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Amino Acid and Peptide Requirement of *Fusiformis necrophorus*

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Uptake of individual amino acids and peptides by *Fusiformis necrophorus* was studied in growing cultures and resting cell suspensions. The cells were able to incorporate 16 of 17 ^{14}C-labeled amino acids into cell protein, the exception being proline. Proline could neither be formed by the cells from any of the other tested amino acids nor be synthesized from glucose or serine when these were used as energy sources. The addition of di- and tripeptides, the octapeptides vasopressin and oxytocin, and the poly (24) peptide ACTH did not stimulate cell growth, but a marked stimulatory effect was noted after the addition of poly-L-proline (mean molecular weight 2,000). It is concluded that cells of *F. necrophorus* (i) possess transport systems for most amino acids but not for proline, (ii) are dependent on exogenous proline in the form of proline-containing peptides for growth, and (iii) may be cultivated in a defined amino acid medium provided the proline requirement is met by the addition of a proline-containing peptide.

A number of microorganisms are known to depend on the availability of small peptides for growth, the peptide providing a supply of amino acids in a readily utilizable form (2). Most bacteria possess a separate transport system for each amino acid, whereas peptides appear to be transferred across the cell membrane by less selective mechanisms, one for di- and tripeptides and another for oligopeptides and those with longer chains (9).

Most of the experience in this field has been gained from *Escherichia coli* and lactobacilli (for a recent review, see reference 9). Of the *Bacteroidaceae*, only *Bacteroides ruminicola* has been studied in some detail with regard to its ability to utilize different sources of nitrogen. This microorganism has the capacity to take up both ammonia and peptides, but it was unable to utilize free amino acids (10). Subsequent studies with ^{14}C-labeled compounds have confirmed that *B. ruminicola* may utilize oligopeptides with a molecular weight of up to about 2,000, but that proline and glutamic acid fail to enter the cell (11). Similarly, *B. melaninogenicus* has only a limited ability to ferment amino acids whereas peptides are readily used (15).

Fusiformis necrophorus has been cultivated with good cell yields, batchwise (16) as well as continuously (14), using complex media with tryptone as the main nitrogen source. It would facilitate studies of the energy metabolism of this microorganism if the cells were grown in a defined medium. This study was therefore designed to define the amino acid and peptide requirements for growth of *F. necrophorus*.

MATERIALS AND METHODS

Strains used. *F. necrophorus* (NCTC 7155) Moore and Holdeman (*Bergey's Manual of Determinative Bacteriology*, 8th edition, in press) was used in this study.

Cultivation methods. The bacteria were grown in 200-ml batch cultures. The growth vessel was a glass flask closed with a rubber stopper. Two glass tubes were inserted through the stopper, one connected to a rubber balloon containing a mixture of N_2 and CO_2 (95 plus 5%, respectively), the other via a filter connected to an outlet. To insure an anaerobic atmosphere, the gas mixture was passed through the vessel to replace the air above the medium before the outlet was closed. The connection to the gas reservoir was left open during cultivation. The medium was inoculated before the rubber stopper was inserted. All cultures were inoculated to a density of approximately 0.1 mg (dry weight)/ml using a culture which had grown overnight in the same medium as used in the batch culture. The cultures were incubated on a magnetic stirrer at 37 C, and samples were taken from a sealed outlet near the bottom of the flask.

Test tube cultures used in the growth stimulation experiments were grown in 10 ml of pre-reduced medium in rubber-stoppered test tubes (140 by 15 mm) which were flushed with N_2 and CO_2 (95 plus 5%, respectively) during inoculation and then incubated at 37 C for 18 h.

Dry weights were determined on 10-ml samples which were washed once in 0.01 M phosphate buffer (pH 7.0) and dried at 100 C before weighing. All dry weights were corrected for the weight of the buffer salts.

Culture media. The complex medium contained per liter: tryptone (Oxoid), 15 g; NaCl, 2.5 g; KH_2PO_4, 3 g; $Na_2HPO_4 \cdot 2H_2O$, 7 g; L-cystein (Merck), 0.5 g; $MgSO_4$, 0.2 g; adenine (Merck), 50 mg; thymine (NBC), 5 mg; guanine (Fluka A.G.), 15 mg; cytosine (Merck), 15 mg; xanthine (Merck), 15 mg; uracil (Merck), 15 mg; B vitamin mixture (ACO, Sweden), 1 ml; trace element solution (5), 0.25 ml; polypropylene glycol P-2000 (Dow Chemicals, Midlands, Mich.), 0.05 ml; and glucose, 10 g. Polypropylene glycol was required to prevent foaming. To the sterilized mixture of tryptone, NaCl, and buffer salts, all other ingredients were added after being sterilized separately. The pH of the medium was 7.0.

An amino acid medium was prepared from the complex medium, tryptone being replaced by amino acids as follows (per liter): L-alanine, 0.1 g; L-arginine, 0.2 g; L-asparagine, 0.3 g; L-cystine, 0.5 g; L-glutamic acid, 0.3 g; glycine, 0.1 g; L-histidine, 0.2 g; L-isoleucine, 0.1 g; L-leucine, 0.1 g; L-lysine, 0.1 g; L-phenylalanine, 0.1 g; L-proline, 0.1 g; L-serine, 0.1 g; L-threonine, 0.1 g; L-tryptophane, 0.1 g; L-valine, 0.1 g; L-glutamine, 0.1 g; L-hydroxyproline, 0.1 g; L-cysteine, 0.5 g. All amino acids were obtained from Merck, sterilized by filtration, and added separately. The following peptides were tested for growth-stimulating effect: L-prolyl-L-tyrosine, L-prolyl-L-glycine, L-prolyl-glycyl-glycine, and poly-L-proline (mean mol wt 2,000) (Sigma Chemical Co.); L-threonyl-L-phenylalanyl-L-proline (kindly provided by R. Jost Institute fur Molekularbiologie der ETH, Zürich, Switzerland); ACTH fragment 1-24 and ACTH fragment 1-11 (kindly provided by W. R. Rittel, Ciba-Geigy AG, Switzerland); and Phe^2-Lys^8-vasopressin and oxytocin (obtained as a gift from Sandoz A.G., Switzerland).

Resting cell suspensions. Resting cell suspensions were prepared from 200-ml cultures grown in the basal medium and harvested after 4 h at an absorbancy (550 nm; light path, 10 mm) of approximately 1.9, corresponding to a cell dry weight of about 0.6 mg/ml. The cells were washed once in freshly prepared complex medium minus tryptone and resuspended in the same medium. Chloramphenicol was added to a concentration of 0.1 mg/ml. Experiments with resting cells were performed in rubber-stoppered test tubes which were flushed with N_2 and CO_2 (95 plus 5%, respectively) and incubated in a water bath at 37 C. The final cell concentration in the resting cell suspensions was approximately 0.2 mg/ml.

Amino acid uptake. Uptake of free amino acids was studied with ^{14}C-uniformly labeled L-amino acids (New England Nuclear Corp.). The following ^{14}C-labeled amino acids were tested in growing cultures: L-alanine, L-arginine, L-asparagine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-tryptophane, and L-valine. The cells were grown in 200-ml cultures using the complex medium with the tryptone concentration reduced to 5 g/liter and with yeast extract (Difco) (5 g/liter) instead of the purines, pyrimidines, and vitamins. When the culture had reached a cell dry weight of approximately 0.4 mg/ml (usually after 3 to 4 h of cultivation), individual ^{14}C-labeled amino acids (0.01 μCi/ml) were added. Samples were then collected after 0, 15, 30, 60, 120, 180, and 240 min for determination of amino acid radioactivity. The samples were handled and the determinations were performed as described by Britten and McClure (1). Membrane filters (Millipore Corp.; type HA) were used for filtering trichloroacetic acid-insoluble material. After the filters had been washed twice with 5% trichloroacetic acid and placed in vials containing 10 ml of dioxane-based scintillation fluid, ^{14}C-activity was determined in a liquid scintillation counter (Nuclear-Chicago Mark II).

The individual ^{14}C-labeled L-amino acids were added to the resting cell suspensions. Samples were removed after 10 s and after 5 min and filtered with Millipore HA filters. The filters were washed three times with a solution containing (per liter): NaCl, 2.5 g; KH_2PO_4, 3 g; $Na_2HPO_2 \cdot 2H_2O$, 7 g; $MgSO_4$, 0.2 g; and a trace element solution, 0.25 ml; and ^{14}C-activity was measured as described above. In growth experiments designed to study the dependence of the cells on exogenous proline, the following ^{14}C-labeled compounds (New England Nuclear Corp.) were used: D-glucose-*U*-^{14}C; L-proline-*U*-^{14}C; and L-serine-*U*-^{14}C; and L-amino acid-*U*-^{14}C mixture containing: L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, and L-valine (NEC-445).

Chemical analyses. Cell protein was precipitated by 5% trichloroacetic acid boiled for 30 min, and then centrifuged at 5,700 rpm for 20 min. The precipitate was washed once in cold 5% trichloroacetic acid. For amino acid analyses, the protein was hydrolyzed by the method of Heathcote and Haworth (4). Salt and peptides were removed from the hydrolysate by ion exchange chromatography using a BioRad AG11A8 column by the method of Heathcote et al. (3). The amino acids were separated by thin-layer chromatography as described by Heathcote and Haworth (4). When the plates had been developed, the individual ninhydrin- or isatin-reactive areas were scraped into test tubes containing 3.5 ml of distilled water. After thorough mixing on a Vortex mixer, the samples were transferred to vials containing 11.5 ml of Aqua-Sol (New England Nuclear Corp.), shaken to obtain a gel, and counted in a liquid scintillation counter. Quantitative protein analysis was performed by the method of Lowry et al. (7).

RESULTS

When grown in the basal medium, *F. necrophorus* 7155 reached a cell dry weight of 1.5 to 2.0 mg/ml in 8 to 10 h. The nitrogen source of the medium was tryptone. In an attempt to compose a defined growth-supporting medium

for *F. necrophorus*, tryptone was replaced unsuccessfully by a mixture of free amino acids. Growth was not supported when tryptone was replaced by acid-hydrolyzed casein (Casamino Acids, 15 g/liter; Difco). These results all indicate that for growth *F. necrophorus* has an obligatory requirement for peptides.

Amino acid uptake. Studies with ^{14}C-labeled amino acids were performed in growing cultures and in resting cell suspensions to determine whether free amino acids could enter *F. necrophorus* cells. All of the ^{14}C-labeled amino acids were studied individually in growing cultures. The results from these experiments demonstrate that, as shown for some of the amino acids in Fig. 1, all amino acids except proline could be taken up by the cells. The ^{14}C-activity of the cell protein increased progressively and to a varying extent during the 3 h after the addition of the labeled amino acids, except in the case of proline. The dry weight of the cells rose from approximately 0.5 to 1.7 mg/ml during this period. The findings suggest that ^{14}C-alanine, ^{14}C-histidine, and ^{14}C-leucine were incorporated less readily than were the other amino acids tested (Fig. 1). To throw more light on the incorporation of alanine, histidine, leucine, and proline into the cells, the uptake of these amino acids was then tested individually in resting cell suspensions, samples being taken after 10 s and after 5 min. The samples were filtered and washed in buffer, and the ^{14}C-radioactivity of the filters was then determined in 5 ml of scintillation fluid. A significant incorporation of histidine was detected after only 10 s at concentrations of 50 to 500 μM labeled histidine (sp act 10 μCi/μmol) but no measurable uptake of either alanine, leucine, or proline. After 5 min, however, marked incorporation was noted for alanine, leucine, and histidine (Fig. 2).

Requirement for exogenous proline. To establish whether cells of *F. necrophorus* require an exogenous source of proline, the following experiments were performed. *F. necrophorus* was grown in a 200-ml batch culture in a modified basal medium (tryptone concentration reduced from 15 to 10 g/liter; glucose concentra-

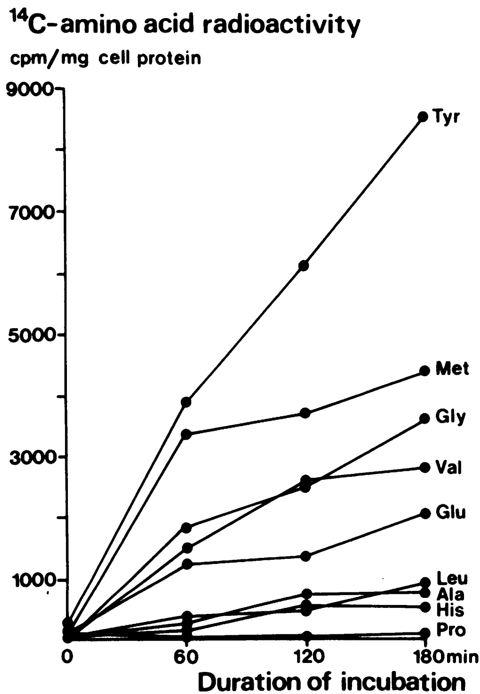

FIG. 1. *Incorporation of ^{14}C-radioactivity into cell protein after incubation of growing cells of F. necrophorus with individual ^{14}C-amino acids. No significant uptake was demonstrable for proline.*

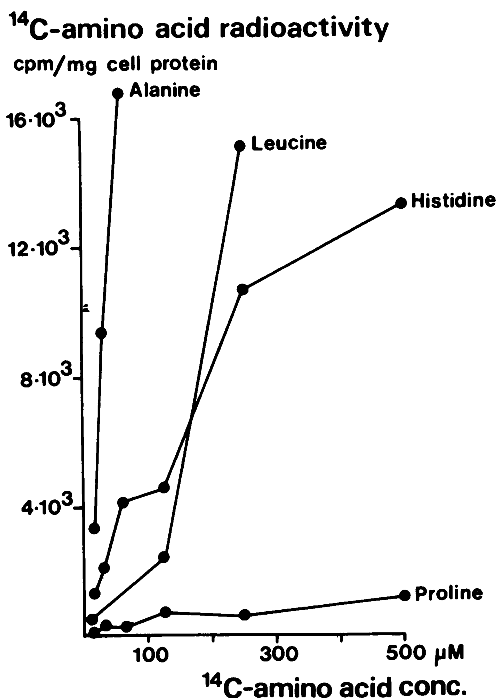

FIG. 2. *Incorporation of ^{14}C-radioactivity into cells of F. necrophorus maintained in resting cell suspensions for 5 min after addition of ^{14}C-labeled alanine, leucine, histidine, and proline, respectively, in rising concentrations. Chloramphenicol was added to a final concentration of 0.1 mg/ml. The cell concentrations were measured as the amount of protein, and the activities are therefore given per milligram of cell protein.*

tion from 10 to 5 g/liter) with the addition of the mixture of ^{14}C-labeled amino acids, described above, including proline (0.02 μCi/ml). After 8 h of cultivation the cells were harvested, the cell protein was analyzed by thin-layer chromatography for the content of ^{14}C-labeled amino acids, and the ^{14}C-radioactivity of the individual spots was determined by liquid scintillator counting. ^{14}C-activity was demonstrated for all the amino acids identified on the thin-layer chromatograms except proline (Fig. 3). The amount of radioactivity found in the spots was approximately five to seven times above the background level. This indicates that none of the added ^{14}C-labeled amino acids was used by the cells for proline synthesis.

Experiments were next performed with ^{14}C-glucose (0.03 μCi/ml) added to the same modified basal medium. The cell protein then contained only minute amounts of ^{14}C-labeled L-glutamic acid and L-phenylalanine (approximately twice the background level), while no radioactivity was detectable for any of the other amino acids (Fig. 4).

Since recent studies have demonstrated that serine may replace glucose as an energy source for *F. necrophorus* (unpublished data), this amino acid was tested as a possible precursor for proline formation. Cells were grown in the basal medium with glucose replaced by serine (10 g/liter) and with the addition of ^{14}C-uniformly labeled serine (sp act, 100 μCi/μmol, 0.02 μCi/ml). The only ^{14}C-labeled amino acid detectable on the thin-layer chromatogram of the hydrolyzed cell protein was serine.

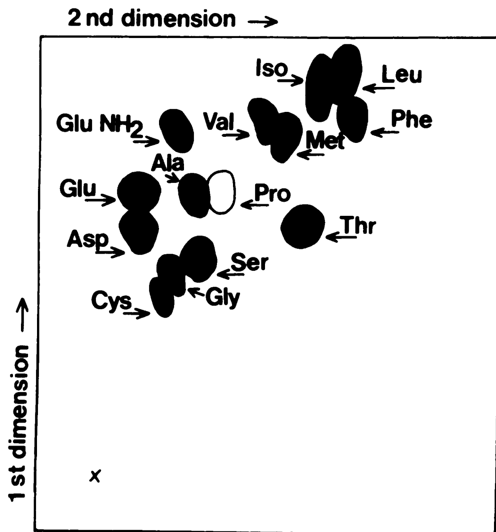

FIG. 3. *Thin-layer chromatogram from a hydrolysate of cells of F. necrophorus grown in a medium containing a mixture of ^{14}C-labeled amino acids. The dark areas mark the spots for those amino acids for which significant radioactivity was detectable. The open area indicates the proline spot which contained no measurable radioactivity.*

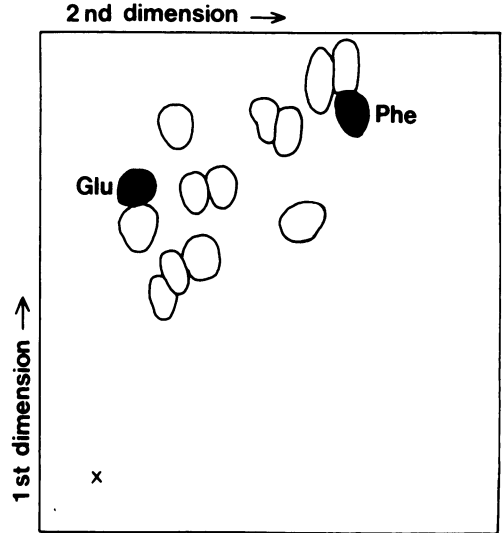

FIG. 4. *Thin-layer chromatogram from a hydrolysate of cells of F. necrophorus grown in a medium containing ^{14}C-glucose. Radioactivity was detectable only in the glutamic acid and phenylalanine spots. For identification of spots see Fig. 3.*

Finally, cells were grown in the basal medium with the addition of ^{14}C-labeled L-proline (0.01 μCi/ml). No measurable radioactivity was found in any of the amino acid spots, including that of proline, on the thin-layer chromatogram of hydrolyzed cell protein.

Growth stimulation experiments. The findings above indicate that proline cannot be synthesized by *F. necrophorus* from either glucose or any of the amino acids tested, which suggests that this species requires exogenous proline. Since the cells are unable to take up proline in its free form, experiments were made to test the growth-stimulating effect of various peptides that contain proline. The basal medium, supplemented with amino acids and with the tryptone concentration reduced (0.5, 1, and 2 mg/ml), and amino acid medium were both used. The peptides were added to a concentration of 0.5 mg/ml. Stimulation of growth was measured as the increase in cell dry weight compared to a control with no peptide added. The cultures were grown for 18 h.

When poly-L-proline (mean mol wt 2,000) was added to the amino acid substrate with different tryptone concentrations, cell growth was stimulated markedly ($P < 0.001$ by paired *t*

test) (Fig. 5). The effect was more pronounced when the concentration of tryptone was low and the medium contained only small amounts of peptides apart from poly-L-proline. In addition, these experiments showed that cells of *F. necrophorus* were able to grow in the amino acid medium, without tryptone, when proline was added in the form of poly-L-proline. Moreover, *F. necrophorus* could be transferred several times in this medium.

Similar experiments were made with several other peptides, including two ACTH fragments (1-24 and 1-11); the 1-24 fragment (containing three proline residues) did not stimulate cell growth significantly, nor did fragment 1-11, which does not contain proline and which was used as a control. Several di- and tripeptides were also tested but did not influence cell growth; neither did the octapeptides Phe^2-Lys^8-vasopressin, Lys^8-vasopressin, and oxytocin (each containing one proline residue).

DISCUSSION

This study demonstrates that *F. necrophorus* has the capacity to utilize all but one of the ^{14}C-labeled amino acids tested, the exception being proline. The rate of uptake of ^{14}C-activity could not be expressed in terms of specific activity since the cells were grown in a complex medium and the exact amount of unlabeled amino acids present was unknown. The results suggested, however, that ^{14}C-labeled alanine, histidine, and leucine were taken up at a lower rate than the other amino acids (Fig. 1). Consequently, the cell uptake of these three amino acids and proline was tested in resting cell suspensions, exposing the cells to considerably higher ^{14}C-activity than in the previous experiments. The results demonstrated that ^{14}C-activity from alanine, histidine, and leucine was readily incorporated (Fig. 2) and that proline was still not taken up in detectable amounts. The inability of *F. necrophorus* to utilize proline as a free amino acid was thus established. The explanation for this finding is not clear. Specific transport systems have been described for a number of individual amino acids in *E. coli* (6), and it is conceivable that *F. necrophorus* lacks the particular system required for transporting proline across the cell membrane.

Subsequent experiments demonstrated conclusively that other amino acids, glucose, and serine, when used separately as energy sources, do not serve as precursors in proline synthesis. Inasmuch as proline is an essential constituent of the cell protein, it follows that *F. necrophorus* has an absolute requirement for exogenous proline. Having noted the failure of proline to enter the bacteria as free amino acid, the question arose whether the cells are able to utilize a proline-containing peptide. This possibility was supported by reports that amino acids and peptides are transported across the cell membrane by different systems (9). Furthermore, separate transfer mechanisms have been reported for dipeptides and oligopeptides, respectively, in *E. coli*. The dipeptide transport system described for *E. coli* requires that both the N-terminal α-amino (or -imino) group and the C-terminal carboxyl group of the peptide are intact, whereas for oligopeptide transport only the former group need be intact (8, 9). Since no comparable information was available regarding dipeptide and oligopeptide transport in *F. necrophorus*, tests were made with L-prolyl-L-tyrosine, L-prolyl-glycine, and L-prolyl-glycyl-glycine. The findings indicate that these proline-containing peptides have no stimulatory effect on growth of *F. necrophorus*, suggesting that they cannot be taken up by the cells. This agrees with the observation of Pittman et al. (11) concerning *B. ruminicola*, for which no utilization of di- and tripeptides was demonstrable. The octapeptides vasopressin and oxytocin did not stimulate growth of *F. necrophorus* either, although they are incorporated by *B. ruminicola* (11). In marked contrast to these results for peptides with short chain lengths, it was found that poly-L-proline (mean mol wt 2,000) exerted a stimulatory effect on cell growth when added to cells growing in a medium containing lowered tryptone concentrations. Moreover, *F. necrophorus* was, in fact,

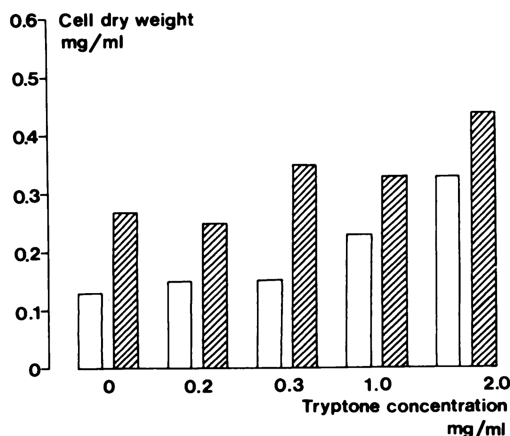

FIG. 5. *Stimulation of cell growth by addition of poly-L-proline (0.5 mg/ml) to cells of F. necrophorus (cross-hatched bars) in comparison to controls (open bars) growing in media with different tryptone concentration.*

able to grow on a defined amino acid medium provided this was supplemented with poly-L-proline. Thus, the failure to cultivate *F. necrophorus* on a synthetic amino acid medium in the present study reflects an absence of a membrane transport system for free proline, coupled with an inability of these cells to synthesize proline. It appears probable that, besides poly-L-proline, other proline-containing peptides of comparable size have a similar growth-stimulating effect. The mechanism whereby poly-L-proline is taken up by the cells may resemble that described for *E. coli* (8), which appears to possess transport systems for di- and oligopeptides with either an α-amino or α-imino group.

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