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Inhibitory Effect of Fumarate on Growth of *Bacteroides fragilis*

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The growth of the strictly anaerobic bacterium *Bacteroides fragilis* on glucose was inhibited in the presence of fumarate. The molar growth yields for glucose were 29.6 and 20.0 g of dry cells/mol of glucose in the absence and presence of fumarate, respectively. In the culture with fumarate, the ratio of lactate production increased and that of succinate was slightly decreased. Fumarate addition also affected the levels of some enzyme activities involved in fermentation of glucose.

Keywords——*Bacteroides fragilis*; fumarate; growth inhibition; glucose metabolism

Introduction

The strictly anaerobic bacterium *Bacteroides fragilis* is one of the major microorganisms in the human intestinal tract[1] and is an infectious pathogen.[2] Properties of this anaerobe have been investigated from a variety of viewpoints.[3] In this bacterium, glucose is degraded *via* the Embden–Meyerhof pathway of glycolysis, though pyrophosphate-dependent 6-phosphofructokinase (EC 2.7.1.90) functions instead of the adenosine triphosphate (ATP)-dependent enzyme.[4] Succinate, acetate, lactate, and propionate are the main products of the fermentation.[5] Heme is required for vigorous growth[6] and is used in the synthesis of cytochromes, which are associated with electron transport for fumarate reduction.[5] In a medium without heme the growth is poor and large amounts of fumarate and lactate are accumulated. This observation strongly suggests that ATP formation is coupled with fumarate reduction to succinate. Propionate formation is catalyzed by methylmalonyl-coenzyme A mutase (EC 5.4.99.2) which is a cobamidoenzyme,[4] and scarcely occurs in the absence of vitamin B_{12}.[7] A large amount of propionate is produced when the cells are grown under a nitrogen atmosphere.[8] In this case the specific activity of the CO_2-fixing enzyme phosphoenolpyruvate carboxykinase (EC 4.1.1.49) is high compared with that in cells grown under a CO_2 atmosphere. 2-Oxoglutarate, which is necessary for ammonia assimilation in this organism,[9] is synthesized by reductive carboxylation of succinate but not *via* isocitrate.[10]

Growth of many kinds of bacteria is known to be stimulated by adding fumarate to anaerobic cultures because of the anaerobic respiration coupled with fumarate reduction.[11] However, when *B. fragilis* was anaerobically grown in a medium containing 20 mM fumarate, inhibition of the cell growth was observed. In this study, in order to understand the action of fumarate we investigated the fermentation products and the activities of some enzymes involved in glucose metabolism of *B. fragilis* in the presence and absence of fumarate.

Materials and Methods

Organism and Cultivation——*B. fragilis* ATCC 23745 was anaerobically grown at 37 C in a complex medium[9] supplemented with L-methionine (10 mg/l) instead of vitamin B_{12}. The medium was filled in a flask stoppered with a rubber septum equipped with glass tubings and was bubbled with CO_2 during growth to maintain the anaerobic condition. Samples were withdrawn periodically through the glass tubing to analyze fermentation products and to

measure optical density. Optical density at 650 nm (OD_{650}) of the culture was measured with a spectrophotometer (Hitachi 100-30). When OD_{650} was higher than 0.5, cultures were diluted with saline for the measurement. The dry weight of the cells was determined after washing the cells with distilled water and drying at 110 °C, and OD_{650} of 0.5 corresponded to 0.20 mg of dry cells/ml.

Assay of Metabolic Products——The supernatant after centrifugation of cultures was assayed for fermentation products. Glucose, acetate, pyruvate, and propionate were determined as described previously.[12] Lactate, fumarate, and succinate were determined by gas chromatography after methylation.[13]

Preparation of Cell-Free Extracts——Cultures were harvested by centrifugation at $10000 \times g$ for 10 min. Harvested cells were washed once with 50 mM Tris–HCl, pH 7.4, and stored at −20 °C until use. Cells suspended in the buffer were disrupted in a sonicator (Tomy UR-150P) and centrifuged at $10000 \times g$ for 10 min. The supernatants obtained were used as crude extracts.

Assay of Enzyme Activities——Enzyme activities were assayed as follows: phosphoenolpyruvate carboxykinase, pyruvate kinase (EC 2.7.1.40), D-lactate dehydrogenase (EC 1.1.1.28) and malic enzyme (EC 1.1.1.40) according to Macy *et al.*[4]; malate dehydrogenase (EC 1.1.1.37) as previously described[14]; and fumarate reductase (EC 1.3.99.1) by monitoring oxidation of dithionite at 315 nm in the presence of fumarate.[15]

Protein was determined by the method of Lowry *et al.*[16] with bovine serum albumin as a standard.

Spectral Measurement——The dithionite-reduced minus air-oxidized difference absorption spectra of the crude extracts were measured with a spectrophotometer (Shimadzu UV-260). The extracts were reduced with several crystals of $Na_2S_2O_4$, and an oxidized sample was prepared by gently shaking the extracts in a cuvette. The amount of cytochrome b was estimated by using the extinction coefficient of 17.5 $mM^{-1} cm^{-1}$.[17]

Results and Discussion

B. fragilis grew well to attain $OD_{650}=2.79$ in the complex medium under a CO_2 atmosphere (Fig. 1A). In the exponential phase of growth (during 1.5—5 h of incubation) the generation time was 1.2 h. On the other hand, the growth of the cells in the medium supplemented with 20 mM fumarate was rather low; the maximal OD_{650} was 2.15 and the generation time was 2.0 h during 1.5—5.5 h of incubation. The culture pH values were the same (6.9) in the media with and without fumarate immediately after inoculation, and fell to pH 5.9 and 5.4 during 11.5 h of incubation, respectively. L-Malate and succinate had no effect on growth of the cells at the concentration of 20 mM. These findings indicate that the cell growth of *B. fragilis* is repressed by fumarate added to the medium. The inhibitory effect of fumarate on the growth was also observed in a defined medium lacking peptone, yeast extract, and beef extract: after 12.5 h of incubation without fumarate OD_{650} was 1.12 and the generation time was 4.4 h, while in the culture with fumarate $OD_{650}=0.450$ and the generation time was 7.5 h.

Metabolic products from glucose and specific activities of some enzymes associated with

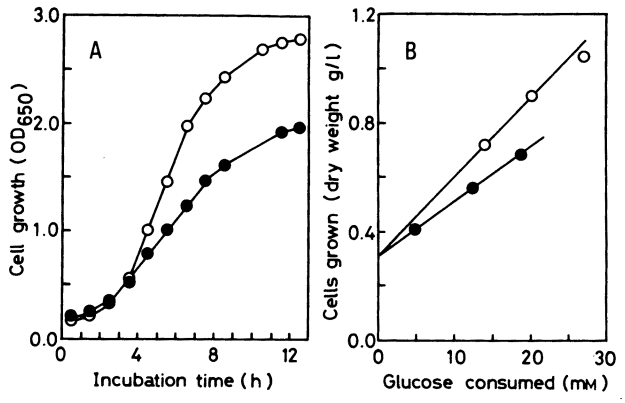

Fig. 1. Growth of *B. fragilis* in the Complex Medium

A) Growth in the absence (○) and in the presence of 20 mM fumarate (●). B) Relations between amounts of grown cells and glucose consumed. Symbols are the same as above.

TABLE I. Fermentation Products of Glucose in *B. fragilis* Grown in the Presence and Absence of Fumarate

Substrate and product	Without fumarate		With fumarate	
	Concentration (mM)	(%)[a]	Concentration (mM)	(%)[a]
Glucose (consumed)	−27.0		−18.6	
Acetate	8.0	14.8	7.5	20.2
Pyruvate	0.3	0.6	0.8	2.2
Lactate	1.9	3.5	5.5	14.8
Fumarate (formed or consumed)	0.3	0.6	−8.1	
Succinate	17.8	33.0	13.5	14.5[b]
Total		52.5		51.7[b]
Cells grown (mg dry weight per ml)	1.03		0.69	

Cultures of 11.5 h incubation with and without 20 mM fumarate were used for analysis. *a*) Carbon recovery as 100 × [product]/2[glucose consumed]. *b*) Estimated by subtracting the amount of succinate formed from exogenous fumarate.

glucose fermentation were determined in the cultures with and without fumarate. Amounts of metabolites in the 11.5 h incubation culture are shown in Table I. Propionate was not detected in either culture; this is presumably due to the absence of vitamin B_{12} in the medium, as shown previously.[7] In the culture without fumarate, 27 mM glucose added was completely consumed and acetate, pyruvate, lactate, fumarate, and succinate were accumulated. Succinate represented 63% of the total amount of products. Carbon recovery of the acids from glucose was 52.5%.

In the culture with fumarate, fumarate was partly consumed during the growth of cells. However, the amount of succinate accumulated was 50% of the total amount of acids produced. This value is about 10% lower than that obtained in the culture without fumarate. On the other hand, the production ratios of acetate and lactate to the amount of glucose consumed were increased by 1.4- and 4.2-fold, respectively, in the fumarate-containing medium. The recovery of glucose carbon was 51.7% in the products when the amount of fumarate consumed was subtracted from that of succinate accumulated.

The relation of the amounts of grown cells to glucose consumption is shown in Fig. 1B. Growth yields on glucose were estimated from the slopes; 29.6 and 20.0 g dry cells/mol glucose in the cultures without and with fumarate, respectively. This finding suggests that energy production during glucose fermentation, including fumarate reduction to succinate, is negatively affected by fumarate added.

B. fragilis is known to have the Embden–Meyerhof pathway for glucose metabolism.[4] Some enzyme activities were determined in crude extracts prepared from cultures in a late logarithmic phase (6 h incubation) and in an early stationary phase (10 h incubation) (Table II). Activities of phosphoenolpyruvate carboxykinase, pyruvate kinase, nicotineamide adenine dinucleotide (NADH)-linked malate dehydrogenase, $NADP^+$-linked malic enzyme, and NAD^+-linked D-lactate dehydrogenase were demonstrated in this organism.[4] Phosphoenolpyruvate carboxykinase, pyruvate kinase, and malate dehydrogenase showed lower activities in the stationary growth phase of both cultures with and without fumarate. The specific activities of pyruvate kinase were rather low in the culture with fumarate, being 50—70% of those in the culture without fumarate. In the fumarate cultures the specific activities of malic enzyme were half in the logarithmic growth phase and 1.5-fold at the stationary phase. The contents of cytochrome b estimated from the α-peak absorption at 560 nm were equal in both cultures and increased during the cell growth to a maximum level.

TABLE II. Enzyme Activities in Crude Extracts Prepared from *B. fragilis* Grown Anaerobically in the Presence and Absence of Fumarate

Enzyme	Specific activity (nmol/min/mg protein)			
	Culture without fumarate		Culture with fumarate	
	6h	10h	6h	10h
Phosphoenolpyruvate carboxykinase	679	250	776	189
Pyruvate kinase	7.53	3.08	5.45	1.71
Malate dehydrogenase	6400	153	7170	149
Malic enzyme	10.5	5.15	5.50	7.71
Lactate dehydrogenase	2.70	2.90	2.29	2.39
Cytochrome b (pmol/mg protein)	5.89	15.8	5.68	15.7

Fumarate reductase activity could not be detected in the extracts. This may be due to instability of the enzyme in air, as reported previously.[5]

In this study, inhibition of the growth of *B. fragilis* was observed in the presence of fumarate. The molar growth yield on glucose decreased by 33%. In the culture with fumarate, the ratio of the amount of lactate produced to the amount of glucose consumed increased and that of succinate produced slightly decreased compared with those in the culture without fumarate. Moreover, the specific activities of malic enzyme and pyruvate kinase were rather low in the logarithmic growth phase of the culture containing fumarate. Like lactate dehydrogenase, malic enzyme was kept at a constant level during growth in the presence of fumarate. These findings suggest that pathways of glucose metabolism were altered in part by the fumarate addition. This presumably caused a decrease in energy production, resulting in the low molar growth yield on glucose. Whether energy transduction coupled with fumarate reduction would be regulated by exogenous fumarate remains to be examined, though the contents of cytochrome b were equal in the presence and absence of fumarate.

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