Uptake and Incorporation of Amino Acids and Peptides by Bacteroides amylophilus

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Bacteroides amylophilus H-18 demonstrated a higher growth yield, a slightly higher growth rate, and a diminished lag period when Tryptose was added to the basal medium. This uptake of labeled amino acids was concentration-dependent, as the contribution of exogenous amino acid to the cell protein increased from 15.4 to 24.1% when the concentration of Casamino Acids in the medium was increased from 1.4 to 2.8 mg/ml. There was considerable redistribution of $^{14}$C-label to other amino acids. Tryptic peptides of casein competed effectively with the amino acids for uptake. The $^{14}$C-label from a protein was incorporated into B. amylophilus H-18 cells presumably after breakdown of the protein by the B. amylophilus H-18 protease.

The well established requirement of Bacteroides amylophilus H-18 for NH$_4^+$ (1, 3, 5, 7) and the evidence that $^{15}$N-NH$_4^+$ contributed to 93% of the cell nitrogen when grown in a Tryptose medium (6) indicated that this bacterium probably incorporated insignificant amounts of organic nitrogen. This dependence on NH$_4^+$ raised the question of the real function of the protease produced by B. amylophilus H-18, since the protease was probably not produced for the hydrolysis of proteins, so that the products might be taken up by the cells. In the present investigation the growth characteristics of B. amylophilus H-18 were studied in minimal and supplemented media, and the incorporation of $^{14}$C-amino acids was followed. The results indicated that organic nitrogen compounds were incorporated to a limited extent.

MATERIALS AND METHODS

The growth medium was based on that of Hungate (8) and contained (grams per liter) K$_2$HPO$_4$, 0.45; KH$_2$PO$_4$, 0.45; (NH$_4$)$_2$SO$_4$, 0.9; NaCl, 0.9; MgSO$_4$, 0.09; CaCl$_2$, 0.09; resazurin, 0.001; Na$_2$S·9H$_2$O, 0.25; ascorbic acid, 0.50; and NaHCO$_3$, 5.0. The mineral and resazurin solutions plus any other additions to the medium were placed in a liter bottle, the volume made up to 900 ml with distilled water. The medium was autoclaved at 120 C for 15 min; the top was tightly closed immediately upon removal from the autoclave to maintain anaerobic conditions. The solutions of sodium bicarbonate (10.0%), sodium sulfide (1.0%), and ascorbic acid (2.0%) were filtered through a membrane filter (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.); these solutions were added to the medium under a stream of oxygen-free CO$_2$. The medium was dispensed into smaller tubes and used for bacterial growth. In the experiments shown in Fig. 1 and 2, L-cysteine·HCl (0.5 g/liter) was used to lower the Eh instead of ascorbic acid and sodium sulfide. All inoculations were made in an atmosphere of CO$_2$. Two media were used: medium M contained the basal salts plus 0.3% (w/v) maltose, and medium MT contained in addition 0.1% (w/v) Tryptose (Difco, Detroit, Mich.). Amino acids were obtained from Nutritional Biochemical Corp., Cleveland, Ohio. $^{14}$C-L-tryptophan was obtained from Nuclear-Chicago Corp., Des Plaines, Ill., whereas other radioactive amino acids and $^{14}$C-maltose were purchased from Schwarz BioResearch Inc., Orangeburg, N.Y., as the uniformly labeled $^{14}$C-product. The radioactive amino acids were checked for purity by thin-layer chromatography (9).

B. amylophilus strain H-18 is a gram-negative, anaerobic, proteolytic microorganism isolated from a sheep rumen (2). Stock cultures were maintained in semisolid MT medium at 4 C for 3-week periods.

Cell density was measured at 660 nm in a Spectronic 20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.). Since the bacterium died rapidly after entering the stationary phase, care was taken to make inoculations from log phase cultures. In the uptake experiments one drop of a log phase culture was inoculated into 6.0 ml of medium.

The uptake of $^{14}$C-amino acids and peptides was studied in 6.0-ml amounts of MT medium. One-milliliter samples were filtered through membrane filters (pore size, 0.45 μm; Millipore Corp.) and were washed once with 2.0 ml of medium M. Radioactivity in the cells was counted in a model 72 scintillation counter (Nuclear-Chicago Corp.) after placing the dried filters in scintillation fluid.

The distribution of $^{14}$C-label in the cell protein was determined by thin layer chromatography (9). The
cells from 6.0 ml of MT medium were harvested by centrifugation at 9,000 × g for 10 min in a refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). They were washed in mineral medium and were suspended in 3.0 ml of 6.0 n HCl. The protein was hydrolyzed for 18 hr at 110 C in sealed evacuated glass vials. The HCl was then removed under vacuum, and the residue was dissolved in distilled water. Samples were quantitatively applied to cellulose thin-layer plates (Cellulose Powder MN300, Macherey Nagel and Co., Germany). The amino acids were separated by a two-dimensional technique (9) using 2-propanol:formic acid:water (40:20:10, v/v) and t-butanol:methylethyl ketone:ammonia:water (50:30:10:10, v/v) solvents. The chromatograms were exposed for 1 to 2 weeks to medical X-ray film (Eastman Kodak Co., Rochester, N.Y.). The films were developed, and the radioactive areas on the chromatograms were located. These areas were loosened and were drawn by vacuum into scintillation fluid, and the radioactivity was counted. The addition of 5 to 60 mg of cellulose caused no increase in quenching under these conditions. Replicate thin-layer plates were sprayed with ninhydrin- cellulose chromagenic reagent (9) to locate and identify the amino acids. Ten µleters of standard solutions of amino acids [2 µg/ml in aqueous 10% (v/v) 2-propanol] were run to verify the positions of the amino acids.

14C-protein was prepared from B. amylophilus H-18 cells grown in 100 ml of MT medium containing 0.5 µCi of 14C-maltose per ml. The log-phase cells were harvested and heated at 80 C for 15 min in 10% trichloracetic acid to remove nucleic acid and were washed in three volumes of acetone. The protein was extracted in 2% Na2CO3. The solution contained 1.4 mg of protein, 0.02 µCi of label/ml.

The cells from a 50-ml mid-log phase in M medium were sedimented at 9,000 × g for 10 min. They were washed twice with distilled water and resuspended in 5.0 ml of distilled water. One-milliliter samples were taken for dry weight, protein determination (10), and amino acid analysis (11) by using a Beckman model 120 C amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.). The results are presented in Table 1 and were used in calculating the contribution of exogenous amino acid to cell proteins.

RESULTS

Effect of organic nutrient on growth. Preliminary experiments demonstrated that although the addition of casein hydrolysate (0.65 to 3.3 mg/ml) to the basal M medium only slightly increased the growth rate, there was a decrease in the lag phase and a slight increase in cell yield. The addition of Tryptose had the same effect. This effect was further investigated by shift-up and shift-down growth conditions (Fig. 1). Again, it was demonstrated that higher cell yields were attained in the Tryptose medium; there was also an indication that slightly higher growth rates were possible in the Tryptose medium. The length of the lag period was less for cells grown initially in MT medium, even when growth was

| Table 1. Amino acid composition of Bacteroides amylophilus H-18 protein* |

| Amino acids   | Mg/100 mg of total amino acids |
|---------------|--------------------------------|
| Alanine       | 7.16                           |
| Arginine      | 5.59                           |
| Aspartic acid | 10.50                          |
| Cysteine      | 0.0                            |
| Glutamic acid | 12.59                          |
| Glycine       | 7.43                           |
| Histidine     | 2.19                           |
| Isoleucine    | 6.46                           |
| Leucine       | 8.25                           |
| Lysine        | 10.08                          |
| Methionine    | 2.58                           |
| Phenylalanine | 4.78                           |
| Proline       | 4.02                           |
| Serine        | 3.12                           |
| Threonine     | 5.02                           |
| Tyrosine      | 4.27                           |
| Valine        | 7.02                           |

* The results are the average of two analyses. An OD660 of 1.0 corresponded to 0.98 mg (dry weight) and 0.44 mg of protein per ml.

Fig. 1. Shift-up and shift-down growth conditions. Cultures of Bacteroides amylophilus H-18 were grown in maltose (M) and maltose Tryptose (MT) media to an OD660 of 0.6. The cells were centrifuged at 9,000 × g for 10 min, washed in M medium, and resuspended to the original volume of 7.0 ml in M medium. From these suspensions, 1.0 ml was added to each of three 4.0-ml amounts of both M and MT media. The average OD660 for each of the four series was plotted against time of incubation. The mean generation times in minutes are in brackets. The inset shows growth over the first 50 min.
continued in M medium, than it was for cells grown initially in M medium and then inoculated into MT medium. These observations suggested that Tryptose supplied greater amino acid pools or maintained beneficial intracellular conditions which allowed the cells to adjust more rapidly to a new environment and that organic nitrogen compounds played a role in the nutrition of B. amylophilus H-18, so its capacity to take up amino acids was investigated.

Uptake of $^{14}$C-label from an amino acid mixture. The first questions to be answered were whether B. amylophilus H-18 was permeable to amino acids and to see if intracellular pools might be established. Five minutes after the addition of $^{14}$C-amino acids (Fig. 2), 14% of the label in the amino acids was taken up by the cells. The cell volume in 1.0 ml of culture was calculated from the dry weight of cells multiplied by five to get the wet weight of cells. This figure of $25 \times 10^{-4}$ ml, when divided into the ratio of label inside to that outside the cells (14/86) gave the degree of concentration as 65-fold. A total cell volume of $5 \times 10^{-4}$ ml was also calculated from the individual volume of a cell (10$^{-12}$ ml) and the number of cells present ($5 \times 10^8$). This represents a 326-fold concentration.

Uptake of individual $^{14}$C-amino acids. The amino acid concentration in the medium was in-

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**TABLE 2. $^{14}$C-amino acid uptake from casein hydrolysate**

| Amino acids | Casein hydrolysate, 1.4 mg/ml | Casein hydrolysate, 2.8 mg/ml |
|-------------|--------------------------------|--------------------------------|
|             | $^{14}$C-label incorporated (%) | Incorporation $^b$ (µg/100 µg of protein) | $^{14}$C-label incorporated (%) | Incorporation $^b$ (µg/100 µg of protein) |
| Arginine    | 8.1                            | 4.7                              | 2.1                            | 4.3                              |
| Aspartic acid | 3.0                            | 3.2                              | 1.4                            | 2.9                              |
| Glutamic acid | 2.0                            | 6.6                              | 3.0                            | 1.3                              |
| Histidine   | 3.2                            | 1.6                              | 0.7                            | 2.6                              |
| Isoleucine  | 2.9                            | 2.4                              | 1.1                            | 3.4                              |
| Leucine     | 2.5                            | 3.5                              | 1.6                            | 1.9                              |
| Methionine  | 1.9                            | 0.9                              | 0.4                            | 1.7                              |
| Phenylalanine | 3.0                            | 2.5                              | 1.1                            | 2.8                              |
| Proline     | 1.0                            | 1.6                              | 0.7                            | 1.2                              |
| Serine      | 3.7                            | 3.1                              | 1.4                            | 2.9                              |
| Tyrosine    | 2.4                            | 2.2                              | 1.0                            | 2.1                              |
| Valine      | 1.8                            | 1.9                              | 0.9                            | 1.4                              |
| Total       | 15.4                           |                                  | 24.1                           |                                  |

$^a$ One-tenth milliliter of a log-phase culture of Bacteroides amylophilus H-18 was inoculated into tubes of 6.0 ml of medium M, containing either 1.4 or 2.8 mg of casein hydrolysate/ml and in addition 0.018 µCi of individual $^{14}$C-amino acids. The cultures were grown to an OD$_{660}$ of 0.5, and the radioactivity in the cells was counted.

$^b$ Calculated from the known content of each amino acid in casein hydrolysate.

$^c$ Calculated from the known cell protein content and the amount of exogenous amino acid incorporated.
Table 3. Uptake and conversion of amino acids

| Amino acids | Incorporated directly (%) | In other amino acids (%) |
|-------------|---------------------------|-------------------------|
| Alanine     | 11                        | 89% Unknown             |
| Arginine    | 45                        | 45% Glu; 4% Orn; 6% unknown |
| Aspartic acid | 7                        | 93% Unknown             |
| Cysteine    | 0                         | 0                       |
| Glutamic acid | 60                       | 15% Pro; 15% Arg; 1% Lys; 8% unknown |
| Glycine     | 2                         | 4% Glu, Thr, Ser; 94% unknown |
| Histidine   | 100                       | 100% Unknown (3)        |
| Isoleucine  | 0                         | 0                       |
| Leucine     | 100                       | 0                       |
| Methionine  | 2                         | 3% Leu, Ile, Val; 95% unknown |
| Phenylalanine | 0                    | 0                       |
| Proline     | 100                       | 0                       |
| Serine      | 1                         | 99% Unknown (4)         |
| Threonine   | 18                        | 42% Gly; 20% Ileu; 20% Tyr |
| Tryptophan  | 18                        | 82% Unknown (6); Asp and Trp |
| Tyrosine    | 76                        | 24% Unknown (2)         |
| Valine      | 85                        | 15% Met, Leu            |

One-tenth milliliter of a log-phase culture of Bacteroides amylophilus H-18 was inoculated into 6.0-ml amounts of medium M, each containing 0.1 mg of an individual 14C-amino acid (0.1 μCi/ml). The cultures were grown to OD660 of 0.5. The cells were sedimented at 9,000 × g and acid hydrolyzed, and the amino acids were separated by thin-layer chromatography. The 14C-amino acids were detected by autoradiography, removed to scintillation fluid, and counted. Radioactive spots which did not correspond to the common amino acids were classified as "unknowns."

Uptake and conversion of amino acids. Preliminary experiments were performed by growing cells in 14C-maltose medium M (reduced by cysteine) to confirm that radioautography could locate 14C-labeled amino acids in protein hydrolysates. All the amino acids, with the exception of cysteine, were found to be labeled. Cysteine was labeled in a medium reduced by ascorbic acid and sodium sulfide. The distribution of 14C-label from individual amino acids was examined (Table 3). The concentration of the amino acid was low (0.1 mg/ml) so that the specific activity was high enough to label other amino acids derived from it. Histidine, leucine, and proline were incorporated without any interconversion to other amino acids. Quite large amounts of arginine, glutamic acid, tyrosine, and valine were incorporated directly. The major portion of alanine, aspartic acid, glycine, isoleucine, methionine, serine, threonine, and tryptophan was converted to other products, often unidentified. We anticipated that those amino acids which were extensively converted to amino acids and other products within the cell would be taken up in greater quantities. There was, however, no correlation between the degree of interconversion and the amount incorporated, as seen in Table 2. No explanation can be offered for the pathways by which some of the derivatives arose.

Uptake of individual 14C-amino acids in the presence of Tryptose. Individual 14C-amino acids were added at a high specific activity and low concentration of cysteine, were found to be labeled. Cysteine was labeled in a medium reduced by ascorbic acid and sodium sulfide. The distribution of 14C-label from individual amino acids was examined (Table 3). The concentration of the amino acid was low (0.1 mg/ml) so that the specific activity was high enough to label other amino acids derived from it. Histidine, leucine, and proline were incorporated without any interconversion to other amino acids. Quite large amounts of arginine, glutamic acid, tyrosine, and valine were incorporated directly. The major portion of alanine, aspartic acid, glycine, isoleucine, methionine, serine, threonine, and tryptophan was converted to other products, often unidentified. We anticipated that those amino acids which were extensively converted to amino acids and other products within the cell would be taken up in greater quantities. There was, however, no correlation between the degree of interconversion and the amount incorporated, as seen in Table 2. No explanation can be offered for the pathways by which some of the derivatives arose.

Table 4. Uptake of 14C-amino acids in the presence of Tryptose

| Amino acids | External concen (mg/ml) | 14C-label incorporated from medium M (%) | 14C-label incorporated from medium MT (%) |
|-------------|------------------------|-----------------------------------------|-----------------------------------------|
| Alanine     | 0.082                  | 16.0                                    | 3.0                                     |
| Arginine    | 0.084                  | 16.2                                    | 2.3                                     |
| Glutamic acid | 0.074              | 16.7                                    | NR b                                   |
| Glycine     | 0.090                  | 10.0                                    | 14.5                                    |
| Histidine   | 0.042                  | 9.2                                     | 3.3                                     |
| Leucine     | 0.064                  | 20.1                                    | 4.4                                     |
| Lysine      | 0.061                  | 3.3                                     | 1.4                                     |
| Methionine  | 0.110                  | 23.4                                    | 11.4                                    |
| Phenylalanine | 0.038              | 17.6                                    | 8.3                                     |
| Proline     | 0.048                  | 20.0                                    | 3.3                                     |
| Serine      | 0.072                  | 5.5                                     | 1.0                                     |
| Threonine   | 0.076                  | 2.9                                     | 1.7                                     |
| Tyrosine    | 0.040                  | 5.3                                     | NR                                      |
| Valine      | 0.074                  | 16.0                                    | 2.9                                     |

Average: 13.4…… 4.8

a One-tenth milliliter of log-phase culture of Bacteroides amylophilus H-18 was inoculated into 6.0-ml amounts of medium M containing 0.083 μCi/ml of individual amino acids at the concentration specified in column one. Tryptose (1.5 mg/ml) was added to a duplicate set of cultures. The cultures were grown to OD660 of 0.6, and the radioactivity in the cells was counted.

b No result.
concentration to media with and without Tryp- 
tose (Table 4). The incorporation of each amino acid, with the exception of glycine, was inhibited by Tryp- 
tose. The average incorporation was re- 
duced from 13.4 to 4.8%. The contribution of the 
amino acids to the bacterial protein, at this low 
concentration in the medium, was small and did 
not exceed 1.0%. It was, therefore, impossible to 
deduce to what extent the Tryphtose peptides 
contributed to the cell protein. The experiment clearly 
demonstrated, however, that competition between 
the peptides and amino acids occurred.

Uptake of \(^{14}\)C-peptides. To determine \(^{14}\)C-pep- 
tide uptake by *B. amylophilus* H-18, 0.1 ml of 
log-phase cells was inoculated into 6 ml of 
medium M containing 0.14 mg of \(^{14}\)C-protein (0.002 
\(\mu \)Ci) per ml. The culture was grown to OD\textsubscript{660} of 
0.6, and the radioactivity in the cells was counted. 
The \(^{14}\)C-protein peptides contributed to 17% of 
the protein synthesized by *B. amylophilus* H-18 in 
this supplemented medium. The \(^{14}\)C label 
incorporated was 24%, protein incorporated was 
34 \(\mu \)g/ml (calculated from 24% of 140 \(\mu \)g of 
protein), and protein incorporated was 17 \(\mu \)g/100 
\(\mu \)g of cell protein (calculated from the protein in- 
corporated and the known protein content of the 
cells).

DISCUSSION

Contrary to expectations that \(\text{NH}_4^+\) would be 
the sole source of cell nitrogen (6), we found that 
some incorporation of \(^{14}\)C-amino acids occurred. 
The degree of concentration of \(^{14}\)C-label within 
the cell was indicative of active transport. The 
possibility of nonspecific adsorption to the cell 
surface cannot be discounted. Subsequent ex- 
eriments (Table 2) failed to demonstrate that 
any individual amino acid contributed to more 
than a small portion of the total cell protein. The 
kinetics of the uptake were not studied, but the 
total contribution of exogenous amino acids to 
cellular protein was increased from 15.4% to 
24.1% when the concentration of the added hy- 
drolysate was increased from 1.4 to 2.8 mg/ml. 
There was a considerable degree of interconver- 
sion to other amino acids before incorporation into 
protein occurred. The role of the protease in 
the nutrition of *B. amylophilus* H-18 is still in 
doubt. There was evidence that tryptic peptides 
competed with \(^{14}\)C-amino acids for incorporation 
and the protease of *B. amylophilus* H-18 has a 
tryptic-type specificity (2). There was also evi- 
dence that \(^{14}\)C-protein in the growth medium was 
incorporated into cell protein and contributed to 
17% of the cell protein. It is presumed that the 
protease played some role in hydrolyzing the pro- 
tein before its uptake and incorporation. This 
would indicate that the protease can play a part 
in the nutrition of the microorganism. The total 
contribution (17%) to the cell protein was, how- 
ever, small, and there is the possibility that \(^{14}\)C-
protein or \(^{14}\)C-peptides adhered to the outer cell 
surface. The obligate \(\text{NH}_4^+\) requirement was not 
replaced by other forms of nitrogen.

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LITERATURE CITED

1. Abou Akkada, A. R., and T. H. Blackburn. 1963. Some ob- 
servations on the nitrogen metabolism of rumen proteo-
lytic bacteria. J. Gen. Microbiol. 31:461–469.
2. Blackburn, T. H. 1968. The protease liberated from Bacte-
rioides amylophilus strain H18 by mechanical disintegra-
tion. J. Gen. Microbiol. 31:37–51.
3. Blackburn, T. H., and P. N. Hobson. 1962. Further studies 
on the isolation of proteolytic bacteria from the sheep rumen. J. Gen. Microbiol. 29:69–81.
4. Britten, R. J., and F. T. McClure. 1962. The amino acid pool 
in Escherichia coli. Bacteriol. Rev. 26:292–335.
5. Hamlin, L. J., and R. E. Hungate. 1956. Culture and physi-
ology of a starch-digesting bacterium (Bacteroides amylo-
philus n.sp.) from the bovine rumen. J. Bacteriol. 72:548–
554.
6. Hobson, P. N., I. J. McDougall, and R. Summers. 1967. The 
nitrogen sources of Bacteroides amylophilus. J. Gen. 
Microbiol. 50:1.
7. Hobson, P. N., and R. Summers. 1967. The continuous culture 
of anaerobic bacteria. J. Gen. Microbiol. 47:53–65.
8. Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic 
bacteria. Bacteriol. Rev. 14:1–49.
9. Jones, K., and J. G. Heathcote. 1966. The rapid resolution of 
naturally occurring amino acids by thin-layer chromatogra-
phy. J. Chromatogr. 24:106–111.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Ran-
dall. 1951. Protein measurement with the Folin phenol re-
agent. J. Biol. Chem. 193:265–275.
11. Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic 
recording apparatus for use in the chromatography of 
amino acids. Anal. Chem. 30:1190–1206.