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Influence of Amino Acids on the Growth of *Bacteroides melaninogenicus*

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Addition of individual amino acids to a Trypticase-yeast extract-hemin medium affected growth rates and final yields of an asaccharolytic strain and a saccharolytic strain of *Bacteroides melaninogenicus*. L-Aspartate or L-asparagine produced maximal growth enhancement for both strains. L-[^{14}C]aspartate was fermented by resting cells of the asaccharolytic strain. L-Cysteine or L-serine also enhanced growth for the saccharolytic strain. However, growth of the saccharolytic strain was inhibited by L-lysine, L-glutamate, L-glutamine, L-isoleucine, L-leucine, and L-proline; growth of the asaccharolytic strain was inhibited by DL-valine and L-serine. Both strains were inhibited by L-histidine, DL-methionine, L-tryptophan, L-arginine, and glycine.

Bacteroides melaninogenicus is indigenous to the oral and intestinal flora of man and certain animals (4, 9, 17, 22) and has been associated with a variety of clinical infections (1, 5, 21, 24). This organism utilizes amino acids as sources of energy, and in addition, some strains ferment a variety of carbohydrates (7, 20). Recent studies with resting-cell suspensions showed that *B. melaninogenicus* can ferment amino acids when they are present as peptides, but may be limited in fermenting free amino acids (23). This suggests that this organism may be more permeable to peptides.

The objective of this investigation was to determine if free amino acids could influence the growth of an asaccharolytic and a saccharolytic strain of *B. melaninogenicus*. We were able to demonstrate that many of the amino acids individually added to a minimal medium were able to influence growth of both strains. Some enhanced growth, and others inhibited it. The addition of L-aspartate in particular produced maximal growth enhancement for both strains. Resting cells of the asaccharolytic strain in the presence of L-[^{14}C]aspartate fermented 80% of the amino acid.

MATERIALS AND METHODS

Organisms. *B. melaninogenicus* ss. *asaccharolyticus* NU B26, an asaccharolytic strain isolated from a human case of acute necrotizing ulcerative gingivitis, and *B. melaninogenicus* ss. *melaninogenicus* ATCC 25261, a saccharolytic strain obtained from the American Type Culture Collection, were used. Neither strain required vitamin K.

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Media. Trypticase-yeast extract-hemin (TYH) medium of the following composition was used: Trypticase (Baltimore Biological Laboratory, Cockeysville, Md.), 3%; yeast extract (Difco Laboratories, Detroit, Mich.), 0.3%; sodium thioglycolate, 0.05%; hemin (equine type III, Sigma Chemical Co., St. Louis, Mo.), 5 μg/ml; and NaCl, 0.5%. Growth responses to individual amino acids were tested in TYH medium with single amino acids (Sigma, reagent grade), added at 0.5% concentration for L isomers and 1.0% for DL mixtures. All media were adjusted to pH 7.0, autoclaved, cooled rapidly, and supplemented with 0.2% sterile $NaHCO_3$.

Cultural methods. Inocula for growth studies were grown anaerobically at 37°C for 18 to 20 h. Cell suspensions were standardized to an absorbance of 0.7 to 0.8 at 600 nm in buffer (0.05 M potassium phosphate, pH 7.0, with 0.05% sodium thioglycolate) using a Bausch and Lomb Spectronic 20 colorimeter.

For growth determinations (repeated four times), matched test tubes (13 by 100 mm) containing 6 ml of medium were closed with rubber serum stoppers immediately after autoclaving and inoculated to an absorbance of 0.03 using a tuberculin syringe. The tubes were incubated at 37°C, and absorbance readings were taken as indicated.

Resting cells were obtained by harvesting log-phase cells and suspending them to a concentration of 35 mg (dry weight) per ml in 0.067 M potassium phosphate buffer (pH 7.5).

Fermentation of L-[^{14}C]aspartate. The fermentation of L-[^{14}C]aspartate was conducted in test tubes (20 by 150 mm) at 37°C. A glass vial containing 0.4 ml of 1 M hydroxide of hyamine in methanol was suspended above the reaction mixture. The tubes were fitted with an entry port covered with a rubber serum stopper to allow addition of 2 M H_2SO_4 to stop the reaction and release $^{14}CO_2$. The system was flushed with nitrogen immediately after adding the components of the reaction mixture.

Analytical methods. Reaction mixtures with rest-

ing cells were quantitatively transferred to centrifuge tubes, and the cells were removed by centrifugation. Total anions were separated from the cations in the reaction mixture with small resin columns (Dowex) as described by Mitruka and Costilow (16). The cations, eluted from the columns with 1 M NH_4OH, were concentrated to dryness and dissolved in water. L-[^{14}C]aspartate was separted and identified by paper chromatography in *n*-butyl alcohol-acetic acid-water (60:15:25). Duplicate samples were used for separation of amino compounds; one paper strip was used for location of the individual amino acids and the other was used for the radioactive assays (6).

The scintillation fluid described by Bray (3) was used for counting the radioactivity in all aqueous samples, and a toluene-based fluid (6) was used for counting the activity in $^{14}CO_2$ collected in hydroxide of hyamine. A Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill., model 2002) was used for counting the radioactivity.

Chemicals. Uniformly labeled L-[^{14}C]aspartic acid was obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Growth response to amino acids. The addition of either L-aspartate or L-asparagine to cultures of either strain in TYH medium produced marked increases in growth rate and total cell yield compared with unsupplemented medium (Tables 1 and 2). The growth responses of both strains at 48 h were dose dependent to L-aspartate over the range of 0.1 to 6.0 mg/ml (Fig. 1). The saccharolytic strain also showed growth stimulation in response to the addition of either L-cysteine or L-serine (Table 2).

Addition of either glycine, L-histidine, L-tryptophan, L-arginine, or DL-methionine to TYH medium produced marked to moderate inhibition of growth of both strains (Tables 1 and 2). DL-Valine or L-serine produced moderate inhibition of growth of the asaccharolytic strain (Table 1). L-Isoleucine, L-leucine, L-proline, L-glutamate, L-glutamine, or L-lysine produced marked to moderate inhibition of growth of the saccharolytic strain (Table 2).

Since L-histidine produced marked inhibition of growth for both strains, their growth responses at 48 h to varying concentrations of this amino acid were determined. Their growth was found to decrease as the concentration of L-histidine increased (Fig. 2).

TABLE 1. *Effect of single amino acid additions to TYH medium on the growth of B. melaninogenicus ss. asaccharolyticus NU B26*

Amino acid added[a]	Amt of growth at:[b]				
	10 h	20 h	30 h	40 h	50 h
None	0.13	0.32	0.48	0.56	0.58
L-Aspartate, L-asparagine	0.17	0.75	1.1	1.3	1.3
L-Histidine	0.04	0.05	0.06	0.09	0.11
DL-Methionine	0.04	0.07	0.16	0.24	0.26
L-Tryptophan	0.09	0.15	0.22	0.30	0.37
DL-Valine	0.08	0.16	0.24	0.30	0.36
L-Arginine, L-serine	0.08	0.16	0.24	0.34	0.50
Glycine	0.09	0.18	0.28	0.37	0.40

[a] L Isomers were added in 0.5% concentration, and DL mixtures were added in 1% concentration.
[b] Optical density at 600 nm.

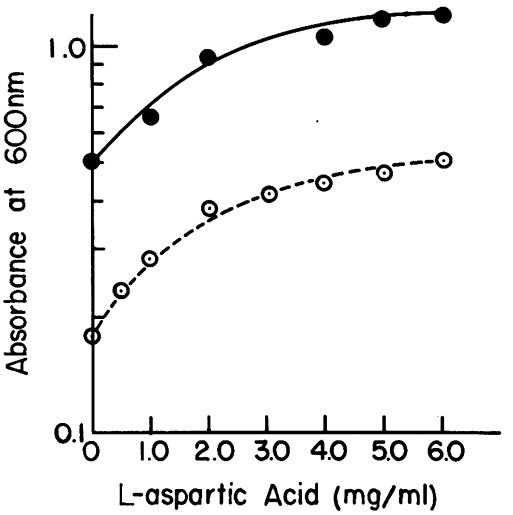

FIG. 1. *Growth responses (48 h) of the saccharolytic (⊙) and asaccharolytic (●) strains of B. melaninogenicus to increasing concentrations of L-aspartate in TYH medium.*

TABLE 2. *Effect of single amino acid additions to TYH medium on the growth of B. melaninogenicus ss. melaninogenicus ATCC 25261*

Amino acid added[a]	Amt of growth at:[b]				
	10 h	20 h	30 h	40 h	50 h
None	0.06	0.10	0.13	0.16	0.20
L-Aspartate, L-asparagine	0.07	0.13	0.20	0.32	0.48
L-Cysteine	0.06	0.11	0.17	0.23	0.30
L-Serine	0.06	0.11	0.16	0.22	0.28
L-Histidine, L-lysine, L-tryptophan, DL-methionine	0.03	0.05	0.06	0.06	0.06
L-Glutamate, L-glutamine	0.05	0.07	0.09	0.10	0.11
Glycine, L-arginine, L-isoleucine, L-leucine, L-proline	0.05	0.07	0.09	0.11	0.13

[a] L Isomers were added in 0.5% concentration, and DL mixture was added in 1% concentration.
[b] Optical density at 600 nm.

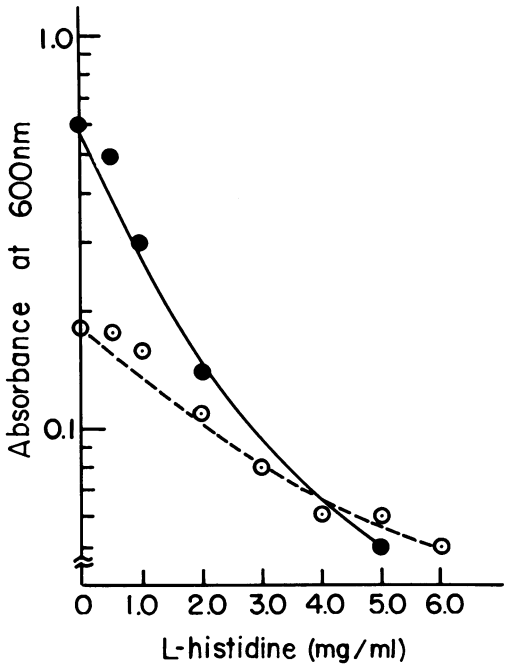

FIG. 2. *Growth responses (48 h) of the saccharolytic (⊙) and asaccharolytic (●) strains of B. melaninogenicus to increasing concentrations of L-histidine in TYH medium.*

Addition of L-alanine, L-hydroxyproline, L-ornithine, L-phenylalanine, or L-threonine to TYH medium did not affect the growth rates or total cell yields of either strain. The asaccharolytic strain showed no change in response to the addition of L-cysteine, L-glutamate, L-glutamine, L-isoleucine, L-leucine, L-lysine, or L-proline. Also, the saccharolytic strain showed no change in response to L-valine.

Growth of the saccharolytic strain in TYH medium was limited. However, the addition of 0.5% glucose to this medium doubled the growth of this organism, suggesting that the limited growth was due to the absence of fermentable sugars. In addition, the effect of each amino acid on growth was not influenced by the presence of glucose in this medium .

Fermentation of L-[^{14}C]aspartate. The stimulation of growth by L-asparate may be due to fermentation. Supporting evidence for this idea was obtained by analyzing the products resulting from the exposure of L-[^{14}C]aspartate to resting-cell suspensions of the asaccharolytic strain (Table 3). Eighty percent of the L-[^{14}C]aspartate added was fermented in 3 h, with 50% of the label appearing in anionic products. Significant amounts of labeled cationic products and $^{14}CO_2$ were also recovered. Recovery of added radioactivity was essentially complete.

DISCUSSION

Many strains of *B. melaninogenicus* grow in media without carbohydrate supplements (20). It has been shown that addition of carbohydrate to basal media did not improve growth of *B. melaninogenicus*, whereas the growth response to Trypticase was proportional to its concentration in the medium (23). Thus, the fermentation of proteinaceous material may be the major energy source in the metabolism of this organism.

In the present study, certain free amino acids added to TYH medium were shown to change the growth rates and final yields of two strains of *B. melaninogenicus*. Some of these amino acids showed variations in effects between the strains. Such results are consistent with the studies showing that these organisms represent a heterogeneous group, both biochemically and serologically (7, 20). It is unlikely that these variations in amino acid effects are directly related to the ability to utilize sugars. However, to make a general statement that the differences in the amino acid responses are related to the overall metabolism of both saccharolytic and asaccharolytic groups, it would be necessary to extend this study by including a greater number of strains representing both groups.

It is interesting to note that Wahren and Gibbons (23) did not find production of labeled volatile fatty acids when their four strains of *B.*

TABLE 3. *Fermentation of L-[^{14}C]aspartate by resting cells of B. melaninogenicus ss. asaccharolyticus NU B26*[a]

Exptl	Radioactivity (cpm)	% Total cpm
L-[^{14}C]aspartate		
Added	7.2599×10^5	100
Fermented	5.8422×10^5	80.5
Products		
CO_2	2.201×10^4	3.0[b]
Anions	3.6876×10^5	50.8
Cations other than L-aspartate	1.9570×10^5	27.0
Total in products[c]	5.8647×10^5	80.8

[a] The reaction mixtures in tubes (20 by 150 mm) contained 150 μmol of potassium phosphate buffer (pH 7.0), 45 μmol of L-[^{14}C]aspartate (specific activity, 16,100 cpm/μmol), 35 mg (dry weight) of cells, and water to 2.8 ml. To trap $^{14}CO_2$, a glass vial containing 0.4 ml of 1 M hydroxide of hyamine in methanol was suspended above the reaction mixture. The reaction was incubated under nitrogen at 37°C for 3 h and was stopped by addition of 0.2 ml of 2 M H_2SO_4.

[b] Values represent percentages of total counts per minute of the L-[^{14}C]aspartate fermented.

[c] Total ^{14}C recovered was 100.3%.

melaninogenicus were grown in the presence of acid-hydrolyzed algal protein uniformly labeled with ^{14}C. However, growth in the presence of denatured algal protein uniformly labeled with ^{14}C did produce labeled volatile fatty acids (23). Other constituents of the growth medium were not included in the report, making it difficult to interpret their results relative to the current findings (23). Wahren and Gibbons interpreted the results as indicating that their strains of *B. melaninogenicus* were more permeable to peptides than free amino acids. The significant stimulation of growth by L-asparate, L-asparagine, L-cysteine, or L-serine in the presence of peptides of Trypticase in this study suggests that for some strains of *B. melaninogenicus* a few amino acids are taken up as readily as peptides.

Of the amino acids tested, L-aspartate and L-asparagine were the only ones that enhanced growth of both strains of *B. melaninogenicus*. Since these amino acids also produced maximal stimulation of growth for both strains, it would be of value to elucidate their metabolic role.

Results obtained in our laboratory from the exposure of L-[^{14}C]asparagine to resting cells of both strains indicate that this amino acid is actively deaminated to L-aspartate (unpublished data). The present study shows L-[^{14}C]aspartate to be actively fermented by resting cells of the asaccharolytic strain. Recovery of radioactivity from the fermentation of L-[^{14}C]aspartate was complete, with 50% of the label appearing as anionic products. We have obtained similar results with the saccharolytic strain of *B. melaninogenicus* (unpublished data).

L-Aspartate might also act as a precursor of heme biosynthesis by reductive reversal of the part of the citric acid cycle leading to succinate, as demonstrated for *Escherichia coli* under anaerobic conditions (8). However, we were unable to grow hemin-starved cells of either strain of *B. melaninogenicus* in TYH medium containing 0.5% L-aspartate in place of hemin (unpublished data).

Since glucose did not influence the enhancement effect of amino acids on the growth of the saccharolytic strain, glucose metabolism apparently does not prevent amino acid fermentation. This is similar to the observation that fermentation of proteinaceous materials by strains of *B. melaninogenicus* and *Fusobacterium nucleatum* was not subjected to repression by glucose (15, 23).

In addition to the growth enhancement produced by a few amino acids, other amino acids were shown to inhibit growth of both strains of *B. melaninogenicus*. Inhibition of bacterial growth by specific amino acids has been shown in autotrophic bacteria (11, 12, 19), as well as in heterotrophic bacteria (2, 10, 13). This sensitivity of *B. melaninogenicus* to certain amino acids may explain why the organism did not grow when Lev et al. (14) attempted to substitute Casamino Acids for Trypticase.

The mechanisms for growth inhibition by these amino acids are not known. Interestingly, the amino acids that inhibited growth of *B. melaninogenicus* in this study are of different chemical groups. For example, the asaccharolytic strain was inhibited by the heterocyclic amino acids, L-histidine and L-tryptophan, by the aliphatic amino acids, glycine and DL-valine, and by DL-methionine, a sulfur-containing compound. A common inhibitory mechanism is unlikely. Also, it is unlikely that competitive inhibition of uptake of required amino acids is occurring, because the growth studies were done in the presence of Trypticase, which has few free amino acids. It is possible that competitive inhibition between amino acids and peptide transport may exist, since it is known that peptides are used by *B. melaninogenicus* (23). However, peptide transport in *E. coli* is independent of amino acid transport (18).

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