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Third System for Neutral Amino Acid Transport in a Marine Pseudomonad

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Uptake of leucine by the marine pseudomonad B-16 is an energy-dependent, concentrative process. Respiratory inhibitors, uncouplers, and sulfhydryl reagents block transport. The uptake of leucine is Na^+ dependent, although the relationship between the rate of leucine uptake and Na^+ concentration depends, to some extent, on the ionic strength of the suspending assay medium and the manner in which cells are washed prior to assay. Leucine transport can be separated into at least two systems: a low-affinity system with an apparent K_m of 1.3×10^{-5} M, and a high-affinity system with an apparent K_m of 1.9×10^{-7} M. The high-affinity system shows a specificity unusual for bacterial systems in that both aromatic and aliphatic amino acids inhibit leucine transport, provided that they have hydrophobic side chains of a length greater than that of two carbon atoms. The system exhibits strict stereospecificity for the L form. Phenylalanine inhibition was investigated in more detail. The K_i for inhibition of leucine transport by phenylalanine is about 1.4×10^{-7} M. Phenylalanine itself is transported by an energy-dependent process whose specificity is the same as the high-affinity leucine transport system, as is expected if both amino acids share the same transport system. Studies with protoplasts indicate that a periplasmic binding protein is not an essential part of this transport system. Fein and MacLeod (J. Bacteriol. 124:1177-1190, 1975) reported two neutral amino acid transport systems in strain B-16: the DAG system, serving glycine, D-alanine, D-serine, and α-aminoisobutyric acid; and the LIV system, serving L-leucine, L-isoleucine, L-valine, and L-alanine. The high-affinity system reported here is a third neutral amino acid transport system in this marine pseudomonad. We propose the name "LIV-II" system.

Marine bacteria require Na^+ for growth (21). In the marine pseudomonad B-16, this requirement has been shown to be a specific requirement for Na^+ for the transport of substrates across the plasma membrane (9). MacLeod and his co-workers investigated, in much detail, the transport of α-aminoisobutyric acid (AIB) in this organism, the role of inorganic cations in this process, and the mechanism of energy coupling (32, 33, 35-37, 40). The transport of fucose (an analogue of galactose) by B-16 and the transport of AIB by *Photobacterium fischeri* were shown to be Na^+-dependent processes, although no detailed study was made (9).

From early data on competition of amino acids with AIB transport in strain B-16 (9), it appeared likely that leucine transport in this organism occurs via a separate, neutral amino acid transport system. A study was begun to investigate this possibility and to characterize leucine uptake in B-16. While initial experiments were in progress, Fein and MacLeod (10) distinguished two systems mediating neutral amino acid transport: one designated the DAG system, capable of transporting glycine, D-alanine, D-serine, AIB, and, to a lesser extent, L-alanine; and a second, designated the LIV system, capable of transporting L-leucine, L-isoleucine, L-valine, and L-alanine. This study was done with mutants defective either in AIB transport or in leucine transport.

In this paper, we report the existence of a third Na^+-dependent neutral amino acid transport system capable of recognizing a variety of hydrophobic amino acids provided that their side chains have a length greater than two carbon atoms. This system has a much higher affinity for its substrates than the two systems described by Fein and MacLeod (10).

MATERIALS AND METHODS

Growth of organism. The organism used was variant 3 of the marine pseudomonad B-16 (ATCC 19855), kindly supplied by R. A. MacLeod of McGill University. It has been classified as *Alteromonas haloplanktis* by Reichelt and Baumann (27). Fresh

cultures were maintained by monthly transfer on agar slopes or plates of a complex growth medium described by Thompson and MacLeod (35), and cells for transport studies were grown in the same complex medium without the agar.

Transport studies. A portion of cells from an overnight culture in the complex liquid medium was transferred to fresh liquid medium, aerated vigorously, and harvested in the mid-logarithmic phase of growth. Generally, cells were washed twice with a buffered complete salts solution consisting of 0.3 M NaCl, 0.01 M KCl, and 0.05 M $MgSO_4$ in 0.05 M tris (hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.2) containing 1 mM phosphate added as H_3PO_4, and suspended to an absorbance of 0.2 at 600 nm in a Spectronic 20 colorimeter. Exceptions to this are noted in the text.

Cells (2.8-ml volumes) were added to tubes containing chloramphenicol (dissolved in ethanol) to a final concentration of 250 μg/ml and preincubated for 15 min at 22°C. The final concentration of ethanol was 50 mM. When potential metabolic inhibitors were used, they were added to the assay tube prior to the preincubation. An isotopically labeled amino acid was added after preincubation, and uptake was followed by the sequential removal and rapid filtration of four 0.7-ml volumes on presoaked (complete salts solution) membrane filters (type HA, 0.45 μm pore size; Millipore Corp.) followed by rapid washing with two 2.5-ml lots complete buffered salts solution at room temperature. When a competing amino acid was present, it was added immediately prior to addition of the labeled substrate. In all cases, the total volume of the assay was made up to 3.0 ml with complete salts solution.

The membrane filters were placed in glass scintillation vials, and 0.1 ml of 10% trichloroacetic acid was added to disrupt the cells, followed by 3.0 ml of absolute ethanol and 10 ml of scintillation phosphor solution {4.0 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-[2]-(5-phenyloxazolyl)benzene per liter of scintillation-grade toluene}. This procedure is almost identical to that of Drapeau et al. (9). Radioactivity of the samples was determined in a Searle Delta 300 liquid scintillation counter.

Fractions were taken at 15, 35, 55, and 75 s of uptake. Initial rates (counts per minute per minute of uptake per 0.7-ml fraction) were calculated from the slope of the initial linear portion of curves of uptake (counts per minute) versus time. Such values were converted to nanomoles of amino acid transported per minute per milligram of protein where necessary.

Protein of the cell suspensions was estimated by the method of Lowry et al. (20). Portions of washed-cell suspensions of known absorbance were centrifuged and suspended in distilled water, and fractions were taken for protein estimation.

Determination of intracellular leucine. Duplicate 3.0-ml volumes of washed cells, suspended to a 0.2 absorbance at 600 nm in complete buffered salts solution, were preincubated with chloramphenicol for 15 min as for the usual transport assay. [^{3}H]leucine (final concentration, 5×10^{-6} M; 500 μCi/μmol) was added and, after 3 min of uptake, all samples were filtered rapidly on membrane filters (diameter, 47 nm; pore size, 0.45 μm; Millipore Corp.) and washed twice with 5.0 ml of complete salts solution. Filters were placed in 10 ml of ice-cold distilled water and vortexed thoroughly, and the suspensions were transferred to centrifuge tubes. They were heated for 10 min in a boiling-water bath and then centrifuged. The supernatants were removed, flash evaporated, dissolved in a small amount of water, and spotted on Whatman no. 1 chromatography paper, along with a marker of L-leucine. Chromatograms were developed for 4 h in acetic acid–*n*-butanol–water (15:60:25, vol/vol/vol), dried, and cut into 1-cm strips. These strips were placed in scintillation vials with 10 ml of scintillant (see above) and counted for radioactivity.

Preparation of protoplasts. Protoplasts were prepared essentially by the method of DeVoe et al. (8). Direct counts of cell forms were determined by using a Neubauer hemocytometer. Each suspension was counted in quadruplicate, with 300 to 400 cells counted for a single determination. Uptake of amino acids by these cell forms was determined as described above.

Preloading of cells with [^{3}H]leucine and [^{14}C]AIB for retention studies. For the leucine study, an hour before harvest, L-[^{3}H]leucine (10 μCi/μmol) was added at a final concentration of 10^{-4} M. Cells were harvested and converted to protoplasts. Whole cells were counted directly, and four 0.7-ml portions were filtered and washed, both immediately after washing of the cells and after they had stood on ice for 1.5 h. The filters were counted for radioactivity as described previously. Protoplasts were counted, and the radioactivity of the fractions was determined in the same way, immediately after formation and after 1.5 h on ice. For the retention study with AIB, cells were harvested, washed, suspended in complete salts solution, and incubated for 30 min with [^{14}C]AIB (1 μCi/μmol) at a final concentration of 10^{-4} M. The cells were washed and converted to protoplasts. Whole cells and protoplasts were counted for cell number and radioactivity retained, as in the leucine retention experiment.

Chemicals. Unlabeled amino acids, amino acid analogues, dicyclocarbodiimide (DCCD), carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP), rotenone, chloramphenicol, lysozyme, and Trizma base [tris(hydroxymethyl)aminomethane] were obtained from Sigma Chemical Co., St. Louis, Mo. Other chemicals were of analytical grade from the British Drug Houses Ltd. The radioactive chemicals, L-[4,5-^{3}H]leucine, L-phenyl[2,3-^{3}H]alanine, L-[G-^{3}H]glutamic acid, L-[U-^{14}C]glutamine, L-[2,5-^{3}H]histidine, L-[4,5(*n*)-^{3}H]lysine hydrochloride, L-[*methyl*-^{3}H]-methionine, L-[5(*n*)-^{3}H]tryptophan, and 2-amino[1-^{14}C]isobutyric acid, were obtained from Amersham/Searle, Arlington Heights, Ill.

RESULTS

Requirement for Na^+ for transport. There is a specific Na^+ requirement for leucine transport by marine pseudomonad B-16. Figure 1, curve A, shows the effect of increasing the Na^+

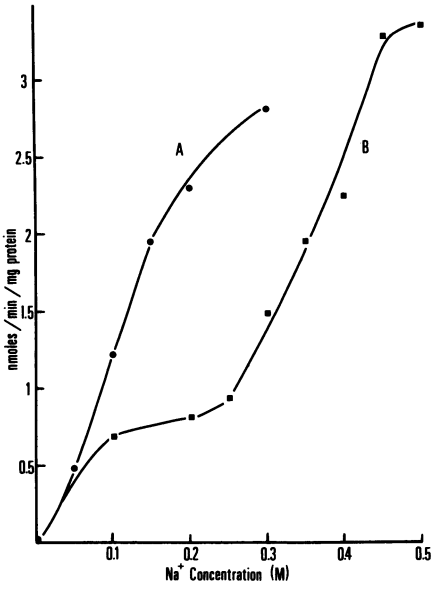

FIG. 1. *Relationship between Na^+ concentration in the assay buffer and leucine uptake of cells washed, prior to assay, in complete salts solution containing 0.3 M Na^+ and no Li^+. Symbols: ●, total $Na^+ + Li^+$ concentration of 0.3 M; ■, total $Na^+ + Li^+$ concentration of 0.5 M. The final concentration of L-[^{3}H]leucine used was 5 × 10^{-6} M.*

concentration on the uptake rate of L-[^{3}H]leucine. In experiments of this type, the cells were washed with complete buffered salts solution containing 300 mM Na^+ (as described in Materials and Methods) prior to suspension, and assay, in various buffered salts solutions containing the specified Na^+ levels, with Li^+ added so that the total concentration of Li^+ plus Na^+ was maintained at 300 mM. The optimum Na^+ concentration was near 300 mM Na^+ under these conditions. No uptake occurred in the absence of Na^+.

To check that Li^+ itself was not affecting the transport system, experiments similar to that shown in Fig. 1, curve A, were done in which Li^+ was omitted but the ionic strength was maintained at the same level by keeping the total concentration of Na^+ plus K^+ equal to 310 mM. A curve similar to that described above for Na^+ was observed. If Na^+ in the assays was constantly maintained at 100 mM while Li^+ and K^+ levels were varied and the total $Li^+ + K^+ + Na^+$ concentration was kept constant at 310 mM, then L-[^{3}H]leucine uptake was the same in all assays, at the level expected for 100 mM Na^+ in the suspension (data not shown).

Because the rate of leucine uptake did not completely reach a plateau at 300 mM Na^+ (Fig. 1, curve A), higher concentrations of Na^+ were used. In these experiments, the total $Li^+ + Na^+$ concentration equalled 500 mM. The initial rate of leucine uptake was, in general, depressed (Fig. 1, curve B) compared with that shown in Fig. 1, curve A, until Na^+ concentrations higher than 400 mM were reached.

To further emphasize the way in which cell preparation and assay conditions can influence the observed relationship between Na^+ concentration and leucine uptake, experiments were done in which, after centrifugation from the growth medium, cells were washed twice in the various salts solutions with appropriate Na^+ and Li^+ concentrations (as in the experiments of Fig. 1) and then suspended and assayed in the same salt solutions with which they were washed. The relationship between Na^+ concentrations and leucine uptake was further distorted compared with Fig. 1 curve A (data not shown).

Kinetics of leucine transport. For determination of leucine transport kinetics (and throughout the remainder of the paper), the Na^+ concentration was maintained at 300 mM, with no added Li^+. Lineweaver-Burk plots of kinetic data from transport assays over a wide range of leucine concentrations (10^{-4} to 10^{-7} M) show an apparent break in the slope of the line at approximately 9 μM, indicating that at least two systems may be responsible for leucine uptake (Fig. 2). The method of Neal (22) was used to derive apparent Michaelis constants from the line of best fit of the lower and upper portions of the graph, i.e., for the lower- and higher-affinity processes. From the experiment shown, and several others, approximate K_m values for the high- and low-affinity systems were 1.9 ± 0.1 ×

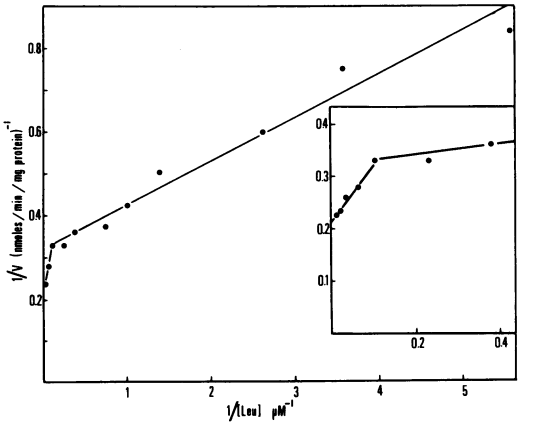

FIG. 2. *Double-reciprocal plot of the initial L-[^{3}H]leucine uptake rate as a function of the extracellular leucine concentration. The inset shows the lower part of the graph on a larger scale.*

10^{-7} and $1.3 \pm 0.1 \times 10^{-5}$ M, respectively. The V_{max} values for these systems varied somewhat from cell batch to cell batch. The two systems showed similar capacities for uptake, with V_{max} values of the high- and low-affinity systems ranging from 1.5 to 2 and from 1 to 1.5 nmol/min per mg of protein, respectively. Thus, in competition studies (reported later) in which substrate levels are low (5×10^{-7} M), the contribution of the low-affinity system is minimized.

State of intracellular leucine. We determined the percentages of leucine taken up and remaining free during the time span used in these studies. Since protein synthesis was inhibited by chloramphenicol, less than 5% of the L-[^{3}H]leucine taken up was found in trichloroacetic acid-precipitable material (data not shown). Of the [^{3}H]leucine in the pool of small-molecular-weight components, over 75% was found to exist as free leucine after 3 min of uptake. A second peak accounted for most of the remaining radioactivity on the chromatogram (Fig. 3). After 5 min of incubation with [^{3}H]leucine, this peak had increased in size and free leucine had decreased to 60% of the total. The amount of leucine taken up in 3 min from a known concentration of external leucine can be calculated as nanomoles per 10^8 cells per 3 min. Of this, 75% can be assumed to be free leucine. By assuming an intracellular volume 1.6 μl/4 $\times$ 10^9 colony-forming units (10), the extent to which the leucine was concentrated by the cells can be approximated. Results from several experiments gave figures of about 3,000-fold concentration or greater within 3 min of uptake from an external solution with an initial leucine concentration of 5×10^{-6} M.

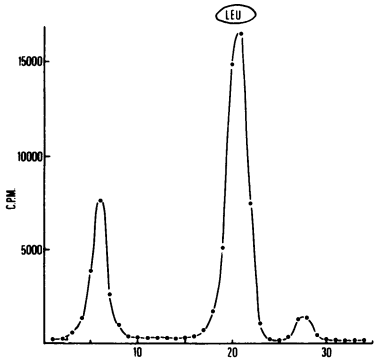

FIG. 3. *Counts per minute versus fraction number after paper chromatography of soluble pool material from cells that had taken up L-[^{3}H]leucine for 3 min. The position on the chromatogram of unlabeled marker leucine as revealed by ninhydrin spray is shown.*

Effects of metabolic inhibitors and sulfhydryl-blocking reagents. Table 1 demonstrates the effects of some metabolic inhibitors and sulfhydryl-blocking reagents. Potassium cyanide at 10^{-2} and 10^{-3} M almost completely inhibited leucine transport, whereas sodium azide at the same concentration showed considerably less inhibition. Rotenone (dissolved in ethanol) had no effect on leucine transport or, as determined previously (33), AIB uptake in membrane vesicles of strain B-16. Uncouplers of respiration, 2,4-dinitrophenol and CCCP, almost completely abolished leucine transport. DCCD, an effective inhibitor of membrane-bound adenosine triphosphatases, had little inhibitory effect at 10^{-4} M and regularly was seen to slightly stimulate leucine transport at a 10^{-5} M concentration.

TABLE 1. *Inhibition of L-[^{3}H]leucine uptake by metabolic inhibitors and sulfhydryl-blocking reagents*

Inhibitor	Concn (M)	Inhibition of uptake rate[a] (%)
KCN	10^{-2}	99
	10^{-3}	98.5
NaN_3	10^{-2}	66
	10^{-3}	25
Rotenone[b]	10^{-5}	5
	10^{-6}	0
2,4-Dinitrophenol[b]	10^{-3}	98
	5×10^{-4}	97
	10^{-4}	45
CCCP[b]	10^{-5}	100
	5×10^{-6}	97.5
	10^{-6}	0
DCCD[b]	10^{-4}	10
	10^{-5}	(−15)[c]
N-ethyl maleimide	10^{-3}	60
	2×10^{-4}	25
Iodoacetate	10^{-2}	100
	5×10^{-3}	99
	10^{-3}	0

[a] Cell suspensions were preincubated with inhibitors for 15 min prior to the addition of L-[^{3}H]leucine (50 μCi/μmol) at a final concentration of 2×10^{-6} M. Assays were run in triplicate. At least two separate experiments were performed.

[b] 2,4-Dinitrophenol, rotenone, CCCP, and DCCD were dissolved in ethanol. The same amount of ethanol was added to assays without inhibitor as a control.

[c] The minus sign indicates percent activation.

The sulfhydryl-blocking reagents *N*-ethyl maleimide and iodoacetate inhibited transport, with *N*-ethyl maleimide being the more effective.

Competition with other amino acids. A number of competition experiments were done to determine the substrate specificity of the high-affinity leucine transport system. After a 15-min preincubation of cells with chloramphenicol (250 μg/ml) to prevent protein biosynthesis, potential competitors to a 2 ×' 10^{-5} M final concentration were added immediately prior to the addition of L-[^{3}H]leucine (final concentration, 5 × 10^{-7} M). Initial uptake rates were determined as described in Materials and Methods. The results (Table 2) suggest that a hydrophobic side chain with more than two carbon atoms, in the α position of an amino acid, is necessary for recognition by this system. Branched-chain hydrophobic amino acids such as L-valine or L-isoleucine, straight-chain amino acids such as L-norvaline and L-norleucine, or aromatic amino acids such as L-phenylalanine and L-tyrosine, all strongly inhibited leucine uptake, suggesting that the transport system easily recognizes all such amino acids. Tryptophan, although possessing a large nonpolar side group, inhibited leucine uptake somewhat less well, possibly because of its comparatively bulky side chain, which might cause steric hindrance. Methionine, with a hydrophobic sulfur-containing side chain, also caused considerable inhibition. However, alanine or glycine showed virtually no inhibition, indicating that a methyl group or H atom is not sufficient for recognition. Figure 4 is a plot of percent inhibition versus numbers of R-chain carbon atoms for the straight-chain amino acids (including L-α-amino-*N*-butyrate). It shows clearly the requirement for a side chain with more than two carbon atoms.

TABLE 2. *Comparison of the inhibition of L-[^{3}H]leucine transport in the marine pseudomonad B-16 by various amino acids*

Competitor	Inhibition of L-[^{3}H]leucine transport[a] (%)
L-Tyrosine	96
L-Phenylalanine	96
L-Isoleucine	95
L-Leucine	94
L-Norleucine	92
L-Valine	86
L-Norvaline	86
L-Tryptophan	74
L-Methionine	65
L-Asparagine	15
L-Serine	13
L-Aspartic acid	12
L-Glutamic acid	11
L-Histidine	10
L-Threonine	9
D-Leucine	8
L-Arginine	7
Glycine	
L-Glutamine	
L-Alanine	<5
Tyramine	
L-Lysine	
L-Proline	
α-AIB	

[a] Percent inhibition was based on uptake in the absence of added inhibitor. L-[^{3}H]leucine (50 μCi/μmol) was at a final concentration of 5 × 10^{-7} M. Inhibitors were added at final concentrations of 2 × 10^{-5} M. Assays were done in duplicate, and three separate experiments were done for each amino acid.

Amino acids with positively or negatively charged side chains showed little inhibition, as did L-glutamine and L-asparagine with their polar, noncharged groups. The presence of a hydroxyl group, as in L-serine and L-threonine, appeared to prevent their recognition by the leucine transport system. The hydroxyl group of L-tyrosine presumably is sufficiently far from the α-atom that it does not interfere with recognition.

The α-carboxyl group appears to be necessary in that tyramine had little effect on leucine uptake, whereas tyrosine was one of the strongest inhibitors of uptake. Proline, in which there is no free α-amino group, also showed little inhibition, indicating a probable need for a free amino group.

The system exhibits strict stereo specificity in that D-leucine did not inhibit L-leucine uptake.

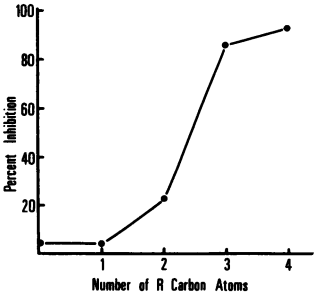

FIG. 4. *Effect of side chain length of neutral amino acids on the extent of L-[^{3}H]leucine transport inhibition. Experimental conditions were as described in the footnote of Table 2. Inhibitors: Glycine, R = 0; L-alanine, R = 1; L-α-amino-N-butyrate, R = 2; L-norvaline, R = 3; and L-norleucine, R = 4. R, Side chain carbon atoms.*

K_i for phenylalanine. Amino acid competition studies indicated a broad specificity for this transport system, showing that the uncharged aromatic amino acids may be sharing the same transport system as the hydrophobic aliphatic amino acids. Inhibition of L-leucine uptake by L-phenylalanine was investigated further. From a Dixon plot (Fig. 5), the K_i for L-phenylalanine was found to be 1.7×10^{-7} M. This was similar to the figure of 1.2×10^{-7} M obtained from Lineweaver-Burk plots of the reciprocals of leucine uptake versus leucine concentration in the presence of several fixed concentrations of phenylalanine.

L-[^{3}H]phenylalanine transport. To demonstrate further that leucine and phenylalanine shared the same transport system, phenylalanine transport was examined. L-[^{3}H]phenylalanine was taken up by an energy-dependent process in which the addition of cyanide or uncouplers prevented uptake (data not shown). We attempted to determine the K_m for phenylalanine transport but were unable to obtain sufficiently accurate initial uptake rates, particularly at low phenylalanine concentrations. From the rough data obtained, the K_m appeared likely to be below the value reported for K_i. The inhibitory activities of various amino acids on phenylalanine uptake were compared to determine whether the specificity of this system resembled that for leucine uptake. The results indicated that the overall inhibition pattern was similar to that for leucine uptake, although the extent of inhibition was lower (Table 3). L-[^{3}H]phenylalanine and inhibitors were used at final concentrations of 5×10^{-7} and 2×10^{-5} M,

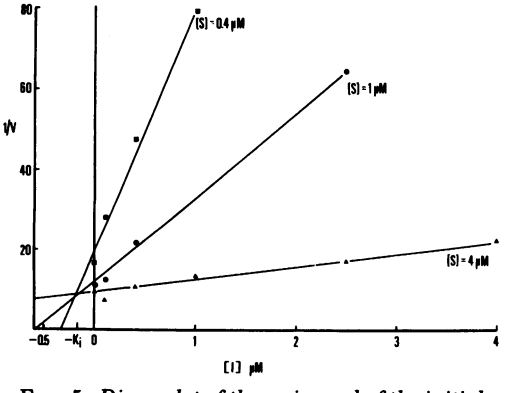

FIG. 5. *Dixon plot of the reciprocal of the initial L-[^{3}H]leucine uptake rate (nanomoles/3 min per 0.7-ml fraction) against the concentration of the inhibitor, phenylalanine, at three fixed concentrations of the substrate, leucine. The point of intersection of the resulting lines corresponds to the negative K_i value on the x axis.*

TABLE 3. *Comparison of the inhibition of L-[^{3}H]phenylalanine transport by various amino acids*

Competitor	Inhibition of L-[^{3}H]phenylalanine transport[a] (%)
L-Phenylalanine	97.5
L-Tyrosine	96.5
L-Isoleucine	75
L-Leucine	60
L-Tryptophan	55
L-Valine	30
L-Methionine	30
L-Serine	8
L-Aspartic acid	8
L-Threonine	
D-Leucine	
L-Arginine	
Glycine	<5
L-Alanine	
Tyramine	
L-Lysine	
L-Glutamine[b]	
α-AIB	

[a] Percent inhibition was based on uptake in the absence of added inhibitor. L-[^{3}H]phenylalanine (50 μCi/μmol) was at a final concentration of 5×10^{-7} M. Inhibitors were added at final concentrations of 2×10^{-5} M.

[b] Glutamine appeared to slightly stimulate uptake.

respectively. As was the case with leucine uptake, phenylalanine and tyrosine showed the most inhibition, followed by the aliphatic amino acids, isoleucine and leucine. Tryptophan, methionine, and valine had intermediate inhibitory activity. Alanine, glycine, serine, threonine, and others showed little inhibition.

Amino acid transport in protoplasts. True protoplasts can be formed from marine pseudomonad B-16 (8). Some cells are lysed during their formation. However, by measuring the uptake of an amino acid per equal number of cell equivalents (obtained by direct counting), the rate of protoplast transport can be compared with that of whole cells. If protoplasts and whole cells have the same uptake rate, then the existence of a periplasmic binding protein as an essential part of the transport system can be ruled out. Such is the case with the α-AIB transport system (8).

Protoplasts were made by the procedure of DeVoe et al. (8). Approximately the same percent conversion of whole cells to protoplasts as reported by these authors was found. The protoplasts appeared to be stable, remaining well rounded, phase dark, and unclumped for at least 1.5 h, as expected for variant 3 of this

organism (11). To further check the stability of the protoplasts, retention studies were done with L-[^{3}H]leucine over 1.5 h. Although leucine was metabolized under the conditions employed and much would have been converted to protein, lysis of the protoplasts would have resulted in the reduction of the amount of labeled material on the membrane filters (pore size, 0.45 μm), and the number of protoplasts counted would have diminished. This was not observed. Whereas this experiment was a check mainly on gross lysis of the protoplasts, the retention of [^{14}C]AIB, a nonmetabolizable substrate, was followed to determine whether the protoplasts were becoming leaky during the experiment. Again, no loss in radioactivity per cell number was observed.

The uptake of L-[^{3}H]leucine and [^{14}C]AIB by protoplasts was compared with that of whole cells. Total uptake varied somewhat with different batches of cells, but the protoplasts always had about the same capacity for leucine transport as whole cells, indicating that a binding protein is not an essential component of the transport system (Table 4).

While these preparations were in use, preliminary studies on the ability of protoplasts to transport other amino acids were done. Only one concentration of substrate was used. Table 5 shows that lysine, histidine, tryptophan, and tyrosine are also taken up by protoplasts and whole cells at approximately the same rate. Methionine was the only amino acid that repeatedly showed a lower uptake rate by protoplasts compared with whole cells. The negatively charged amino acids glutamic acid and aspartic acid were transported at a much greater rate by protoplasts. Glutamine also was transported in protoplasts at almost twice the rate in whole cells.

DISCUSSION

Transport of amino acids in bacteria has been found to be complex, often involving several systems with different specificities for the transport of a particular amino acid (14, 23). Two neutral amino acid systems have been reported for the marine pseudomonad B-16 (10). Evidence has been presented here for a third system.

Initial characterization of leucine uptake by B-16 showed that leucine was accumulated by an active transport process in that unmetabolized leucine was concentrated within the cell, and energy was required for the process. The respiratory inhibitor cyanide was seen to have a greater inhibitory effect than azide. A similar result was seen by Drapeau et al. (9) for the inhibition of AIB uptake and probably reflects differences in the affinity of the terminal oxidase for the two inhibitors. Uncouplers at low concentrations also inhibited uptake. DCCD did not inhibit transport. These data indicate that leucine transport is coupled to energy derived from respiration. The action of DCCD, the adenosine triphosphatase inhibitor, was interesting in that the lower concentration used (10^{-5} M) stimulated transport slightly but reproducibly. The stimulation of active transport and other energy-linked functions by low levels of DCCD has been observed in cells and membrane vesicles of *Escherichia coli* mutants lacking the Mg^{2+}-Ca^{2+}-activated adenosine triphosphatase, or in wild-type membrane vesicles stripped of the adenosine triphosphatase, and is believed to be due to DCCD decreasing the permeability of the membranes to protons (4, 29, 30). Whole cells of *Ectothiorhodospira halophila* also showed stimulation of active transport in the presence of DCCD (28). Perhaps the adenosine triphosphatase of B-16 is not tightly bound and the membrane is thus slightly leaky to protons, or else DCCD is causing stimulation by some other mechanism. Thiol reagents inhibited transport, with *N*-ethyl maleimide

TABLE 4. *Comparison of leucine and AIB transport in protoplasts and whole cells*

B-16	Uptake[a] of:		
	10^{-4} M leucine	10^{-4} M AIB	10^{-6} M leucine
Whole cells	0.33	0.16	0.185
Protoplasts	0.36	0.145	0.18

[a] Uptake is expressed as nanomoles per minute per 10^8 cell equivalents. Specific activity of L-[^{3}H]leucine was 10 μCi/μmol; that of [^{14}C]AIB was 2 μCi/μmol.

TABLE 5. *Comparison of uptake of various amino acids by protoplasts of the marine pseudomonad B-16*

Amino acid	Uptake[a]
L-[^{3}H]leucine	100
L-[^{3}H]methionine	50
L-[^{3}H]tryptophan	90
L-[^{3}H]histidine	90
L-[^{3}H]tyrosine	110
L-[^{3}H]lysine	110
L-[^{3}H]glutamic acid	400–600
L-[^{3}H]aspartic acid	300
L-[^{14}C]glutamine	200

[a] Expressed as percent uptake of an equal number of whole cells. All amino acids were at final concentrations of 2×10^{-5} M. Tritiated amino acids had a specific activity of 25 μCi/μmol; [^{14}C]glutamine had a specific activity of 2.5 μCi/μmol. Figures are rounded to the nearest 10%.

showing greater inhibition than iodoacetate, a result similar to that found by Sprott and MacLeod (33) for inhibition of AIB transport in membrane vesicles of B-16.

The high-affinity system is a further example of an Na^+-dependent uptake system in the marine pseudomonad B-16. The changes observed in the relationship of Na^+ concentration to leucine uptake, depending on the method of washing of the cells and the ionic strength of the suspension buffer, are not understood but demonstrate the need for careful description of the system used for transport assay. Several effects of Na^+ and Li^+ are known (40, 35). Although Na^+ is essential for transport, AIB uptake increased at low concentrations of Na^+ when the ionic strength was increased by the addition of Li^+ to the suspension medium to a total Li^+ + Na^+ concentration of 0.2 M (the figure then regarded as optimal for AIB transport, although more recent papers have shown an optimum of about 0.3 M or higher [10, 11]). In the same study Wong et al. (40) showed that, although Li^+ was partially effective in replacing Na^+ in preventing the loss of [^{14}C]AIB from cells preloaded with the isotope, at concentrations greater than 0.2 M, leakage of [^{14}C]AIB was enhanced. If the Na^+ concentration was increased to 0.5 or 1.0 M, leakage of AIB again occurred. Similar effects of high Li^+ concentrations and high ionic strength probably contribute to the shapes of the curves seen in Fig. 1. The ways in which ions such as Na^+ and Li^+ alter membrane porosity are not known (40). In our studies, the use of 300 mM Na^+ without Li^+ allowed direct comparison with the results of Fein and MacLeod (10).

Leucine uptake, as revealed by kinetic studies, involved at least two systems: a high-affinity system with an apparent K_m of 1.9×10^{-7} M and a low-affinity system with an apparent K_m of 1.3×10^{-5} M. The low-affinity system is probably the LIV system detected by Fein and MacLeod (10) and reported to transport L-leucine, L-isoleucine, L-valine, and L-alanine. No K_m for leucine was reported by these authors since most of their data were obtained from L-alanine transport studies. We concentrated on the characterization of the high-affinity system, previously unreported for this organism.

The high-affinity system shows an unusual specificity for bacterial systems. Clearly, the L form of the amino acid and a relatively large (greater than two carbon atoms) side chain (either straight, or branched, or aromatic) are the major requirements for recognition, with free α-carboxyl and α-amino groups probably being necessary as well. This specificity differs markedly from that of the LIV system of Fein and MacLeod (10). The LIV system has L-alanine as a major substrate, and the transport of L-[^{3}H]alanine is strongly inhibited by L-serine and L-threonine, as well as by L-leucine, L-isoleucine, L-valine, and their straight-chain analogues, and by α-AIB to some extent. It is not inhibited by L-phenylalanine. In contrast, the high-affinity system reported in this paper, is not inhibited by L-alanine, L-serine, L-threonine, or α-AIB. L-Phenylalanine and L-tyrosine are the most inhibitory of all the amino acids tested, being more effective than L-leucine itself in inhibiting L-[^{3}H]leucine uptake.

Indeed, the K_i for phenylalanine inhibition was found from both Dixon and Lineweaver-Burk plots to be about $1.4 \pm 0.3 \times 10^{-7}$ M, slightly lower than the K_m for leucine transport. The strong competitive nature of the inhibition revealed by these experiments is evidence of a common carrier for phenylalanine and leucine, and complements the results found in the specificity studies. Although an accurate value for the K_m of phenylalanine transport could not be obtained, the rough data did indicate a value somewhat lower than the K_i. No evidence for a low-affinity component of uptake was seen. Competition studies with other amino acids on L-phenylalanine transport demonstrated a pattern of side chain specificity similar to that for leucine transport, although the extent of inhibition was not as great as with leucine uptake, the percent inhibition values being considerably lower. Amino acids with relatively large and aliphatic side chains showed strong inhibition of phenylalanine uptake, and amino acids such as alanine, serine, and threonine inhibited to a very small extent. The difference in extent of inhibition of phenylalanine uptake as compared to that of leucine uptake is probably due in part to the slightly higher affinity of the carrier for phenylalanine and, possibly, the existence of a separate high-affinity system for the uncharged aromatic amino acids. The increased inhibition of phenylalanine transport by tryptophan compared with that by valine supports the idea of a second system.

In other bacterial species, aliphatic amino acids are usually transported by one or more systems distinct from those serving the aromatic amino acids (14, 23). Transport systems for leucine, isoleucine, and valine have been reported in *E. coli* K-12 (6, 12, 13, 26), *Pseudomonas aeruginosa* (17), *Staphylococcus aureus* (31), and *Bacillus subtilis* (19). The uptake pattern may be extremely complex, as in *E. coli* K-12, which has been reported to have six systems involved in the transport of the branched-chain amino acids (12, 13). In none of these

studies has inhibition by aromatic amino acids been reported.

In *E. coli* B/r, a transport system somewhat similar to the LIV-I system of *E. coli* K-12, but of broader specificity, has been distinguished (34). This system recognizes alanine, cysteine, homoserine, threonine, and tyrosine in addition to the branched-chain amino acids, and thus is one example of an aromatic amino acid apparently sharing a transport system with aliphatic amino acids. However, it differs substantially from the high-affinity hydrophobic amino acid system in marine pseudomonad B-16, in that the B-16 system does not recognize the small side chain of alanine, nor does it recognize threonine or serine, or indeed any amino acid with a polar side chain.

In many bacteria, the aromatic amino acids phenylalanine, tyrosine, and tryptophan are taken up by one or more systems specific for them (1, 2, 5, 7, 18). In none of these cases, is substantial inhibition shown by the branched-chain amino acids, in contrast to the high-affinity system of B-16 discussed in this paper.

Indeed, the B-16 system is more similar in specificity to those systems found in eucaryotic organisms. The L system of Erlich ascites cells and other mammalian tissues transports a wide variety of neutral amino acids with large hydrophobic side chains, including phenylalanine, tyrosine, tryptophan, and branched-chain amino acids (24). Inhibitory effects of methionine, valine, leucine, isoleucine, and phenylalanine on labeled valine and phenylalanine uptake in yeasts were seen by Halvorson and Cohen (15). In germinated conidia of *Neurospora crassa*, system I for neutral amino acids transports phenylalanine, tryptophan, and leucine (25, 39), and a binding protein showing the same affinities has been detected (38). However, isoleucine and valine do not compete with phenylalanine transport by system I in this organism.

The significance of the apparent broad specificity of the B-16 high-affinity system may be related to the need for marine organisms to efficiently garner nutrients from their dilute environment. Since no other marine bacterium has been studied with respect to amino acid transport, we do not know whether this statement can be applied generally.

In bacterial transport systems, periplasmic binding proteins are sometimes found associated with the uptake of amino acids, sugars, and ions (23). Transport by such systems is sensitive to osmotic shock, and the mechanism of energy coupling appears to differ from that of the shock-resistant transport systems (3). In leucine transport in *E. coli*, the LIV-I system is associated with a periplasmic binding protein, but the LIV-II system is not (26). The LIV system in strain B-16 apparently does not have a binding protein associated with it, since L-alanine uptake occurs in isolated membrane vesicles (33); the DAG system also does not have a periplasmic binding protein (8). No periplasmic binding protein appears necessary for the functioning of the high-affinity transport system reported here, since protoplasts showed the same ability to transport leucine as whole cells. Similar results were found for tryptophan, tyrosine, and lysine. Methionine was the only amino acid of the 10 tested for which the protoplast uptake rate was substantially below that in whole cells. This indicates that methionine may be taken up by more than one system, one of which may require a binding protein for activity. The results obtained with glutamic acid and aspartic acid were interesting in that they showed severalfold increases in the uptake rate of protoplasts compared to that of whole cells. One possible explanation of this is that, whereas in whole cells these negatively charged amino acids must pass a negatively charged peptidoglycan layer, in protoplasts such a barrier is not present. (This does not explain why glutamine is taken up more rapidly by protoplasts.) Kahane et al. (16) reported a similar finding with membrane vesicles of an *E. coli* glutamate-utilizing mutant, in that vesicles transported glutamate more than 10-times faster than whole cells. No data were given for this. The results obtained in the present study were preliminary—only one concentration of amino acid was used in each case.

In summary, a third Na^+-dependent neutral amino acid transport system, lacking a periplasmic binding protein, has been identified in the marine pseudomonad B-16. It differs in its kinetics and specificity from both the DAG and LIV systems described previously by Fein and MacLeod (10). These authors did not detect the high-affinity system in their study probably for the following reasons. With the DAG mutants in which both the LIV system and the high-affinity leucine uptake system should have been present, alanine was used as a substrate in the kinetic and specificity experiments. Since alanine is not a substrate for the high-affinity system, this system was not detected. No kinetic or specificity studies were done on either the wild type or the LIV mutants with leucine as a substrate, since these mutants were used to help characterize the DAG transport system and α-AIB was the substrate used. Furthermore, the LIV mutants may have been double mutants lacking both leucine uptake systems since ultraviolet irradiation (known to

cause deletions) was used, and the selection by the tritium suicide technique was for cells that had little ability to transport leucine. Some residual leucine uptake was observed in the LIV mutants. This may have been due to uptake by the DAG system, which their data indicate has some affinity for leucine at high concentrations.

From the data of Fein and MacLeod (10) and the present paper, leucine transport appears to occur via at least two systems, with the DAG system possibly also transporting leucine at very high concentrations. We propose that the high-affinity system reported here be called the LIV-II system (and, consequently, that of Fein and MacLeod, the LIV-I system), although the specificity of this system appears much broader than that of other LIV systems described.

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