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Mutational Separation of Transport Systems for Branched-Chain Amino Acids in *Pseudomonas aeruginosa*

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Several types of *Pseudomonas aeruginosa* mutants defective in the transport systems for branched-chain amino acids were isolated by selection for resistance to 5',5',5'-DL-trifluoroleucine, a leucine analog, under certain conditions. Mutants resistant to trifluoroleucine in the absence of Na^+ were defective in the high-affinity system. These mutants fell into two classes. One class showed a defect in the production of a periplasmic binding protein for leucine, isoleucine, valine, alanine, and threonine, and the other showed normal production of the binding protein as determined by a binding assay and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Properties of the former class of mutants have been partly described (T. Hoshino and M. Kageyama, J. Bacteriol. **141**:1055–1063, 1980). Mutants selected for resistance to trifluoroleucine with Na^+ and an excess amount of alanine showed a defect in the low-affinity system. Membrane vesicles prepared from such a mutant lost the transport activity for leucine. A mutant which showed increased activity of the low-affinity system with a defect in the high-affinity system was obtained from strain PML1453 (high-affinity system defective) by selecting for utilization of isoleucine as a carbon source.

The transport of the branched-chain amino acids L-leucine, L-isoleucine, and L-valine in *Pseudomonas aeruginosa* is mediated by two kinetically distinguishable systems, LIV-I and LIV-II (8). LIV-I, with a high affinity, is specific for alanine and threonine in addition to branched-chain amino acids. In contrast, LIV-II, with a lower affinity, is specific only for branched-chain amino acids and is operative only when Na^+ is present. These facts enabled us to assay separately the transport activities for branched-chain amino acids by the two systems in whole-cell suspensions (8). Several lines of evidence suggest the distinct molecular organization of LIV-I and LIV-II: (i) osmotic shock treatment of cells causes a preferential decrease in the LIV-I system alone (8), (ii) the binding protein for leucine, isoleucine, valine, alanine, and threonine (LIVAT-BP) which was recovered in and purified from the osmotic shock fluid shows properties similar to those of the LIV-I system in specificity and affinities for substrates (10), and (iii) membrane vesicles prepared from *P. aeruginosa* cells have an Na^+-dependent transport system for branched-chain amino acids, properties of which correspond to those of the LIV-II transport system (8, 9). However, no conclusive findings have been obtained for the molecular organization of the LIV-I and the LIV-II transport systems. To clarify this problem, genetic approaches may be valuable.

In this report we describe selection procedures with 5'-5'-5'-trifluoro-DL-leucine (TFL), a leucine analog, that enabled us to isolate various types of mutants defective in the LIV transport systems. The properties of the representatives of mutants isolated by these procedures are also described.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study were *P. aeruginosa* PML14 (prototroph) and its derivatives (10). Bacteria were grown aerobically at 37°C in a synthetic medium (D-medium) (8) with 0.5% D-glucose as an energy source unless otherwise indicated.

Mutagenesis. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was performed according to the procedure of Fargie and Holloway (5), with modifications described previously (10).

Preparation of shock fluids and membrane vesicles. Preparation of the shock fluids of *P. aeruginosa* cells was performed by basically the same procedure as described previously (10). Cells grown in 250 ml of D-medium were harvested at the midexponential phase and subjected to cold-shock treatment (10). The resulting fluid after centrifugation was dialyzed overnight against 10 mM Tris-hydrochloride, pH 7.3, containing 0.02% NaN_3 and stored at −20°C until used.

For obtaining membrane vesicles, cells were grown aerobically in another synthetic medium, G-medium (9), with 0.5% D-glucose as an energy source. Preparation of membrane vesicles by osmotic lysis of spheroplasts was carried out according to a previously described method (9).

Assays of transport and binding activities. Transport

activities of LIV-I and LIV-II were assayed separately with [^{14}C]leucine as a substrate at 37°C as described previously (8). LIV-I activity was assayed in the absence of Na^+, and LIV-II activity was assayed in the presence of 20 mM NaCl and 10 mM L-alanine. Transport assays for proline and asparagine were done similarly, with [^{14}C]proline (45 μCi/μmol) and [^{14}C]asparagine (120 μCi/μmol), respectively.

Transport activities by membrane vesicles were assayed with L-malate as an energy source as described previously (9).

Binding activities of shock fluids were assayed by the equilibrium dialysis method (4), using dialysis bags filled with 0.3 ml of each sample (10).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a discontinuous buffer system was carried out according to the method of Laemmli (11) with the modifications of Mizuno and Kageyama (16).

Other methods. Elimination of Na^+ from agar powder (Iwai Kagaku Co., Tokyo, Japan) was performed by washing the powder with 1 M KCl as described previously (10). Agar plates used in this study were all prepared with this washed agar. The concentration of contaminated Na^+ derived from the washed powder in agar plates was estimated to be 0.03 mM. When needed, NaCl was added to plates to make the final concentration 20 mM.

Protein content was determined by the method of Lowry et al. (13), with bovine serum albumin as a standard.

Chemicals. ^{14}C-labeled amino acids were all purchased from Radiochemical Centre, Amersham, United Kingdom. Acrylamide was from Eastman Kodak Co., Rochester, N.Y. Sodium dodecyl sulfate and Coomassie brilliant blue were products of E. Merk, Darmstadt, West Germany. Nitrosoguanidine was purchased from Aldrich Chemical Co., Milwaukee Wis., and TFL was from Fairfield Chemical Co., Blythewood, S.C. All other compounds used were of reagent grade.

RESULTS

Effect of TFL on growth of and LIV transport activities in *P. aeruginosa*. Cell growth of *P. aeruginosa* PML14 in the synthetic D-medium was completely inhibited by 5 μg of TFL per ml (27 μM) with or without Na^+. In nutrient broth, however, no inhibition of cell growth was observed. *P. aeruginosa* PML14 cells grew normally in nutrient broth even with 100 μg of the analog per ml. Growth inhibition by TFL in D-medium without Na^+ was suppressed by 50 μM leucine, isoleucine, or valine and also, though less effectively, by alanine or threonine. In contrast, in the presence of Na^+, growth inhibition by TFL was suppressed by the branched-chain amino acids alone. Since the Na^+-dependent system, LIV-II, is not responsible for alanine or threonine transport (8), the above findings suggest that TFL affected the growth of *P. aeruginosa* after being transported into cells via the LIV-I system alone or via both systems in the absence or presence of Na^+, respectively.

The effects of TFL on amino acid transport in *P. aeruginosa* cells were investigated by competition experiments (Fig. 1). Leucine transport by LIV-II was completely abolished by the addition of a 130-fold amount of TFL (final concentration, 2.7 mM). TFL also showed an inhibitory effect on LIV-I. The addition of this amount of TFL, however, decreased leucine transport via LIV-I by only 20 to 30%. In contrast, no effect of TFL on the transport systems for proline and asparagine was observed. These findings indicate that TFL was taken up by cells specifically via the LIV transport systems and caused inhibition of the growth of cells.

Isolation of mutants altered in the LIV transport systems. *P. aeruginosa* has two transport systems for branched-chain amino acids, LIV-I and LIV-II, which can be analyzed separately under appropriate conditions. LIV-I alone is active without Na^+, LIV-II alone is functional with Na^+ and an excess of alanine, and both systems are operative with Na^+ (8). These properties of the LIV transport systems enabled us to isolate mutants with a specified defect in the

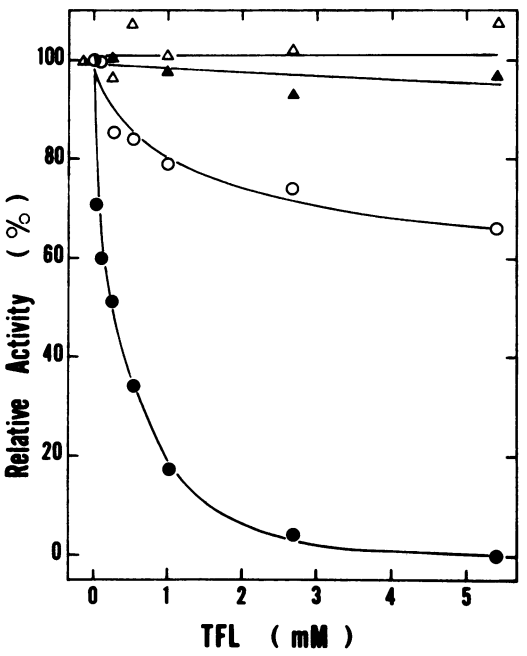

FIG. 1. Effect of TFL on amino acid transport in *P. aeruginosa*. Initial rates were determined with 20 μM ^{14}C-amino acids in the presence of TFL at the indicated concentrations. Relative activity is shown as compared with the control (without TFL). Control values of initial rates of transport of leucine by LIV-I (○) and by LIV-II (●), of proline (△), and of asparagine (▲) were 35, 41, 30, and 1.9 nmol/mg of protein, respectively.

LIV transport systems by selecting for resistance to TFL.

(i) Isolation of LIV-I-defective mutants. LIV-I-defective mutants were selected for resistance to TFL in the absence of Na^+. Nitrosoguanidine-mutagenized cells (10^7) were plated on a D-medium-based agar plate containing 50 μg of TFL per ml and grown at 37°C for 48 h. Colonies appeared at a frequency of 10^{-5}. Such colonies were streaked out for single colonies and checked for resistance to 10 μg of TFL per ml on agar plates. About half of those tested (67 colonies) showed stable resistance to TFL. These were examined for LIV-I and LIV-II activities, and 15 mutant strains had lost LIV-I activity but retained normal levels of LIV-II activity. The other TFL-resistant strains showed partial defects in LIV-I activity. Four of those mutant strains, PML1451, PML1453, PML1458, and PML1459 were chosen for further study. The properties of PML1451 and PML1453 were partly described previously (10).

(ii) Isolation of LIV-II-defective mutants. LIV-II-defective mutants were selected for resistance to TFL in the presence of Na^+ and an excess of alanine. Nitrosoguanidine-mutagenized cells (10^5) were plated on a plate containing 10 μg of TFL per ml, 20 mM NaCl, and 10 mM alanine. After growth for 48 h, colonies appeared at a frequency of 4×10^{-4} on the plate. Forty colonies were streaked out and tested for resistance to TFL. Eight colonies were actually resistant to TFL with Na^+ and alanine, but sensitive to TFL in the absence of alanine. Among these, six colonies showed almost complete loss of LIV-II activity. The other two colonies showed partial loss of the activity. Strain PML1461 was chosen for detailed characterization.

(iii) Isolation of mutants defective in both LIV-I and LIV-II. Mutants defective in both LIV-I and LIV-II were isolated by selecting for resistance to 10 μg of TFL per ml in the presence of 20 mM NaCl. Colonies resistant to TFL appeared at a frequency of 10^{-6}. Among 12 colonies tested in the uptake assay, only 2 were defective in both LIV-I and LIV-II. The other 10 colonies showed considerable levels of LIV transport activities. Strain PML1463 was chosen for further characterization.

(iv) Isolation of revertants to TFL-sensitive phenotype. The wild-type strain PML14 is able to grow with L-isoleucine as a sole carbon source with a doubling time of about 4 h. This suggests that cultivation with isoleucine as a carbon source in the absence of Na^+ would distinguish revertants to the LIV-I-positive phenotype from LIV-I mutants. In fact, such revertants, PML1452 and PML1454, have been isolated from PML1451 and PML1453, respectively, and partly characterized (10). In the process of this selection, however, another type of mutant was also isolated from PML1453. This mutant strain, PML1455, was TFL sensitive in the absence of exogeneously added Na^+ and showed a defect in LIV-I activity.

The TFL sensitivity patterns of the mutant strains are summarized in Table 1. All strains except PML1455 showed the expected response to TFL, indicating that functioning of only one of the LIV transport systems is enough to confer TFL sensitivity on *P. aeruginosa* cells. In this respect, strain PML1455 behaved abnormally. This strain, with a defect in the LIV-I transport system, showed a TFL-sensitive phenotype in the absence of Na^+.

Transport phenotypes of mutants. The activities of several transport systems in mutant strains were investigated (Table 2). Strains PML1451, PML1453, PML1458, and PML1459 showed very low levels of leucine uptake by LIV-I yet retained normal levels of leucine transport by LIV-II and of proline transport, indicating that these mutants have specific defects in LIV-I. In contrast, strains PML1452 and PML1454, TFL-sensitive revertants of PML1451 and PML1453, respectively, showed normal levels of LIV-I, LIV-II, and proline transport. Strain PML1455, another TFL-sensitive revertant of PML1453, showed an enhanced LIV-II level (about fivefold higher than that of wild-type PML14) and a defect in the LIV-I transport system. Strain PML1461 completely lost the LIV-II activity and retained normal

TABLE 1. TFL sensitivity of LIV transport mutants[a]

Strain	System present	Growth with:		
		10 μg of TFL/ml	10 μg of TFL/ml + 20 mM NaCl	10 μg of TFL/ml + 20 mM NaCl + 10 mM alanine
PML14	LIV-I, LIV-II	−	−	−
PML1451	LIV-II	+	−	−
PML1452	LIV-I, LIV-II	−	−	−
PML1453	LIV-II	+	−	−
PML1454	LIV-I, LIV-II	−	−	−
PML1455	LIV-II	−	−	−
PML1458	LIV-II	+	−	−
PML1459	LIV-II	+	−	−
PML1461	LIV-I	−	−	+
PML1463	Neither	+	+	+

[a] A colony was suspended in 0.5 ml of D-medium and grown for several hours at 37°C. The cell suspension was streaked on a D-medium-based agar plate containing supplements as indicated and grown for about 40 h.

TABLE 2. Uptake of L-amino acids by LIV transport mutants

Strain	Uptake (nmol/mg of protein per min) of:		
	Leucine[a] via:		Proline[b]
	LIV-I	LIV-II	
PML14	28	38	46
PML1451	3.2	32	41
PML1452	38	37	42
PML1453	3.6	36	41
PML1454	36	40	38
PML1455	3.5	190	26
PML1458	5.3	25	36
PML1459	1.5	34	36
PML1461	33	0.9	45
PML1463	1.1	1.9	36

[a] Assayed at a final concentration of 20 μM.
[b] Assayed at a final concentration of 50 μM.

TABLE 4. Leucine-binding activities of shock fluids of LIV transport mutants

Strain	Binding activity[a] (nmol/mg of protein)
PML14	1.00
PML1451	0.22
PML1452	1.25
PML1453	0.21
PML1454	1.28
PML1455	0.21
PML1458	1.18
PML1459	1.29
PML1461	1.51
PML1463	0.88

[a] Assayed at 1 μM leucine with crude shock fluids by equilibrium dialysis.

levels of LIV-I and proline transport, indicating a specific defect in LIV-II. Concomitant losses of LIV-I and LIV-II was found in strain PML1463, with a normal level of proline transport (Table 2).

Leucine transport by whole cells was tested in detail. Figure 2 shows double-reciprocal plots of leucine uptake in the presence of Na^+ by representative strains. Wild-type strain PML14 gave a biphasic curve, confirming earlier work (8). Two sets of K_m and V_{max} values were obtained by analyzing the data by the method of Neal (18). Kinetic analyses were further performed under LIV-I (without Na^+) and LIV-II (with Na^+ and an excess of alanine) assay conditions. Each set of K_m and V_{max} values obtained under the two conditions corresponded quite well to a low and a high K_m constituent in the presence of Na^+ (Table 3), respectively. In contrast to the wild-type, PML14, mutants gave straight lines even in the presence of Na^+ (Fig. 2). Strains PML1453 and PML1459 showed the normal kinetic properties of LIV-II with a defect in LIV-I (Table 3). The kinetics of leucine uptake with Na^+ by these mutants did not follow Michaelis-Menten kinetics, however (Fig. 2). This effect was probably caused by residual LIV-I activity, for a quite straight line was obtained by adding an excess of alanine. Strain PML1461 showed normal kinetic properties of LIV-I with a complete loss of LIV-II (Fig. 2 and Table 3). These findings clearly show that two systems, LIV-I and LIV-II, operate independently. Kinetic analyses further demonstrated that the enhanced LIV-II activity in strain PML1455 was caused by an increase in V_{max}, but not by a change in its affinity for substrates.

Properties of shock fluids of mutants. Our previous reports (8, 10) suggested that LIVAT-BP, found in the osmotic shock fluid of *P.*

TABLE 3. Kinetic parameters of leucine transport by LIV transport mutants[a]

Strain	Kinetic parameter					
	Without NaCl		With NaCl		With NaCl and alanine	
	K_m (μM)	V_{max} (nmol/mg of protein per min)	K_m (μM)	V_{max} (nmol/mg of protein per min)	K_m (μM)	V_{max} (nmol/mg of protein per min)
PML14	0.34	30	0.37 7.6	26 45	11	50
PML1453		0[b]	8.3	59	12	45
PML1454	0.26	31	0.30 6.9	35 52	12	48
PML1455		0[b]	10	280	8	270
PML1459		0[b]	10	50	11	38
PML1461	0.19	23	0.19	23		0[b]
PML1463		0[b]		0[b]		0[b]

[a] Initial rates of leucine uptake were determined without NaCl, with 20 mM NaCl, and with 20 mM NaCl and 10 mM alanine.
[b] Transport activity was too low to determine the kinetic parameters.

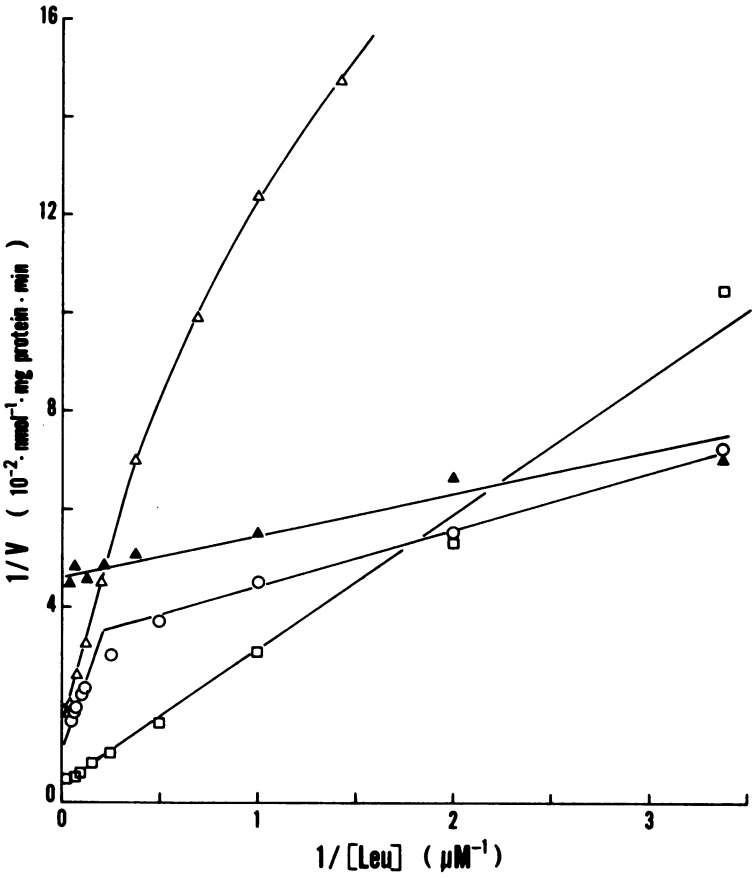

FIG. 2. Double-reciprocal plots of leucine (Leu) uptake by strains PML14 (○), PML1453 (△), PML1455 (□), PML1461 (▲) in the presence of 20 mM NaCl. V, Initial rate.

aeruginosa, is required for LIV-I. The osmotic shock treatment of cells caused a preferential decrease in LIV-I activity (8). The properties of LIVAT-BP were very similar to those of the LIV-I system in substrate specificity and affinity (10). To examine whether there are any defects in the LIVAT-BPs of mutant strains, shock fluids were prepared from these mutants and characterized (Table 4 and Fig. 3). Two LIV-I-defective strains, PML1451 and PML1453, showed little binding activity for leucine, as described previously (10). Revertants PML1452 and PML1454 regained the activity, further confirming previous results (10). Other two LIV-I-defective mutants, PML1458 and PML1459, however, retained normal levels of leucine-binding activity. Shock fluids of strains PML1461 (LIV-II defective) and PML1463 (LIV-I and LIV-II defective) also showed normal levels of leucine-binding activity. The properties of the mutants' shock fluids were further investigated by sodium dodecyl sulfate-polyacrylamide gel

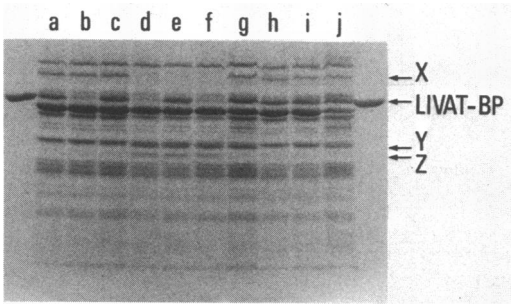

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of shock fluids of LIV transport mutants of *P. aeruginosa* PML14. Samples containing about 4 μg of protein from strains PML14 (a), PML1451 (b), PML1452 (c), PML1453 (d), PML1454 (e), PML1455 (f), PML1458 (g), PML1459 (h), PML1461 (i), and PML1463 (j) were loaded on a 12% polyacrylamide gel. Purified LIVAT-BP from strain PML14 (0.7 μg) was loaded on both end lanes.

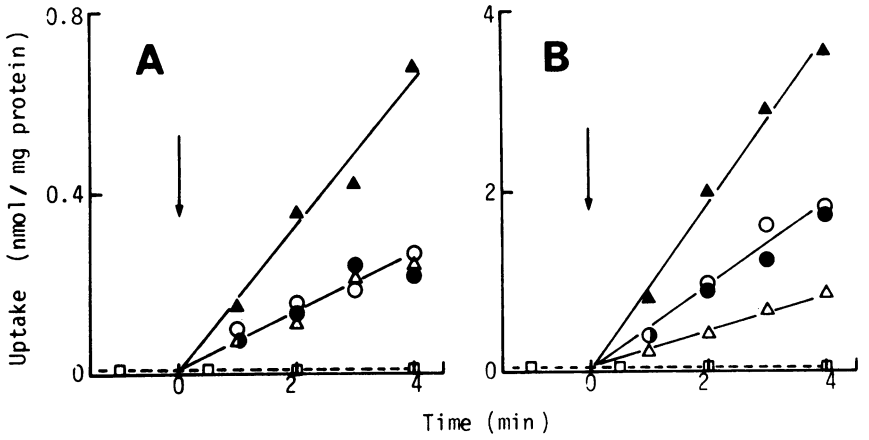

FIG. 4. Transport of proline (A) and asparagine (B) by membrane vesicles of representative LIV transport mutants. After preincubation with 50 μM [^{14}C]proline or [^{14}C]asparagine at 25°C for 2 min, reactions were initiated by the addition of 20 mM potassium malate at the time indicated by the arrow. Squares show accumulation levels of substrate without malate by each strain. Symbols: ○, PML14; ●, PML1453; △, PML1455; ▲, PML1461.

electrophoresis (Fig. 3). The LIVAT-BP was one of the major proteins in the shock fluid of wild-type strain PML14 (Fig. 3a), and the only one that is responsible for leucine binding by the shock fluid (10). Strains PML1451 and PML1453, LIV-I-defective mutants, were defective in the LIVAT-BP band, although very low levels of staining were still found (Fig. 3b and d). Strains PML1452 and PML1454, revertants of PML1451 and PML1453 to an LIV-I-positive phenotype, regained the band (Fig. 3c and e), as described previously (10), confirming that LIVAT-BP is required for LIV-I activity. In contrast, other LIV-I-defective mutants, PML1458 and PML1459, showed normal levels of LIVAT-BP (Table 4 and Fig. 3g and h), suggesting that additional component(s) other than LIVAT-BP are necessary for LIV-I activity. Strain PML1455, an LIV-I-defective mutant with enhanced LIV-II activity, was still defective in the LIVAT-BP band (Fig. 3f). Therefore, this protein seems to have nothing to do with the LIV-II transport system. In the shock fluids of several mutants the appearance of two additional polypeptides, bands Y and Z, with concomitant disappearance of band X, was observed (Fig. 3d through f). In addition, band Y alone was observed in some mutants (Fig. 3g and j). These bands, however, are probably not involved in the LIV transport systems. No correlation of the phenomena with the LIV transport phenotypes of mutants were found.

Properties of transport activities by membrane vesicles. In previous reports (8, 9), we demonstrated the similarity of the transport system for branched-chain amino acids in membrane vesicles to LIV-II in whole cells and suggested that LIV-II alone is responsible for branched-chain amino acid transport by membrane vesicles. Transport activities by membrane vesicles of the following strains with different LIV transport phenotypes were investigated: PML14 (wild type), PML1453 (LIV-I defective), PML1455 (LIV-II enhanced), and PML1461 (LIV-II defective) (Fig. 4). Without malate, none of the vesicle preparations of these strains showed accumulation of proline (Fig. 4A) or asparagine (Fig. 4B). The addition of malate, however, caused marked accumulation of these amino acids by every strain, although some differences in effectiveness between preparations were observed. These findings indicate that all vesicle preparations retained the ability to actively accumulate the amino acids. Activities for leucine transport by membrane vesicles were investigated (Fig. 5). Membrane vesicles prepared from the wild-type strain, PML14 (Fig. 5A), or the LIV-I-defective mutant PML1453 (Fig. 5B) showed respiration-dependent leucine uptake in the presence of Na^+. Strain PML1461, an LIV-II-defective strain, however, showed no accumulation of leucine even when sodium malate was added (Fig. 5D). Membrane vesicles prepared from PML1455, an LIV-I-defective strain with enhanced LIV-II activity, showed marked leucine transport with the addition of sodium malate as an electron donor (Fig. 5C). The initial rate of leucine uptake by the vesicles from PML1455 was threefold higher than that of those from strains PML14 or PML1453. The activity of leucine uptake by vesicles thus paralleled that of LIV-II in whole cells.

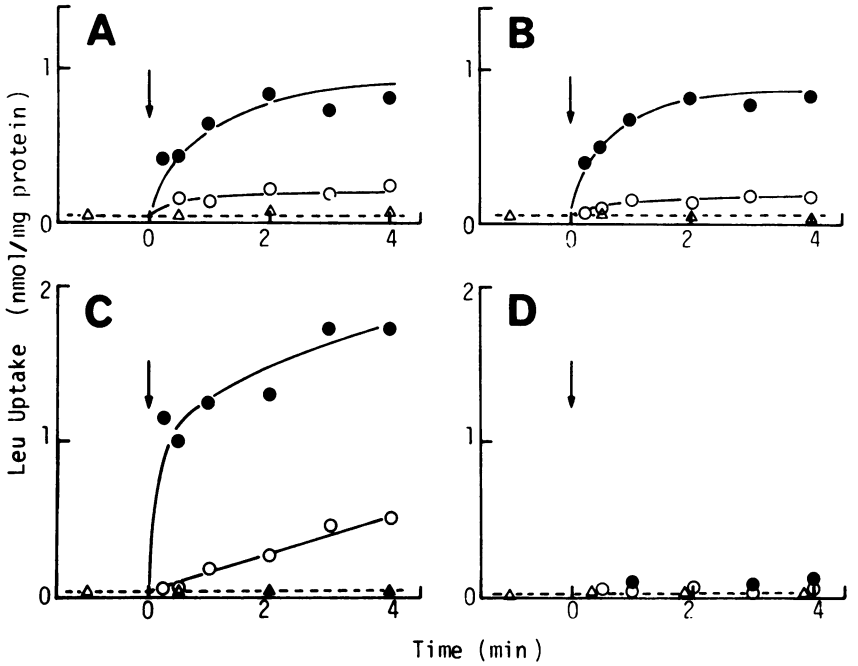

FIG. 5. Transport of leucine (Leu) by membrane vesicles of PML14 (A), PML1453 (B), PML1455 (C), and PML1461 (D). After preincubation with 20 μM [^{14}C]leucine at 25°C for 2 min, reactions were initiated at the time indicated by the arrow by the addition of 20 mM potassium malate (○) or 20 mM sodium malate (●). Triangles indicate uptake with no addition.

DISCUSSION

Mutants defective in amino acid transport usually have been isolated as cells having a phenotype of substrate analog resistance (1, 21) or of a requirement for a high concentration of certain amino acids (14, 17). Selection for resistance to L-valine (2, 6, 7) or for a requirement for a high concentration of leucine (3) or isoleucine (22, 23) has been used to isolate *Escherichia coli* mutants defective in the transport systems for branched-chain amino acids. Since *P. aeruginosa* is naturally resistant to valine (15), application of the selection for valine resistance to this species was impossible. Selection for resistance to TFL, an artificial leucine analog, has proved very useful for isolating *P. aeruginosa* mutants defective in the transport systems for branched-chain amino acids. A functional LIV-I or LIV-II alone was enough to cause the inhibition of cell growth by TFL. Thus, three phenotypically distinguishable types of mutants were isolated. Mutants defective in LIV-I, those defective in LIV-II, or those in both LIV transport systems were selected for resistance to TFL without Na^+, with Na^+ and an excess of alanine, or with Na^+ alone, respectively (Table 1).

Mutants defective in LIV-I showed leucine uptake with the normal kinetic properties of LIV-II, and, in reverse, normal LIV-I activity was found in strain PML1461, which has a defect in LIV-II. In addition, enhancement of LIV-II activity was found in strain PML1455. These results indicate that the two systems operate independently. These findings, however, do not exclude the possibility that one or more genes or components are shared by the two systems. Guardiola et al. (6), in fact, found such a pleiotropic mutation in the gene *brnR*, involved in coding for the LIV transport systems in *E. coli*. *E. coli* mutants defective in both LIV-I and LIV-II have been isolated by Anderson and Oxender (3). We have found a similar mutant in *P. aeruginosa*. Strain PML1463, selected for resistance to TFL in the presence of Na^+, had lost both LIV transport systems. However, reversion to an LIV-I-positive phenotype with a defect in LIV-II was observed in this strain at a frequency of 10^{-6} (data not shown), suggesting that the simultaneous loss of LIV-I and LIV-II was not caused by a pleiotropic mutation, but rather by double mutations. Therefore, we have no evidence so far showing that common components are required for the LIV transport systems in *P. aeruginosa*.

Transport activity for branched-chain amino acids in membrane vesicles corresponded to the LIV-II found in whole cells (Fig. 5). Membrane

vesicles prepared from strain PML1453 (LIV-I defective) retained normal activity for leucine uptake as shown by wild-type strain PML14, whereas vesicle preparations from strain PML1461 (LIV-II defective) had completely lost the activity. Membrane vesicles prepared from strain PML1455, having enhanced LIV-II activity with a defect in LIV-I, showed enhanced leucine uptake, further demonstrating such a parallelism.

As shown in this study, TFL-resistant mutants defective in LIV-I fell into two classes. One class, respresented by strains PML1451 and PML1453, has already been shown to have a defect in the production of LIVAT-BP (10). Revertants of this class of mutants to an LIV-I-positive phenotype were further isolated and were found to regain normal production of LIVAT-BP, suggesting that the LIVAT-BP found in the shock fluid is required for the activity of LIV-I in *P. aeruginosa* (10). In this study, however, the other class, represented by strains PML1458 and PML1459, was found to produce normal levels of LIVAT-BP as determined by binding assay or by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of osmotic shock fluids. These findings suggest that additional component(s) other than LIVAT-BP are necessary for the activity of LIV-I. Anderson and Oxender (2) demonstrated that at least three genes, *livH*, *livJ*, and *livK*, are required for the normal activity of the *E. coli* LIV-I (8). These genes are closely linked on the chromosome of *E. coli* K-12 and recently have been cloned into λ phage and subsequently into a plasmid vector (19, 20). Genes *livJ* and *livK* were shown to code for the LIV- and leucine-specific binding proteins, respectively. Strains with the *livH* mutation were found to retain functional binding proteins, but were missing the high-affinity LIV-I activity. The *livH* gene product has not yet been identified (2). The similar properties of the *livH* mutant and strains PML1458 and PML1459 may suggest that a gene analogous to *livH* codes for a necessary component of LIV-I in *P. aeruginosa*. In *E. coli*, expression of the high-affinity transport systems (LIV-I and a leucine-specific system) is under the control of *livR* and *lstR*, genes for negatively acting regulatory elements (12). In *P. aeruginosa*, however, such a regulatory gene has not yet been identified. However, more information on the biochemical and genetic properties of the mutants is needed to clarify the genetic and the molecular organization of the LIV transport systems in *P. aeruginosa*.

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