B	1, 26, 31, 65, 88, 112, 120, 121	Exudate (pus)	Perineal infections
C	11b, 29, 309, 310, 450	Exudate (pus)	Skin infections and submucous abscesses
D	2, 4, 6, 13, 15, 16, 34, 92, 280	Exudate (pus)	Post-operative wound infections
E	12, 21a, 50, 101	Exudate (pus)	Intra-abdominal wound sepsis
F	5, 99, 102, 272, 273	Exudate (pus)	Otitis
G	18, 39, 94	Endocervix	Infections of female genital tract
H	49, 131	Blood	Septicaemia
U	19, 23, 82, 98, 123, 21b	Not known	Not known

plates in an atmosphere of 5% (v/v) CO₂, 5% (v/v) H₂ in N₂ at 37 °C using an anaerobic cabinet (model 1028, Forma Scientific). Biochemical tests were done using the API 20A anaerobic system as described by Knivett *et al.* (1983). Volatile and non-volatile end products of metabolism were analysed by GLC (Elhag *et al.*, 1986) with a Shimadzu gas chromatograph (model GC-9A). All strains were examined using these tests and only strains which accurately conformed to the description of *B. fragilis* (Holdeman *et al.*, 1984) were included in this study.

Preparation of cell-free extracts. Cells were harvested from blood agar plates (three per strain) after 2 d growth under the conditions described above and suspended in 0.5 ml 0.1M-HEPES/NaOH buffer, pH 7.5. The cell suspension was shaken with Ballotini beads (grade 12) for 12 min in a Mickle tissue disintegrator at 4 °C. After low speed centrifugation (6000 *g*) to remove the beads, the remaining unbroken cells and cell debris were removed by centrifugation at 37000 *g* for 10 min. The supernatants, which contained between 30–50 mg protein ml⁻¹, were used for studies of the enzymes.

Enzyme activity and pH optimum. These were measured for only a limited number of strains to obtain the optimum conditions for electrophoretic visualization. Details of these procedures have been reported previously (Reeves *et al.*, 1971; Shah & Williams, 1982). All assays were done at 25 °C using a Beckman DU-7 spectrophotometer.

Electrophoresis and staining of enzymes. Cell-free extracts were electrophoresed on Cellogel support strips, 5.7 × 14 cm (Reeve Angel Scientific), as described previously (Shah & Williams, 1982) with minor modifications. Electrophoresis was done on an LKB Multiphor unit (model 2117) with a LKB Multitemp II thermostatic circulator (model 2219) at 10 °C. Electrophoresis was allowed to proceed for 1.5 h at 15 V (cm length of gel)⁻¹ in 0.04 M-barbitone/acetic acid buffer, pH 8.6. NADP was used for measurements of G6PDH activity; NAD was used for MDH. The electron acceptors phenazine methosulphate and thiazolyl blue tetrazolium-MTT (BDH) were used to visualize enzymes (Shah & Williams, 1982).

Statistical methods. Cluster analysis was used to calculate the similarities of the strains which produced single and multiple bands both of G6PDH and MDH. Before analysis, the mobility distances were standardized to give 'unit free' distances between the enzymes. The usual Euclidean distances were used to calculate the distances while the single linkage method was used for clustering of the enzymes. The Biomedical Package (BMDP, 1985) was used for this analysis.

RESULTS AND DISCUSSION

The presence of two pentose phosphate pathway enzymes, G6PDH and 6PGDH, in *B. fragilis*, *B. thetaiotaomicron*, *B. distasonis*, *B. ovatus*, *B. vulgatus*, *B. eggerthii* and *B. splanchnicus* (referred to as the '*B. fragilis* group') has been reported previously (Shah & Williams, 1982). These enzymes were not present in other species of the genus *Bacteroides* and therefore appeared to be of diagnostic value for species of the '*B. fragilis* group' (Shah & Collins, 1983). MDH and GDH were also reported amongst species of the '*B. fragilis* group' (Shah & Williams, 1982) and other *Bacteroides* species (Shah *et al.*, 1976, 1983, 1985; Shah & Hardie, 1979; Shah & Collins, 1981, 1987; Haapasalo *et al.*, 1985). The correlation between the presence of MDH and GDH and other chemotaxonomic characteristics was so significant that the occurrence of these enzymes was considered to be one of the major criteria for defining the genus *Bacteroides sensu stricto* (Shah & Collins, 1983, 1987).

Fig. 1. Standardized electrophoretic mobilities of MDH vs G6PDH of strains which produced only single bands of both enzymes. ···, 87% similarity; ---, 81% similarity; —, 77% similarity.

Table 2. Electrophoretic mobilities of MDH and G6PDH of the 27 strains that had single bands only

Strain no.	Electrophoretic mobility (cm)		Strain no.	Electrophoretic mobility (cm)	
	MDH	G6PDH		MDH	G6PDH
D2	4.0	5.8	A69	4.0	5.6
F5	3.9	5.5	U82	3.6	5.0
D6	4.5	6.0	G94	4.2	5.8
C11b	4.2	5.3	U98	4.1	5.8
A11c	4.0	5.3	F99	4.2	5.5
D15	3.9	5.2	E101	4.5	5.5
D16	4.4	6.1	F102	3.9	5.0
U19	4.5	5.7	U123	4.3	4.5
A20	3.7	4.8	H131	4.3	6.1
E21a	4.1	5.4	F272	3.5	5.0
U23	3.8	5.5	D280	4.2	5.4
A32	3.9	5.3	C309	4.2	5.4
D34	3.8	5.3	C450	4.1	5.9
A43b	3.9	5.7			

The restriction of G6PDH and MDH to the '*B. fragilis* group' was recently pursued by Rogemond & Guinet (1985). Antigenic extracts of MDH and G6PDH were detected by crossed immunoelectrophoresis in all species of the '*B. fragilis* group' except *B. vulgatus* and *B. distasonis* which lacked G6PDH antigenic specificity. Both MDH and G6PDH showed more electrophoretic heterogeneity (unpublished data; Shah & Williams, 1982) than 6PGDH and GDH and were therefore used in the present study to probe the enzyme polymorphism of strains of *B. fragilis* for subsequent epidemiological studies of *B. fragilis* virulence.

Of the 52 strains used in this study, all possessed high levels of both G6PDH [>120 nmol NADP reduced (mg protein) $^{-1}$ min $^{-1}$] and MDH [>150 nmol NAD reduced (mg protein) $^{-1}$ min $^{-1}$].

Fig. 2. Electrophoretic mobilities of enzymes of strains which possessed multiple bands of at least one enzyme. SB, DB, TB and QB refer to single, double, triple and quadruple bands respectively.

Fig. 3. Dendrogram of 25 strains of *B. fragilis* which possessed multiple electrophoretic bands of MDH or G6PDH or both.

min^{-1}]. All strains of *B. fragilis* tested for the presence of G6PDH required NADP as coenzyme whereas the MDH enzyme was NAD dependent.

Twenty-seven strains produced only single bands of both G6PDH and MDH. The electrophoretic mobilities of these strains are given in Table 2. In all cases G6PDH migrated faster than MDH. The enzyme mobilities for MDH ranged between 3.5 and 4.5 cm whereas the G6PDH enzymes migrated between 4.5 and 6.1 cm (Table 2). However, the electrophoretic similarity between strains were not clearly discernible from these results. For each strain a standardized migration distance was derived for both MDH and G6PDH and the data were subjected to cluster analysis (Fig. 1). At the 87% level of similarity, three clusters were formed, and arbitrarily labelled A, B and C (Fig. 1). When the similarity level was decreased to 81%, cluster A encompassed strains G94 and D2, strain E21a was included in cluster B, while strains A11c and D34 were circumscribed with cluster C. In addition, new clusters labelled D, E and F were formed. At the 77% similarity level strains E101 and U19 clustered together and three other clusters were formed (Fig. 1) which were amalgamations of A and E, B and C and an enlargement of D. Strains F102, A20 and particularly U123 appeared to be atypical and did not amalgamate with any of the above clusters.

Of the strains used in this study, 25 gave multiple bands of one or both enzymes. The exact relative mobilities of all strains compared to reference strains NCTC 8560 and NCTC 9343 are shown in Fig. 2. Ten strains produced single bands of MDH and double bands of G6PDH. Of the eight strains from perineal infections (labelled B) five fell into this category while the three remaining strains also had multiple bands of G6PDH (Fig. 2). Eight strains had the reverse enzyme pattern of multiple MDH bands with a single G6PDH band. Only four strains possessed double bands of both enzymes. Strains B112, G39 and B120 were atypical in possessing three or more bands of one or both enzymes (Fig. 2).

Simple comparisons between strains and their inter-relationships with each other were not easily perceptible: hence a cluster analysis of the enzyme patterns was done. Fig. 3 shows a dendrogram of the 25 strains. The dendrogram was subdivided into six clusters at the 72% level of similarity. Strains which possessed a single MDH-double G6PDH enzyme pattern clustered in phenon I which also contained three reference strains (Fig. 3). Phenon II formed a compact cluster which contained only the strains which possessed double bands of both enzymes. Strains

which had a double MDH–single G6PDH pattern were recovered in phenon III, with strain F273 showing the lowest affinity with the group. Strains B112, G39 and B120 were resolved separately (Fig. 3); they were also different in their enzyme electrophoretic patterns from all other strains studied.

The results of the present study confirmed the suspected heterogeneity of *B. fragilis* strains. However, these electrophoretic variants may result from as little as one amino acid substitution in the enzyme molecule which represents only a very small part of the bacterial genome. The stability of these electrophoretic groups must therefore be confirmed by other well established chemotaxonomic criteria such as lipid composition and DNA:DNA hybridization. The electrophoretic patterns, however, provide a useful basis for selecting strains for DNA:DNA hybridization. This approach has been successful in resolving major taxonomic dilemmas among other species of the genus *Bacteroides*, for example the controversy surrounding *B. oralis* and similar taxa (Shah *et al.*, 1982, 1985; Shah & Collins, 1981; Watabe *et al.*, 1983; Haapasalo *et al.*, 1986). Among the pigmented asaccharolytic species two taxa were clearly discerned using these methods (Shah *et al.*, 1976), which led to the recognition of *B. gingivalis* and *B. asaccharolyticus* (Coykendall *et al.*, 1980; Finegold & Barnes, 1979). The combined use of G6PDH, 6PGDH, MDH and GDH for discriminating amongst species of the '*B. fragilis* group' has already been established (Shah & Williams, 1982); however, the full potential of MDH and G6PDH for use in assessing the heterogeneity of *B. fragilis* should await the outcome of DNA:DNA hybridization experiments.

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