Pathways of glutamate catabolism among *Fusobacterium* species

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Glutamate is a major source of energy for *Fusobacterium* species but its mode of catabolism has not hitherto been elucidated. Cell suspensions of *F. nucleatum* and *F. varium*, as representative species from the oral cavity and gastrointestinal tract, respectively, both decarboxylated position-labelled glutamate but by different pathways. $^{14}\text{CO}_2$ was released only from C-5 by *F. nucleatum* whereas *F. varium* decarboxylated glutamate at either C-1 or C-5. In both species, 2 mols of glutamate fermented yielded 2 mols of acetate and 1 mol of butyrate, suggesting the possibility of three metabolic pathways: the 2-oxoglutarate, mesaconate and 4-aminobutyrate pathways. Enzymes representative of the three pathways were assayed for in cell-free extracts of fusobacteria. All species tested possessed high levels of both glutamate dehydrogenase and 2-oxoglutarate reductase, indicating the presence of the 2-oxoglutarate pathway. Enzymes representative of the mesaconate pathway were detected in *F. sulci*, *F. ulcerans*, *F. mortiferum* and *F. varium*, while the latter two species also possessed the 4-aminobutyrate pathway. The pathways of glutamate catabolism therefore bore no relationship to the site of isolation of the fusobacteria tested but instead correlated with their chemotaxonomic properties. Thus, *F. varium*, *F. mortiferum*, *F. ulcerans* and *F. sulci*, which possess a peptidoglycan structure based on diaminopimelic acid, have either two or three pathways for glutamate catabolism whereas *F. nucleatum* and other species that have a lanthionine-based murein metabolized glutamate solely by the 2-oxoglutarate pathway.

Introduction

Fusobacterium species colonize the mucous membranes of man and animals and are now regarded as important opportunistic pathogens (Moore *et al.*, 1984). Their successful colonization and survival is probably influenced by their ability to utilize substrates effectively at these sites. Previous studies have so far revealed that carbohydrates, as energy sources, are poorly utilized and their fermentation is limited to only a few species such as *F. varium* (Gharbia & Shah, 1988*b*). Species were shown to assimilate 30–100% of the available glucose in the medium (Gharbia & Shah, 1988), but [^{14}C]glucose incorporation experiments revealed that this is used for the biosynthesis of various cellular components (Gharbia & Shah, 1989*b*) and storage glycopolymers (Robrish *et al.*, 1987). The latter can, however, be metabolized in the absence of other fermentable substrates (Robrish & Thompson, 1988).

Peptides, supplied as protein hydrolysates (Gharbia & Shah, 1988) and amino acids (Gharbia & Shah, 1989; Gharbia *et al.*, 1990; Shah & Gharbia, 1989) comprise the major sources of energy for all *Fusobacterium* species.

Among the amino acids used, acidic (anionic) and basic (cationic) monomers, particularly glutamate, are taken up at high concentrations. Furthermore, amino acid uptake profiles are significantly altered by incorporating high levels of glutamate in the culture medium. These observations, together with the fact that glutamate constitutes more than 50% of the pool of free amino acids in human dental plaque (Singer & Kleinberg, 1983), suggest that this amino acid is likely to be a major source for *F. nucleatum* *in vivo*. Preliminary results indicated that there might be significant differences in the method by which glutamate is catabolized by *Fusobacterium* species. In the present study we therefore determined the pathway by which glutamate is catabolized by *Fusobacterium* species using enzyme assays, experiments with radio-labelled glutamate and metabolic end-product determinations. Species which generally colonize the oral cavity (e.g. *F. nucleatum*, *F. alocis*, *F. periodonticum* and *F. sulci*), the gastrointestinal tract (e.g. *F. varium*, *F. gonidiaformans*, *F. mortiferum* and *F. necrogenes*), the skin (e.g. *F. ulcerans*) and others which are frequently isolated from animals (e.g. *F. russii* and *F. necrophorum*) were tested.

Methods

Bacterial strains and cultivation. The following strains were used: *F. nucleatum*, ATCC 25586; *F. alocis*, ATCC 3364; *F. gonidiaformans*, ATCC 25563; *F. naviforme*, NCTC 25832; *F. necrophorum*, NCTC 10575; *F. necrogenes*, ATCC 25556; *F. periodonticum*, ATCC 33693; *F. russii*, ATCC 25533; *F. sulci*, ATCC 35585; *F. ulcerans*, NCTC 12111; *F. varium*, VPI 0499A; and *F. mortiferum*, NCTC 25557. All strains were maintained by weekly subculture on 2.5% (v/v) blood agar plate, in an atmosphere of 10% (v/v) CO₂ and 5% (v/v) H₂ in N₂ at 37 °C.

Determination of glutamate fermentation pathways with [¹⁴C]glutamate. *F. nucleatum* and *F. varium* strains were used as representative species. Both were grown in a medium containing (% w/v): glucose, 0.5; yeast extract, 0.5; trypticase, 1.0; NaCl, 0.5; and sodium thioglycollate, 0.1. Cells were harvested after 24 h by centrifugation at 10000 g for 15 min, and washed twice in phosphate-buffered saline (50 mM potassium phosphate containing 0.85% NaCl), pH 7.5, under anaerobic conditions. Fermentation experiments were done with cell suspensions prepared by resuspending the washed cells at 80–100 times the concentration in the original culture in O₂-free 50 mM-potassium phosphate buffer (pH 7.0) containing 0.1% sodium thioglycollate. Samples (0.5 ml) of such cell suspensions were incubated with sodium [¹⁻¹⁴C]- or [⁵⁻¹⁴C]glutamate (20 µmol; 370 kBq) in a total volume of 2 ml in tubes stoppered with vaccine caps under an atmosphere of N₂. Durham tubes containing hyamine hydroxide were placed in each O₂-free tube to trap released CO₂. All tubes and reagents were held at 37 °C prior to and after initiating the reactions. At 30 min intervals, one reaction mixture was stopped by the addition of 1 M-HCl (100 µl), and the tube was allowed to stand for 30 min at room temperature. The Durham tube was then removed and the exterior was rinsed thoroughly with distilled water and the tube and contents were placed in a scintillation vial containing 6 ml of the scintillation fluid Cocktail T (BDH). The cell suspension itself was centrifuged and to 1 ml of the supernatant, 2 ml methanol and 0.5 ml 50% (v/v) H₂SO₄ were added. The contents were mixed and held at 50 °C for 30 min, after which 1 ml of distilled water and 0.5 ml chloroform were added to the mixture, and the contents centrifuged at 1000 g for 10 min. The chloroform layer was withdrawn, and mixed with 6 ml of the scintillation fluid, and the samples counted in a model SL30 liquid scintillation counter (Intertechnique, UK).

Determination of enzyme activities. Cell-free extracts, prepared as described previously (Gharbia & Shah, 1988a) were centrifuged at 20000 g for 15 min to remove the particulate fraction. The supernatants were kept in sealed vials under an O₂-free atmosphere of N₂ at 4 °C, and assayed as quickly as possible to ensure minimal loss of activity. All assays were completed the same day, and substrates used were freshly prepared. All assays were done at 25 °C using a Gilford model 240 spectrophotometer. The Lowry procedure was used for protein estimation with bovine serum albumin fraction V (Sigma) as standard. All substrates were from Sigma unless otherwise stated.

2-Oxoglutarate reductase (EC 1.1.99.2). 2-Oxoglutarate reductase activity was determined spectrophotometrically by following changes in absorbance at 340 nm. The reaction mixture for assaying enzymic reduction of 2-oxoglutarate contained the following components: 0.5 mM-potassium phosphate buffer, pH 7.5, 10 mM-NADH, and 100 µl or 50 µl of cell extract. The reaction was initiated with 100 µl 7.5 mM-2-oxoglutarate, in a total volume of 1 ml. The pH optimum for 2-oxoglutarate was determined over the pH range of 5–11. The Michaelis constant was estimated for the above reaction mixture using increasing concentrations of 2-oxoglutarate (9.3×10^{-6} – 6×10^{-4} M). Interference from glutamate dehydrogenase, activity was minimized by ensuring that NH₄⁺ was eliminated from the reaction mixture.

The reverse reaction was measured in a reaction mixture containing 0.1 M-Tris/HCl, pH 8.5; 10 mM-2-hydroxyglutarate, 100 µl; 10 mM-NAD⁺, 100 µl; 0.1 M-freshly neutralized hydroxylamine, 50 µl; and 50 µl of cell extract, in a total of 1 ml.

Glutamate dehydrogenase (EC 1.4.1.2). Glutamate dehydrogenase activity was measured by following changes in absorbance at 340 nm, both in the direction of glutamate oxidation and in the direction of glutamate synthesis. Optimal pH values for these reactions were determined. In the oxidation assay the following mixture was used: 0.1 M-Tris/HCl buffer, pH 8.5; 40 mM-glutamate, 100 µl; 10 mM-NAD⁺, 100 µl; and 50 µl of cell extract, in a total volume of 1 ml. The formation of glutamate from 2-oxoglutarate was determined in a reaction mixture containing 0.1 M-Tris/HCl buffer, pH 7.0; 0.5 M-NH₄Cl, 50 µl; 40 mM-2-oxoglutarate, 100 µl; 10 mM-NADH, 100 µl; and 50 µl of cell extract.

Reaction rates for 2-oxoglutarate reductase and glutamate dehydrogenase were corrected for NADH oxidase activity by subtracting the reaction rate observed in reaction mixtures before the addition of the C₅-substrates. One unit of enzyme activity was defined as the amount of enzyme that catalysed the oxidation of 1 µmol NADH min⁻¹ or the reduction of 1 µmol NAD⁺ min⁻¹. Specific activity was defined as the amount of substrate (in µmol) converted min⁻¹ (mg protein)⁻¹.

3-Methylaspartase (EC 4.3.1.2). 3-Methylaspartase was measured by following the conversion of 3-methylaspartate to mesaconate, which results in the formation of a double bond and therefore an increase in absorbance at 240 nm. The pH optimum for the reaction was determined, then the Michaelis constant was estimated using the following reaction mixture: 0.05 M-Tris/HCl buffer, pH 7, 500 µl; 150 mM-KCl, 60 µl; 15 mM-MgCl₂, 60 µl; and 100 µl of cell extract. The reaction was initiated by the addition of sodium-L-threo-β-methylaspartate at a concentration ranging from 0.1×10^{-6} – 24×10^{-4} M. One unit of enzyme activity was defined as the amount of enzyme required to catalyse an increase in A₂₄₀ of 0.04 min⁻¹, equivalent to 1.039×10^{-2} µmol mesaconate min⁻¹ (Barker *et al.*, 1958).

4-Hydroxybutyrate dehydrogenase (EC 1.1.1.61). This enzyme was assayed by following the reduction of NAD⁺ at 340 nm. The pH optimum for 4-hydroxybutyrate oxidation was determined over the range pH 6–10.5, in the following reaction mixture: 0.1 M-Tris/HCl buffer, pH 7, 700 µl; 20 mM-NAD⁺, 100 µl; and 100 µl of cell extract. The reaction was initiated with 100 µl 20 mM-4-hydroxybutyrate. The Michaelis constant was estimated in the above reaction mixture at pH 6.5 using 4-hydroxybutyrate concentrations ranging from 3×10^{-5} – 8×10^{-3} M. Specific activity is expressed as described for glutamate dehydrogenase.

Acetyl-CoA:hydroxybutyrate-CoA transferase. This enzyme was assayed by monitoring the increase in absorbance at 365 nm due to a complex reaction leading to the formation of crotonyl-CoA. The reaction mixture consisted of 0.1 M-Tris/HCl buffer, pH 5–11.5; 78 mM-cysteine, 50 µl; 10 mM-ATP, 50 µl; 2.6 mM-coenzyme A, 50 µl; and 100 µl of cell extract in a total of 1 ml. The reaction was initiated with 4-hydroxybutyrate using separately a series of concentrations ranging from 1.4×10^{-6} – 7×10^{-4} M.

Acid end-product analysis. Volatile and non-volatile fatty acid end-products of metabolism were analysed by GLC as described previously (Shah *et al.*, 1976).

Results

Glutamate fermentation pathways in *F. nucleatum* and *F. varium*

The fermentation of glutamate by both *F. nucleatum* and *F. varium* yielded butyrate and acetate as the only acidic

Fig. 1. Percentage release of $^{14}\text{CO}_2$ from [1- ^{14}C]- (●) and [5- ^{14}C]- (□) glutamate by cell-suspensions of *F. nucleatum* (a) and *F. varium* (b).

end-products. Both strains produced 2 mols of acetate and 1 mol of butyrate per 2 mols of glutamate added. When [5- ^{14}C]glutamate was used as the substrate, over 95% of the total radioactivity was recovered in the CO_2 liberated by *F. nucleatum*, and 86% by *F. varium*. However, when similar experiments were done with [1- ^{14}C]glutamate, only 0.3% of the radioactivity was found in the CO_2 released by *F. nucleatum* and 14.6% by *F. varium*. The decarboxylation of all the [^{14}C]glutamate added to a cell suspension of *F. nucleatum* required 3.5 h for completion, while *F. varium* required only 2.5 h. The time course of $^{14}\text{CO}_2$ release by both species is shown in Fig. 1.

Enzymes specific for each pathway

The above experiments suggested that at least two pathways for glutamate fermentation were used by *F. varium*, since both [1- ^{14}C]- and [5- ^{14}C]glutamate were decarboxylated to produce $^{14}\text{CO}_2$. To test for the presence of the 4-aminobutyrate pathway, a cell-free extract of *F. varium* was assayed for the presence of 4-hydroxybutyrate dehydrogenase and acetyl-CoA:hydroxybutyrate-CoA transferase. Low levels of both were detected. The optimal pH for both reactions was 6.5, and the Michaelis constant (K_m) was 1×10^{-3} M for the oxidation of 4-hydroxybutyrate, whereas the K_m for its conversion to 4-hydroxybutyrate-CoA was 6×10^{-4} M. To determine which pathway led to the decarboxylation at C-5 of glutamate, cell-free extracts were screened for

Table 1. Comparison of the properties of glutamate catabolic enzymes of *F. nucleatum* and *F. varium*

GDH, Glutamate dehydrogenase; ORG, 2-oxoglutarate reductase; MA, 3-methylaspartase; HBD, 4-hydroxybutyrate dehydrogenase; HT, acetyl-CoA:hydroxybutyrate-CoA transferase.

Species	Enzyme	Coenzyme	pH optimum	Specific activity* [$\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$]
<i>F. nucleatum</i>	GDH	NAD $^{+}$	9.0	34.0
		NADP $^{+}$	9.0	1.0
	GDH	NADH	7.5	16.0
		NADPH	7.5	1.0
	OGR	NADH	9.0	28.0
		NADPH	9.0	2.5
		NAD $^{+}$	7.5	15.0
		NADP $^{+}$	7.5	0.02
	MA	—	—	0.0
	HBD	NAD $^{+}$	—	0.0
<i>F. varium</i>	GDH	NAD $^{+}$	10.5	20.0
		NADH	8.5	10.0
	OGR	NADH	9.5	18.0
		NAD $^{+}$	8.5	9.7
	MA	—	9.5	3.4
	HBD	NAD $^{+}$	6.5	0.2
	HT	CoA + ATP	6.5	0.06

* Values are means of three experiments.

Table 2. Distribution of enzymes representative of glutamate catabolic pathways

GDH, Glutamate dehydrogenase; ORG, 2-oxoglutarate reductase; MA, 3-methylaspartase; HBD, 4-hydroxybutyrate dehydrogenase; HT, acetyl-CoA:hydroxybutyrate-CoA transferase. Enzyme specific activities of $<0.001 \mu\text{mol min}^{-1}$ (mg protein) $^{-1}$ were considered negative.

Species	GDH	ORG	MA	HBD	HT
<i>F. nucleatum</i>	+	+	—	—	—
<i>F. alocis</i>	+	+	—	—	—
<i>F. gonidiaformans</i>	+	+	—	—	—
<i>F. naviforme</i>	+	+	—	—	—
<i>F. necrophorum</i>	+	+	—	—	—
<i>F. necrogenes</i>	+	+	—	—	—
<i>F. periodonticum</i>	+	+	—	—	—
<i>F. russii</i>	+	+	—	—	—
<i>F. sulci</i>	+	+	+	—	—
<i>F. ulcerans</i>	+	+	+	—	—
<i>F. varium</i>	+	+	+	+	+
<i>F. mortiferum</i>	+	+	+	+	+

enzymes representing both the mesaconate and 2-oxoglutarate pathways. Cell-free extracts of *F. varium* possessed 3-methylaspartase activity with an optimal pH of 9.5. The K_m for the deamination of 3-methylaspartate was 1.6×10^{-4} M. In addition, high levels of glutamate dehydrogenase and 2-oxoglutarate reductase were detected in the cell-free extracts indicating the presence of the 2-oxoglutarate pathway. The pH optimum for

Fig. 2. Glutamate catabolism via (a) the 2-oxoglutarate pathway, (b) the mesaconate pathway and (c) the 4-aminobutyrate pathway.

glutamate oxidation was 10.5, and pH 9.0 for the reverse reaction. The reduction of 2-oxoglutarate occurred within a narrow alkaline pH range (8.0–10.5) with an optimum at 8.5. The K_m value for 2-oxoglutarate reduction was 3.26×10^{-5} M, and 1.8×10^{-5} M for glutamate synthesis.

F. nucleatum possessed higher levels of both glutamate dehydrogenase and 2-oxoglutarate reductase than *F. varium*. The optimum for glutamate oxidation by glutamate dehydrogenase was 9.0, while glutamate formation from 2-oxoglutarate in the presence of NH_4Cl had a pH optimum of 7.5. The reduction of 2-oxo-

(c) 4-Aminobutyrate pathway

Fig. 2. (continued)

glutamate to 2-hydroxyglutamate had an optimum at pH 9.0. The Michaelis constant for glutamate formation was 4.7×10^{-3} M, and for 2-oxoglutarate reduction in the presence of NADH, 5.3×10^{-5} M. However, none of the enzymes representative of the mesaconate or the 4-aminobutyrate pathways were detected in *F. nucleatum*. The properties of these enzymes in *F. nucleatum* and *F. varium* are summarized in Table 1.

When the type strains of other *Fusobacterium* species were screened for the presence of enzymes representative of the three pathways, three patterns were revealed (Table 2). *F. mortiferum* was similar to *F. varium* in possessing all three pathways, while *F. sulci* and *F. ulcerans* lacked enzymes of the 4-aminobutyrate pathway. All other strains tested possessed enzymes of the 2-oxoglutarate pathway only.

Discussion

Different pathways for the anaerobic and aerobic metabolism of glutamate have been postulated. Anaerobic bacteria generally ferment glutamic acid to acetate, butyrate, NH₃, CO₂ and H₂ (Barker, 1981). The fact that anaerobes produce only 1 mol of CO₂ per mol of glutamate degraded, whereas aerobes theoretically produce 3 mols of CO₂ using the tricarboxylic acid cycle, suggests that a different pathway is used for the metabolism of glutamic acid. Several studies have been done to determine the possible pathways of glutamate catabolism in anaerobic bacteria (see, e.g., Buckel &

Barker, 1974). Both *Clostridium tetanomorphum* and *Peptostreptococcus aerogenes* were studied extensively, and although both decarboxylate glutamate at position C-5 and produce acetate and butyrate, different metabolic intermediates were recovered (Buckel & Barker, 1974; Lerud & Whitely, 1971; Whitely, 1957). In the present study, *F. nucleatum* decarboxylated glutamate at C-5, possessed enzymes of the 2-oxoglutarate pathway (Fig. 2a) and lacked key intermediates of the mesaconate pathway (see Fig. 2b). *F. varium*, however, possessed key intermediates of both pathways, but in addition decarboxylated [1-¹⁴C]glutamate, indicating the presence of the 4-aminobutyrate pathway (Fig. 2c).

All species tested in the present study possessed high levels of both glutamate dehydrogenase and 2-oxoglutarate reductase, indicating that the 2-oxoglutarate pathway for glutamate catabolism predominates among *Fusobacterium* species. Several studies have been done to determine the role and distribution of this pathway among glutamate-fermenting anaerobes (Buckel & Barker, 1974; Lerud & Whitely, 1971; Horler *et al.*, 1966). *Acidoaminococcus fermentans*, *Micrococcus aerogenes* and *Peptostreptococcus aerogenes* degrade glutamate solely via this pathway, in which the successive intermediates are 2-oxoglutarate, 2-hydroxyglutamate and probably vinylacetyl-CoA and crotonyl-CoA (Fig. 2a). The latter is converted to acetate, butyrate and H₂. Although several studies were undertaken to purify the intermediates of this pathway, the enzyme responsible for the conversion of 2-hydroxyglutamate to crotonyl-CoA

via vinylacetyl-CoA still requires investigation. Studies by Buckle & Barker (1974) suggested a role for a dehydratase which required the presence of coenzyme-A and acetyl phosphate. In addition pre-incubation with NADH, FeSO₄, MgCl₂ and dithiothreitol under anaerobic conditions was necessary to attain maximum activity. More detailed analysis requires purification of this enzyme and synthesis of the intermediates of the 2-oxoglutarate pathway.

The 4-aminobutyrate pathway interacts with the 2-oxoglutarate pathway in that both require glutamate dehydrogenase for the initial reaction and use the same enzymes for conversion of vinylacetyl-CoA (Fig. 2c). The 4-aminobutyrate pathway was detected only in *F. varium* and *F. mortiferum*, both of which are normally isolated from the gastrointestinal tract.

Enzymes representative of the mesaconate pathway (Fig. 2b) were detected in the cell-free extract of four species (Table 2). The properties of 3-methylaspartase (the key enzyme for this pathway) were investigated in *F. varium*. 3-Methylaspartase had an alkaline pH optimum, similar to that of glutamate dehydrogenase and 2-oxoglutarate. In the initial coenzyme-B₁₂-dependent reaction, the linear carbon chain of glutamate is rearranged to the branch-chain of 3-methylaspartate (Fig. 2b). Elimination of NH₃ plus addition of H₂O yields citramalate, which subsequently is cleaved to acetate and pyruvate. The reducing equivalents produced by the subsequent oxidative decarboxylation of pyruvate to CO₂ and CoA are used either to synthesize butyryl-CoA from 2 molecules of acetyl-CoA or to form molecular hydrogen. ATP is generated from the thioester, probably via an acyl phosphate.

A good method to differentiate between the 2-oxoglutarate and the mesaconate pathways should be by the pattern of labelled end-products derived from the fermentation of [¹⁴C]glutamate. According to Fig. 2(a, b), C-1 of glutamate should be incorporated into acetate and butyrate in the 2-oxoglutarate pathway, but into acetate only in fermentation via the mesaconate pathway (Buckle & Barker, 1974). This can be tested on the basis of the differential labelling patterns of the butyrate obtained with [4-¹⁴C]glutamate. Butyrate formed via this pathway will be derived from C-1 and C-3 (Fig. 2b), whereas butyrate formed via 2-oxoglutarate will be labelled at C-4. Future studies will aim to use [4-¹⁴C]glutamate to test the relative contribution of each pathway to glutamate catabolism in *F. varium* and related species.

The results of this study reveal that glutamate is used as an energy source by all the *Fusobacterium* spp. tested, that is by species found in the oral cavity and in the colon of man and animals. Whereas there is no ecological distribution of species based on the pathway by which

glutamate is catabolized, a chemotaxonomic grouping of species is clearly evident. Thus species such as *F. varium*, *F. mortiferum*, *F. ulcerans* and *F. sulci* that possess a peptidoglycan structure based upon diaminopimelic acid have two or three pathways for glutamate catabolism (Table 2). The remaining species, which have a lanthionine-based mucopeptide (Gharbia & Shah, 1990), metabolize glutamate solely by the 2-oxoglutarate pathway (Table 2). The conjecture that this may reflect phylogenetic differences between these species is currently being tested by 16S rRNA sequence analysis.

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