Transport of Aromatic Amino Acids by *Brevibacterium linens*

P. BOYAVAL,† EVELYNE MOREIRA, AND M. J. DESMAZEAUD

Laboratoire de Microbiologie laitière et Génie alimentaire, INRA-CNRZ, 78350 Jouy-en-Josas, France

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Whole metabolizing *Brevibacterium linens* cells were used to study the transport of aromatic amino acids. Kinetic results followed the Michaelis-Menten equation with apparent $K_m$ values for phenylalanine, tyrosine, and tryptophan of 24, 3.5, and 1.8 μM. Transport of these amino acids was optimum at pH 7.5 and 25°C for phenylalanine and pH 8.0 and 35°C for tyrosine and tryptophan. Crossed inhibitions were all noncompetitive. The only marked stereospecificity was for the t. form of phenylalanine. Transport was almost totally inhibited by carbonyl cyanide-m-chlorophenylhydrazine. Iodoacetate and N-ethylmaleimide were much more inhibitory for tryptophan transport than for transport of the other two aromatic amino acids.

Many aromatic amino acid transport systems have been characterized in gram-negative bacteria. *Salmonella typhimurium* (2) and *Escherichia coli* K-12 (7) have four transport systems for these amino acids. One is common for the three, and there is one specific system for each. *Yersinia pestis* (27) has a general system which is fairly specific for tyrosine, a specific system for tryptophan, and a third relatively specific system for phenylalanine but which also transports tryptophan and tyrosine. Most workers in this field have extended their findings by isolating and studying mutants deficient in one of the transport systems (17, 20, 29). In some cases, the genes responsible for permease synthesis have been localized on the chromosome map. Ames and Roth (3) mapped the loci of histidine permease and the aromatic permease in *S. typhimurium*.

These systems have been studied to a lesser extent in gram-positive bacteria. *Arthrobacter pyridinolis* has two systems for phenylalanine transport (19), and *Bacillus subtilis* has a common system for the transport of phenylalanine and tyrosine (12).

In spite of the importance of *Brevibacterium linens* in the food industry and its considerable economic value, it has not been studied to a great extent. The genus *Brevibacterium* remains classed "genera incertae sedis" in *Bergey's Manual of Determinative Bacteriology* (8). In addition to its intense proteolytic action (13), this microorganism participates in the formation of flavoring compounds or their precursors (25). Among these substances, phenol and indole, which arise from the catabolism of aromatic amino acids by this bacterium (22), are found in large quantities in certain cheeses.

The present work was undertaken to determine the general properties of the aromatic amino acid transport system(s) in *Brevibacterium linens*. This study is in the broader context of the better knowledge of this bacterium to domesticate its action and clarify its taxonomy.

**MATERIALS AND METHODS**

Organism and culture. *B. linens* 47 is an avirulent gram-positive bacterium isolated from French camembert cheese in our laboratory. It was grown at 26°C with shaking in medium R (0.7% Bacto-tryptone [Difco Laboratories, Detroit, Mich.], 0.5% yeast extract [Difco], 0.5% sodium lactate, 0.5% NaCl, and 0.25% K$_2$HPO$_4$). The pH was adjusted to 7.0 before autoclaving for 20 min at 120°C. Growth was monitored by direct microscopic counting, by turbidity measurement at 650 nm (Cary 219 spectrophotometer), and by pH determination. Cultures were routinely checked for contamination by streaking on agar.

Stock cultures. Cultures were maintained on agar slants of medium R at 4°C and were transferred monthly. Individual colonies were inoculated into 100 ml of medium R in 500-ml Erlenmeyer flasks to start cultures.

Preparation of cells for uptake assays. Cells in late-exponential growth were harvested by centrifugation at 2,500 × g for 5 min. The pellet was washed twice with 0.1 M sodium phosphate buffer (pH 8.0) containing 5 g of NaCl per liter. The cells were resuspended to a final density of 2 to 3 optical density units (650 nm) in a prewarmed Erlenmeyer flask with sodium phosphate buffer containing 5 g of NaCl and 8.33 ml of sodium lactate per liter. Cells were always equilibrated in terms of temperature and aerobiosis by shaking for at least 30 min (Tecnam SB16 shaker, 120 strokes per min). The pH was always 8.0 and the temperature 25°C unless indicated otherwise. Each experiment was performed with a freshly prepared suspension.

Transport assays. The method of Britten and McClure (6) was used with some modifications: chloramphenicol was not routinely included in the reaction
mixtures, and the reaction was started by adding 14C-labeled amino acid. At the indicated intervals, 150-μl samples were removed and rapidly filtered through a membrane filter (HA, 0.45 μm pore size; Millipore Corp., Bedford, Mass.). The filters were immediately washed twice with 4.0 ml of prewarmed (26°C) 0.1 M sodium phosphate buffer (pH 8.0) with 5 g of NaCl per liter (7). The filters were then dried under an infrared lamp and placed in scintillation vials containing 8 ml of cocktail (Ready-solv Hp/b; Beckman Instruments, Fullerton, Calif.). Radioactivity was determined in a Beckman LS 9000 liquid scintillation spectrometer with a counting efficiency better than 93% for 14C. The rate of transport was expressed as micromoles of substrate taken up per minute per milligram of dry cell weight.

Inhibition experiments. Before the uptake reaction was started by the addition of 14C-amino acid, cell suspensions were incubated for 10 min in 0.1 M sodium phosphate (pH 8.0) containing 0.5% sodium lactate, 0.5% NaCl, and the metabolic inhibitor studied. Inhibitor concentrations were those used by Moran (21). At various times, 150 μl of suspension was removed during a 30-min incubation and processed as above.

TCA-insoluble components. The method of Brown (7) was used with some modifications. At the same time as portions were sampled for the determination of total uptake, an equal volume was transferred to cold 5% (wt/vol) trichloroacetic acid (TCA). The samples were left for 30 min with periodic vigorous shaking and were filtered through 5% TCA-saturated membrane filters. After two washes with 4 ml of cold 3% TCA, the filters were dried and radioactivity was determined as above.

Dry weights were determined by centrifuging cells at 2,500 × g for 5 min. After two washes with distilled water, 5 ml of the suspensions was dried at 100°C to constant weight.

Inhibition by unlabeled amino acids and structural analogs. Reactions were started by adding appropriate quantities of cells to reaction mixtures containing the 14C-amino acid and the analog or the 13C-amino acid, as indicated. Samples were filtered after a 2-min incubation, and radioactivity was determined. Similar to the findings of Krulwich et al. (19), control experiments showed that the solvents used to dissolve the analogs and the peptides, ethanol, ether, and dimethylformamide had no effect on transport at the concentrations used (less than 1%).

Chemicals. [U-14C]phenylalanine (specific radioactivity, 450 mCi/mmol), [U-14C]tyrosine (specific radioactivity, 388 mCi/mmol), and [2-14C]tryptophan (specific radioactivity, 49 mCi/mmol) were purchased from the C.E.A. (Saclay, France).

Carbonyl cyanide-m-chlorophenylhydrazone (CCCP), iodoacetic acid (IAA), 2,4-dinitrophenol (DNP), and p-hydroxymercuribenzoate (PCMB) were purchased from Sigma Chemical Co., St. Louis, Mo.

All unlabeled amino acids and structural analogs were purchased from Sigma, and the dipeptides were purchased from Cyclo Chemicals, Los Angeles, Calif.

RESULTS

Uptake of aromatic amino acids. The initial rates of uptake of aromatic amino acids by whole B. linens cells were directly proportional to cell concentration (data not shown). This incorporation was reproducible under the conditions used, i.e., with metabolizing cells in the absence of protein synthesis inhibitors.

Energy sources required for phenylalanine uptake. With freshly grown cells, there was no significant difference in uptake as a function of the various carbon sources tested. The energy reserves of the cells were depleted by starvation for 3 h in 0.1 M phosphate buffer (pH 8.0) at 26°C with vigorous shaking. Uptake was then determined as described after adding a carbon source. The five energy sources tested all resulted in a greater initial velocity than the controls (Fig. 1).

Effect of pH on transport. Transport of each of the amino acids was sensitive to pH (Fig. 2). Uptake peak widths did not exceed 2 pH units, and optima were located at pH 8.0 for tyrosine and tryptophan and at 7.5 for phenylalanine.

The maximum incorporated quantities of phenylalanine (0.27 mmol/mg dry weight) were greater than those of tyrosine (0.09 mmol/mg dry weight) for identical exogenous concentrations.

Effect of temperature on transport. Amino acid incorporation by the cells was highly temperature dependent (data not shown). The optimal

![Graph](https://example.com/graph.png)

Fig. 1. Effect of the carbon source on phenylalanine transport by B. linens. The cells were previously incubated for 3 h at 26°C with vigorous shaking in the absence of an exogenous carbon source. The cells were then incubated for 5 min with the indicated carbon source before 0.1 mM L-[U-14C]phenylalanine was added. Symbols: △, control (no carbon source); ■, glucose; □, sodium lactate; ○, glycerol; ▽, lactose; ▼, galactose.
temperature was 25°C for phenylalanine, but transport was significant between 15 and 45°C. The optima for tyrosine and tryptophan were around 35°C, and the peaks were narrower than in the case of phenylalanine. Uptake dropped rapidly around 40 to 45°C and was absent at 55°C.

Changes in the initial rates of aromatic amino acid uptake as a function of temperature are shown as an Arrhenius plot in Fig. 3. This type of representation shows two activation energies for phenylalanine and tyrosine transport. They are 6.3 and 5.7 kcal/mol for phenylalanine and tyrosine between 40 and 15°C and are 40 and 43 kcal/mol between 15 and 4°C. The transport of tryptophan exhibits a single activation energy of 6.6 kcal/mol.

**Transport kinetics.** Uptake kinetic parameters were determined. Linear regression lines were calculated with three independent experiments for each amino acid. The Michaelis constants ($K_m$) calculated for phenylalanine, tyrosine, and tryptophan were 25, 3.4, and 1.8 μM. Maximal velocities ($V_{max}$) of phenylalanine, tyrosine, and tryptophan transport were 0.5, 0.3, and 0.01 nmol/min per mg dry weight.

**Crossed inhibitions between the amino acids.** The six possible pairs were tested to determine inhibition. Tryptophan noncompetitively inhibited tyrosine transport with an inhibition constant ($K_i$) of 0.23 mM (Fig. 4). Similarly, phenylalanine and tyrosine noncompetitively inhibited tryptophan transport (Fig. 4). All the combinations resulted in noncompetitive inhibition.

**Effect of nonaromatic amino acids and structural analogs on transport.** Other amino acids, as well as structural analogs of phenylalanine, tyrosine, and tryptophan, were tested to determine the specificity of the transport systems. Tyrosine and tryptophan transports were inhibited when the other amino acids (l form) were present in molar ratios of 100:1 and 20:1, respectively. In the measurement conditions used, i.e., 2 min of contact, tyrosine transport was inhibited 50% by the branched-chain amino acids valine and leucine and 40% by glycine and methionine. Tryptophan transport was found to be sensitive to proline and to l-glutamine, l-cysteine, and l-serine, three uncharged polar side chain amino acids. This may explain their similar effects.

The transport of L-phenylalanine was not inhibited by 100-fold higher concentrations of the 18 natural l-amino acids. On the other hand, L-phenylalanine transport was inhibited in the same conditions by the dipeptides glycyL-L-phenylalanine (44%) and L-phenylalanylglucose (13%). Two other dipeptides, L-tyrosyl-L-leucine and glycyl-L-tyrosine, had no effect on phenylalanine transport (6 and 0% inhibition, respectively).

Inhibitions by structural analogs led to the
determinations of the groups in each molecule participating in the uptake phenomenon. Certain analogs were potent inhibitors of tyrosine transport while having little or no effect on phenylalanine. This was the case for m-fluoro-DL-tyrosine (82 and 0% inhibition, respectively) and L-β-3,4-dihydroxyphenylalanine (59 and 17%, respectively). This confirms the greater specificity of phenylalanine transport.

Among the phenylalanine analogs, the most potent was p-fluoro-DL-phenylalanine. The fluo-
line atom is thus not too great a steric hindrance for the recognition of these compounds. Trypto-
han transport was affected only slightly (27%) by this inhibitor (Table 1).

An interesting finding was that another type of ring structure did not change the recognition of the compound to any great extent. Thus, β-2-thienylalanine was a fairly potent inhibitor of phenylalanine (42%) and tyrosine transport (67%). In addition, tryptophan analogs were not very active, whereas β-2-thienylalanine inhibited tryptophan transport only 7%. An α-carboxyl group seems to be important, since there was very little inhibition by tyramine or DL-phenyl-
ethylamine. The α-amino group is also important, since phenylalanine transport was not inhibited excessively by L-β-phenyllactic or mandelic acid. In addition, p-hydroxyphenyl-
pyruvic, p-hydroxyphenylacetic, and DL-p-hydroxyphenyllactic acids were ineffective on ty-
rosine transport.

Compounds which inhibited phenylalanine and tyrosine transports were tested against tryptophan (Table 1) and were found to be relatively inactive, with maximum inhibition of 27% for p-
fluoro-DL-phenylalanine and 3-hydroxy-DL-kynurenine. The structural analogs of tryptophan, on the other hand, inhibited its transport more

FIG. 3. Arrhenius plots relating temperature to initial rates of phenylalanine (●) and tyrosine (☆) uptake at pH 8.0. Transport velocity was given in nanomoles per minute per milligram of dry weight. The temperatures were included for reference points only. The slopes were calculated by a least-squares analysis.

FIG. 4. (A) Crossed competition between tyrosine and tryptophan. (B) Crossed competition between tryptophan and phenylalanine or tyrosine.
than 38% (Table 1). It would appear that the \(\alpha\)-amino and \(\alpha\)-carboxyl are less important in this case than for the transport of the other two aromatic amino acids, since these functional groups were absent from potent inhibitors. The length of the carbon chain on the indole ring also seems to be of secondary importance, since indole alone inhibited tryptophan transport 60%.

After L-amino acid transport stabilized (after about 20 min), the unlabeled L or D forms of the same amino acid were added at a 10-fold higher concentration. In the case of phenylalanine, the L and D forms behaved quite differently (Fig. 5). Thus, the L form led to a decrease in the quantity of radioactivity incorporated, whereas the D form had no effect on incorporation, with the exception of the dilution effect. Release of the labeled amino acid tended to stabilize after 10 to 15 min at 0.7 nmol/mg. This value corresponds to the quantity of TCA-insoluble cellular material determined when the unlabeled amino acid was added. In agreement with Brown (7), we consider that these samples represent the incorporation of radioactive amino acids into tRNA and proteins. This shows the considerable degree of stereospecificity of phenylalanine transport. The transports of tyrosine and tryptophan, on the other hand, were similarly affected (Fig. 5) by the D and L forms.

### Effects of metabolic inhibitors of aromatic amino acid transport
Transport was measured in the presence of various inhibitors to determine the energy source used in the process (Fig. 6).

### Table 1. Inhibition of \[^{14}\text{C}\]tryptophan uptake by structural analogs of tryptophan, phenylalanine, and tyrosine

<table>
<thead>
<tr>
<th>Analog</th>
<th>% Inhibition of rate of uptake</th>
</tr>
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<tbody>
<tr>
<td>5-Fluoro-DL-tryptophan</td>
<td>71</td>
</tr>
<tr>
<td>6-Fluoro-DL-tryptophan</td>
<td>54</td>
</tr>
<tr>
<td>5-Methyl-DL-tryptophan</td>
<td>49</td>
</tr>
<tr>
<td>5-Hydroxy-DL-tryptophan</td>
<td>51</td>
</tr>
<tr>
<td>Indole</td>
<td>60</td>
</tr>
<tr>
<td>DL-Indole-3-lactic acid</td>
<td>71</td>
</tr>
<tr>
<td>Tryptophol</td>
<td>68</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>60</td>
</tr>
<tr>
<td>Indole-3-acetic acid</td>
<td>38</td>
</tr>
<tr>
<td>Indole-3-propionic acid</td>
<td>78</td>
</tr>
<tr>
<td>Indole-3-pyruvic acid</td>
<td>70</td>
</tr>
<tr>
<td>p-Fluro-DL-phenylalanine</td>
<td>27</td>
</tr>
<tr>
<td>(\beta)-2-Thienylalanine</td>
<td>3</td>
</tr>
<tr>
<td>3-Hydroxy-DL-kyurenine</td>
<td>27</td>
</tr>
<tr>
<td>DL-(\alpha)-Phenylethylamine</td>
<td>11</td>
</tr>
<tr>
<td>Phenylpyruvic acid</td>
<td>19</td>
</tr>
<tr>
<td>p-Hydroxynaphthylpyruvic acid</td>
<td>20</td>
</tr>
<tr>
<td>Tyramine</td>
<td>0</td>
</tr>
</tbody>
</table>

* Unlabeled analogs (0.1 mM) and \[^{14}\text{C}\]tryptophan (2.5 \(\mu\)M) were added together to cell suspension. Cells were filtered after a period of 2 min.
were PCMB, which forms mercaptides with sulfhydryls, CCCP, which cancels proton coupling by rendering the membrane permeable to protons (4, 14) but which may also act at the level of sulfhydryls, and DNP (10 mM). CCCP inhibited the three transports to the same extent. PCMB appeared to be less active toward tryptophan than toward phenylalanine and tyrosine transports.

**DISCUSSION**

Our results enable us to conclude that the transport of aromatic amino acids by *B. linens* is determined by three high-affinity permeases. The hypothesis of the existence of these three high-affinity transport systems is based on the differential behavior of transport in terms of concentration-dependent kinetics, pH and temperature optima, structural and stereospecificity, and responses to metabolic inhibitors and sulfhydryl reagents. The six possible combinations between the aromatic amino acids demonstrated only noncompetitive inhibition, which is a rather infrequent finding. To our knowledge, the only example of this type of behavior is the case of phenylalanine inhibition of tryptophan transport in *Bacillus megaterium* (5). A common transport system for the three aromatic amino acids is frequently observed in gram-negative bacteria, e.g., *E. coli* K-12 (23) and *S. typhimurium* (2). This common system could not be detected in *B. linens* under the experimental conditions used.

We observed no great differences among the carbon compounds tested as energy sources for amino acid transport. Lactate was chosen for two reasons: (i) its usual presence in the natural medium of the bacterium (1), and (ii) to obtain maximal levels of amino acid incorporation. After the starvation period, there was some decrease in the quantity of amino acids absorbed, probably as a result of the treatment the cells underwent during preparation.

Fluorinated analogs were found to be the most powerful inhibitors of aromatic amino acid transport in *B. linens*. This agrees with findings in other bacteria, notably *P. aeruginosa* (18) and *Y. pestis* (26). These results show that the presence of fluorine does not disturb molecular recognition by the permeases to any great extent.

The importance of the α-amino and α-carboxyl groups was shown for phenylalanine and tyrosine. The size of the aromatic ring appears to be sufficient for its recognition by the tryptophan permease, which requires a relatively voluminous molecule. Substitutions by small groups (methyl, hydroxyl) on the indole ring have little effect on the recognition of the molecule.

A high degree of specificity of phenylalanine transport has been reported for *E. coli* (10), where only L-phenylalanine could displace previously incorporated phenylalanine. Stereospecificity in *B. linens* is of the same order as in *Y. pestis* (26) for phenylalanine transport, as well as for tyrosine transport. In the latter case, both the D and L forms are transported by both bacteria. Tryptophan appears to be transported by *B. linens* in both conformations, which is not the case for *Pseudomonas acidovorans* (24).

A correlation is to be noted between the fact that phenylalanine can be used as sole carbon source by *B. linens* (J. Richard, personal communication) and that the pH and temperature optima for phenylalanine transport are the same as for the growth of this strain (pH 7.5 to 8.0, 25°C). This type of correlation was not observed for tyrosine and tryptophan.

Arrhenius plots of phenylalanine and tyrosine transports were biphasic. The break in the curve at 15°C reflects the gel-liquid phase transition temperature of membrane lipids (11). 1-Aspar-
tate transport via the general amino acid transport system in *Neurospora crassa* shows a break at 17°C when an Arrhenius plot is constructed (30), whereas phenylalanine transport in *E. coli* ML308 shows a break between 31 and 32°C (28). Below this temperature, the more rigid structure of the membrane, due to the presence of unsaturated fatty acids, interferes with transport functions (9). Jähning and Bramhall (15) interpreted this break as the result of compensation of the high activation energy by entropy. This compensation leads to a continuous rate of permeation at the point of phase transition.

Tryptophan transport was more dependent on sulphydryl groups than was transport of the other two amino acids, which differs from *Y. pestis* (26). The transporters of the latter are insensitive to sulphydryl group inhibitors.

The three permeases were inactivated by CCCP and DNP, consistent with the prime energy source being proton conduction.

Current work in our laboratory is involved with characterizing each system separately by searching for mutants defective for certain permeases (isolation based on the capacity to grow in the presence of toxic analogs of the β-2-thienylalanine type after undergoing various mutagenic treatments).

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LITERATURE CITED