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Identification of the LIV-I/LS System as the Third Phenylalanine Transporter in *Escherichia coli* K-12

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In *Escherichia coli*, the active transport of phenylalanine is considered to be performed by two different systems, AroP and PheP. However, a low level of accumulation of phenylalanine was observed in an aromatic amino acid transporter-deficient *E. coli* strain (Δ*aroP* Δ*pheP* Δ*mtr* Δ*tna* Δ*tyrP*). The uptake of phenylalanine by this strain was significantly inhibited in the presence of branched-chain amino acids. Genetic analysis and transport studies revealed that the LIV-I/LS system, which is a branched-chain amino acid transporter consisting of two periplasmic binding proteins, the LIV-binding protein (LIV-I system) and LS-binding protein (LS system), and membrane components, LivHMGF, is involved in phenylalanine accumulation in *E. coli* cells. The K_m values for phenylalanine in the LIV-I and LS systems were determined to be 19 and 30 μM, respectively. Competitive inhibition of phenylalanine uptake by isoleucine, leucine, and valine was observed for the LIV-I system and, surprisingly, also for the LS system, which has been assumed to be leucine specific on the basis of the results of binding studies with the purified LS-binding protein. We found that the LS system is capable of transporting isoleucine and valine with affinity comparable to that for leucine and that the LIV-I system is able to transport tyrosine with affinity lower than that seen with other substrates. The physiological importance of the LIV-I/LS system for phenylalanine accumulation was revealed in the growth of phenylalanine-auxotrophic *E. coli* strains under various conditions.

It has been reported that *Escherichia coli* has five distinct transport systems (AroP, Mtr, PheP, TnaB, and TyrP) for the accumulation of aromatic amino acids (36). A general amino acid permease, encoded by the *aroP* gene, transports three aromatic amino acids with high affinity (8, 11, 21, 36). The closely related PheP protein transports phenylalanine in preference to tyrosine but does not exhibit tryptophan uptake activity (10, 34–36). Mtr and TyrP are specific for tryptophan and tyrosine, respectively (19, 36, 42, 51, 52), and TnaB is a low-affinity, tryptophan-specific transporter encoded in the tryptophanase operon together with the *tnaA* gene (13, 36, 43).

In a previous study, we cloned the tyrosine transporter *tutB* gene of *Erwinia herbicola* and used *E. coli* cells to determine the properties of its product (23). In the course of that study, we found that the aromatic amino acid transporter-deficient *E. coli* strain TK1135 (Δ*aroP* Δ*pheP mtr24* Δ*tna* Δ*tyrP*) (23) has the ability to accumulate phenylalanine in an energy-dependent manner, although the initial rate of uptake, as well as the steady-state level, was quite low. This finding prompted us to examine the basis for this activity and whether this transport activity is physiologically important in *E. coli*. Here, we present evidence indicating that a branched-chain amino acid transporter, the LIV-I/LS system (1–3, 18, 24, 28, 29, 32, 37, 38, 45, 50), acts as the third phenylalanine transporter, plays a significant role in the accumulation of phenylalanine, and has a broader substrate specificity than previously reported.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are derivatives of *E. coli* K-12. The strains and plasmids are listed in Table 1 with their characteristics. The *aroP*, *pheP*, *tna*, and *tyrP* genes were disrupted as described previously (23), and the *mtr* gene was disrupted, using mtr-1 and mtr-2 (Table 1) as primers, by the method described by Datsenko and Wanner (12). Disruption of the *brnQ* gene was carried out as follows. The *brnQ* gene was amplified by PCR using KOD polymerase (Toyobo, Japan) with the genomic DNA of MG1655 as the template and brnQ-F and brnQ-R (Table 1) as the primer pair, and the amplified DNA fragment was ligated with the 3.5-kb *Nsi*I (blunt-ended)-*Nru*I fragment of pACYC177 (6). The internal region of the *brnQ* gene was then removed by *Eco*RV-*Pvu*II digestion and replaced with the Flp recognition target (FRT)-flanked kanamycin resistance gene (FRT-*kan*$^+$-FRT), which was amplified by PCR using pKD13 (12) as the template and pKD13-1 and pKD13-4 (Table 1) as the primer pair. The resulting Δ*brnQ*::(FRT-*kan*$^+$-FRT) gene was introduced into strain MG1655 harboring pKD46 (12) by electroporation and allowed to integrate into the chromosome through a double-crossover event. Elimination of the *kan* gene from the integrated locus was carried out as described previously (12), with the aid of plasmid pCP20 carrying the Flp recombinase gene (7). Disruption of the *livHMGF* region and the *livJ-yhhK-livKHMGF* gene cluster was performed similarly. In these cases, primer pair livH-F and livF-R and primer pair liv-F1 and liv-R1 (Table 1) were used for amplification of the *livHMGF* and *livJ-yhhK-livKHMGF* genes, respectively. The internal 2.9-kb *Bgl*II-*Pvu*II region in the *livHMGF* cluster and the 6.4-kb *Pvu*II region within the *livJ-yhhK-livKHMGF* cluster were deleted and replaced with the FRT-*kan*$^+$-FRT gene. Integration into the chromosome and subsequent elimination of the *kan*$^+$ gene were carried out as described above.

After confirmation of the correct recombination event by Southern hybridization analysis (41) and/or genomic PCR analysis with primers designed to anneal external regions that had been used for the homologous recombination event, the disrupted gene was transferred to other strains by P1 transduction (26).

Media and chemicals. Luria-Bertani (LB) (26) broth was routinely used for the cultivation of *E. coli* strains. M63-glucose (26) was used as the minimal medium, and, when necessary, phenylalanine and pantothenate were added as growth requirements to final concentrations of 10 μM to 1 mM and 5 μg/ml, respectively. Ampicillin, tetracycline, and kanamycin were used at final concentrations of 100, 15, and 30 μg/ml for LB medium and 50, 7.5, and 15 μg/ml for

TABLE 1. Strains, plasmids, and oligonuclotides used in this work

Strain, plasmid, or oligonucleotide	Characteristic(s) or sequence	Source or reference
Strains		
MG1655	λ⁻ *rph-1*	Laboratory stock
NK6024	λ⁻ Δ(*gpt-lac*)*5 pheA18*::Tn*10 relA1 spoT1 thi-1*	B. J. Bachmann
TK1135	MG1655 Δ*aroP mtr24* Δ*pheP* Δ*tna* Δ*tyrP*	23
TK1170	MG1655 Δ*aroP* Δ*mtr* Δ*pheP* Δ*tna* Δ*tyrP*	This study
TK1173	TK1170 *pheA18*::Tn*10*	This study
YG74	MG1655 Δ*aroP* Δ*livHMGF* Δ*pheP*	This study
YG106	MG1655 Δ*aroP* Δ*brnQ* Δ*pheP*	This study
YG108	MG1655 Δ*aroP* Δ*brnQ* Δ*livHMGF*	This study
YG109	MG1655 Δ*brnQ* Δ*livHMGF* Δ*pheP*	This study
YG195	MG1655 Δ*aroP* Δ*brnQ* Δ*mtr* Δ*pheP* Δ*tna* Δ*tyrP*	This study
YG198	MG1655 Δ*aroP* Δ*brnQ* Δ*livHMGF* Δ*mtr* Δ*pheP* Δ*tna* Δ*tyrP*	This study
YG201	MG1655 Δ*aroP* Δ*brnQ* Δ*livHMGF* Δ*pheP*	This study
YG208	MG1655 *pheA18*::Tn*10*	This study
YG210	YG106 *pheA18*::Tn*10*	This study
YG211	YG108 *pheA18*::Tn*10*	This study
YG212	YG109 *pheA18*::Tn*10*	This study
YG213	YG201 *pheA18*::Tn*10*	This study
YG228	MG1655 Δ*aroP* Δ*brnQ* Δ(*livJ-yhhK-livKHMGF*) Δ*pheP*	This study
YG256	YG228 Δ*tyrP*	This study
Plasmids		
pACYC177	p15A replicon *bla*⁺ *kan*⁺	6
pCP20	pSC101 replicon (Ts) *bla*⁺ *cat*⁺ Flp(λR*p*) *cI857*	7
pKD13	*oriR*γ *bla*⁺ FRT-*kan*⁺-FRT	12
pKD46	*oriR101 repA101*(Ts) *bla*⁺ *araC*⁺ *gam*⁺-*bet*⁺-*exo*⁺ (*araBp*)	12
pMBO131	Mini-F replicon *cat*⁺	30
pMW118	pSC101 replicon *bla*⁺ *lacZ*α⁺	Nippon Gene
pYG218	pSC101 replicon *bla*⁺ *livH*⁺ *M*⁺ *G*⁺ *F*⁺	This study
pYG237	Mini-F replicon *kan*⁺ *livJ*⁺	This study
pYG239	Mini-F replicon *kan*⁺ *livK*⁺	This study
pYG249	Mini-F replicon *kan*⁺; the 1.7-kb *Nhe*I-*Bam*HI fragment containing the *kan*⁺ gene was recovered from pACYC177, blunt ended, and then ligated with the 4.8-kb *Sca*I-*Xho*I (blunt-ended) fragment of pMBO131	This study
Oligonucleotides		
brnQ-F	5′-GATTAGCCATGTCTTTTTCACGGAA-3′ (for cloning the *brnQ* gene; upstream end)	
brnQ-R	5′-ATGCTTTGATCCCGTCGAGAATAC-3′ (for cloning the *brnQ* gene; downstream end)	
livH-F	5′-AGGTTACCTTATGTCTGAGCAG-3′ (for cloning the *livHMGF* genes; upstream end)	
livF-R	5′-CGGTTTCATCGTTTATCTCTCTT-3′ (for cloning the *livHMGF* genes; downstream end)	
liv-F1	5′-CCAATCCCCACGCAGATTGTTAATAAACTG-3′ (for cloning the *livJ-yhhK-livK* genes and the *livJ-yhhK-livKHMGF* genes; upstream end)	
liv-R1	5′-GTGAGGGAAAATGGGAGATGGGGC-3′ (for cloning the *livJ-yhhK-livKHMGF* genes; downstream end)	
liv-R2	5′-CAATCATATAAACCTCGCCGTGGG-3′ (for cloning the *livJ-yhhK-livK* genes; downstream end)	
mtr-1	5′-CACCGTCGCTGCTTGGCGGCGTGGTGATTATCGGCGGCACGTGTAGGCTGGAGCTGCTTC-3′ (for disruption of the *mtr* gene; upstream end)	
mtr-2	5′-GCTGCCAAAGCGTTTACGCGATGCACGGGCTAACAGCGCCATTCCGGGGATCCGTCGACC-3′ (for disruption of the *mtr* gene; downstream end)	
pKD13-1	5′-GTGTAGGCTGGAGCTGCTGCTTC-3′ (for amplifying the kanamycin resistance gene; upstream end)	
pKD13-4	5′-ATTCCGGGGATCCGTCGACC-3′ (for amplifying the kanamycin resistance gene; downstream end)	

the minimal medium, respectively. For the disk inhibition assay, disks were impregnated with 1 mM concentrations of various amino acids and then put onto the plates. L-(U-^{14}C)-isoleucine (314 mCi/mmol, 0.05 mCi/ml), L-(U-^{14}C)-leucine (306 mCi/mmol, 0.05 mCi/ml), L-(U-^{14}C)-valine (256 mCi/mmol, 0.05 mCi/ml), and L-(U-^{14}C)-tyrosine (434 mCi/mmol, 0.05 mCi/ml) were purchased from Amersham Pharmacia Biotech. L-(U-^{14}C)-phenylalanine (496 mCi/mmol, 0.1 mCi/ml) and L-(side chain-3-^{14}C)-tryptophan (58.1 mCi/mmol, 0.02 mCi/ml) were from Perkin-Elmer Life Sciences Inc. The chemicals were all obtained commercially and not purified further.

Genetic techniques. Standard genetic techniques were used essentially as described by Sambrook and Russell (41). The method used for generalized transduction involving the P1 phage was that described by Miller (26).

Cloning of the *liv* gene cluster. The chromosomal locus including the *liv* gene cluster consists of the *livJ*, *yhhK*, *livK*, *livH*, *livM*, *livG*, and *livF* genes in that order. While the *livJ* and *livK* genes encode periplasmic binding proteins, the *livH*, *livM*, *livG*, and *livF* genes specify membrane channel components (1, 45). The function of the *yhhK* gene has not been clarified yet. The DNA fragment containing the *livJ-yhhK-livK* region was amplified by high-fidelity PCR using KOD polymerase (Toyobo, Japan) with the genomic DNA of MG1655 as the template and liv-F1 and liv-R2 (Table 1) as the primer pair. To clone the *livJ* gene, the amplified fragment was digested with *Aat*I to remove the *yhhK* and *livK* genes and then inserted into the *Sal*I (blunt-ended) site of pYG249. The *livK* gene was recovered by *Bgl*II digestion of the amplified fragment, blunt ended, and then inserted into the *Sal*I (blunt-ended) site of pYG249. The amplified *livK* and *livJ* genes were entirely sequenced to ensure that no misincorporation of nucleotides had occurred during the PCR amplification.

The genes for the *livHMGF* cluster were cloned as follows. The DNA fragment containing the *livJ-yhhK-livKHMGF* gene cluster was amplified by high-fidelity PCR with the genomic DNA of MG1655 as the template and liv-F1 and liv-R1 (Table 1) as the primer pair. After insertion of the fragment into the *Pvu*II site of pMW118 (Nippon Gene, Tokyo, Japan), the 2.3-kb *Eco*RV fragment containing the *livJ-yhhK-livK* genes was removed and the remaining large fragment carrying the *livHMGF* genes was circularized by self-ligation. Although the *livKHMGF* genes constitute an operon and are usually transcribed in one unit, it

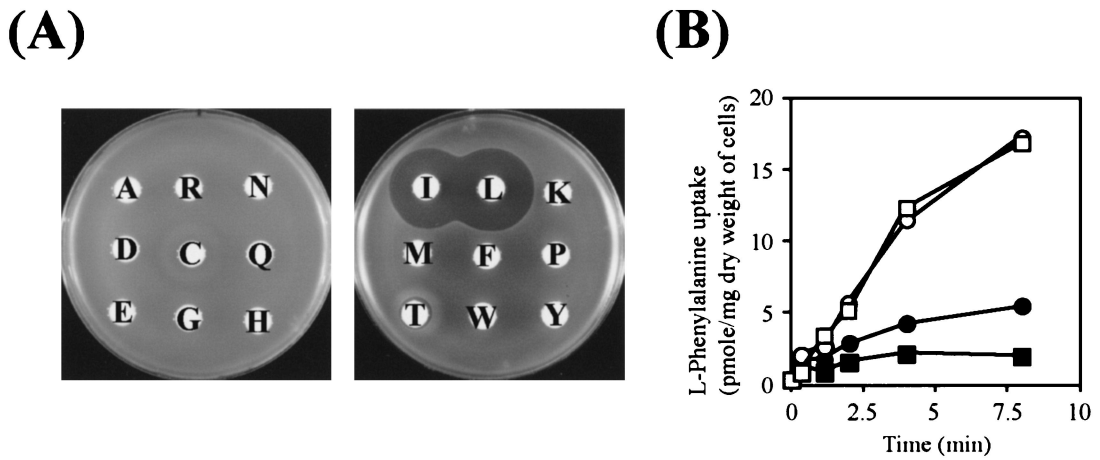

FIG. 1. Inhibition of phenylalanine uptake by branched-chain amino acids. (A) Growth inhibition of phenylalanine-auxotrophic (Phe$^-$) *E. coli* strain TK1173 (Δ*aroP* Δ*mtr* Δ*pheP* Δ*tna* Δ*tyrP pheA18*::Tn*10*) in the presence of branched-chain amino acids, as observed on disk assaying. Cells were grown in LB medium, washed twice with M63 minimal buffer, mixed with the top agar, and then overlaid on M63 minimal solid medium containing 100 μM phenylalanine. Disks were impregnated with 1 mM concentrations of various amino acids (indicated by a one-letter code). (B) Phenylalanine uptake activity of *E. coli* strain TK1170 (Δ*aroP* Δ*mtr* Δ*pheP* Δ*tna* Δ*tyrP*). L-Phenylalanine was added to cell suspensions to a final concentration of 50 μM in either the absence (□) or presence of 5 μM glutamate (○), leucine (■), or valine (●). Samples were withdrawn at the indicated times. The experiments were repeated three times with essentially the same results; the data for a representative experiment are shown.

has been shown that a weak internal promoter present just upstream of the *livH* gene can direct synthesis of the downstream genes (1). Sequence analysis of the amplified fragment revealed a two-base discordance compared to data reported by Blattner et al. (4) at a locus downstream of the stop codon of the *livF* gene, which would have no substantial effect on the properties of the LIV-I/LS system. The resulting plasmid, pYG218, was introduced into strain YG201 (Δ*aroP* Δ*brnQ* Δ*livHMGF* Δ*pheP*) and examined for the ability to complement the chromosomal *livHMGF* lesion with respect to phenylalanine transport.

Transport assays. Transport assays were performed as described previously (23, 51), with slight modifications as follows. Cells grown in minimal medium were harvested at mid-exponential phase and then washed twice with M63-glucose containing 60 μg of chloramphenicol/ml to stop protein synthesis. The assay was initiated by adding the cell suspension to the reaction mixture containing various concentrations of labeled substrates in the presence or absence of cold competitive inhibitors. The rate of nonspecific diffusion was determined using energy-starved cells that had been prepared by incubating cells in the presence of 100 μM carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) for 30 min prior to starting the assay. The uptake of substrates was expressed as picomoles per milligram of dry cells as a function of time.

RESULTS AND DISCUSSION

It has so far been considered that in *E. coli*, the active transport of aromatic amino acids across the inner membrane is mediated by five distinct permeases, AroP, Mtr, PheP, TnaB, and TyrP (8, 10, 11, 13, 19, 21, 34–36, 42, 43, 51, 52) and that among them, the AroP and PheP systems are responsible for the accumulation of phenylalanine in cells. However, as described above, in the course of studying tyrosine transporter TutB of *E. herbicola* through the use of *E. coli* cells (23), a low level of accumulation of phenylalanine was observed in the aromatic amino acid transporter-deficient strain TK1135 (Δ*aroP* Δ*pheP mtr24* Δ*tna* Δ*tyrP*) (data not shown). At first we speculated that this activity might be due to altered specificity of the mutant Mtr protein, i.e., Mtr24 (20), although the nature of the *mtr24* allele has not been elucidated. This possibility, however, was ruled out by the observation that an *E. coli* strain, TK1170 (Δ*aroP* Δ*pheP* Δ*mtr* Δ*tna* Δ*tyrP*), accumulated as much phenylalanine (Fig. 1B) as strain TK1135 in an energy-dependent manner. Even though the initial rate of uptake and the steady-state level of phenylalanine in cells were not so high compared to those with known phenylalanine transport systems, AroP and PheP, reported previously (5, 49) (Fig. 2), this activity seems to be important for cells to accumulate phenylalanine because a phenylalanine-auxotrophic (Phe$^-$) strain could be obtained by transducing TK1170 (Δ*aroP* Δ*pheP* Δ*mtr* Δ*tna* Δ*tyrP*) with a P1 phage lysate prepared from strain

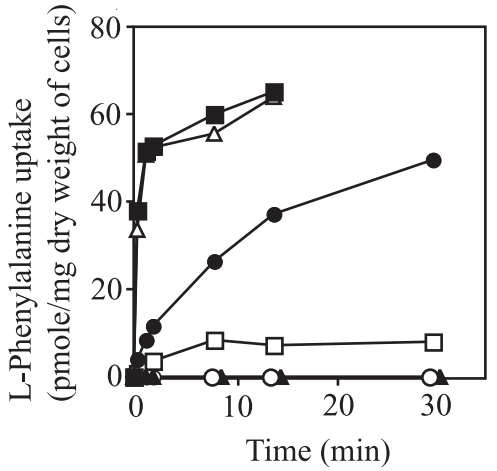

FIG. 2. The LIV-I/LS system as the third phenylalanine transporter in *E. coli*. L-Phenylalanine uptake was measured in various *E. coli* cells, including YG74 (Δ*aroP* Δ*livHMGF* Δ*pheP*) (BrnQ) (▲), YG106 (Δ*aroP* Δ*brnQ* Δ*pheP*) (LIV-I/LS) (□), YG108 (Δ*aroP* Δ*brnQ* Δ*livHMGF*) (PheP) (●), YG109 (Δ*brnQ* Δ*livHMGF* Δ*pheP*) (AroP) (△), and YG201 (Δ*aroP* Δ*brnQ* Δ*livHMGF* Δ*pheP*) (○) cells, and compared to that in wild-type strain MG1655 (■). Cell suspensions were incubated in the presence of 1 μM L-(U-^{14}C)-phenylalanine, and samples were withdrawn at the times indicated. The experiments were repeated three times with essentially the same results; the data for a representative experiment are shown.

NK6024 (*pheA18*::Tn*10*) (PheA, chorismate mutase-prephenate dehydratase) and subsequent selection with Tn*10* as a marker. These findings suggested that *E. coli* might have at least one additional phenylalanine transporter.

Inhibition of phenylalanine uptake by branched-chain amino acids. The question is what system is involved in this transport: a protein encoded by an undefined open reading frame (function unknown ORF) or a defined small molecule transporter with broad substrate specificity? We found by using a disk inhibition assay (Fig. 1A) that the growth of the aromatic transporter-negative Phe$^-$ strain TK1173 (Δ*aroP* Δ*pheP* Δ*mtr* Δ*tna* Δ*tyrP pheA18*::Tn*10*) in minimal medium supplemented with phenylalanine was severely inhibited in the presence of isoleucine and leucine. In this assay, valine and serine were omitted since both cause a serious growth defect by blocking the synthesis of intermediates required for the synthesis of other amino acids (17, 25, 44). While large clear zones of inhibition were observed around the disks impregnated with isoleucine and leucine, a small inhibition zone was also observed around the disk impregnated with threonine (Fig. 1A), which is discussed later. No significant inhibition zones appeared around the other 15 amino acids tested. The results in Fig. 1A suggested that phenylalanine might be accumulated in cells through a branched-chain amino acid transport system. This notion was further supported by a transport assay with L-(U-^{14}C)-phenylalanine. Whereas the presence of glutamate (Fig. 1B) in the assay mixture did not affect phenylalanine uptake, the addition of valine and leucine decreased the phenylalanine uptake activity even with a low concentration (5 μM valine and leucine each versus 50 μM phenylalanine). These results strongly suggested that the active transport of phenylalanine into *E. coli* cells with the Δ*aroP* Δ*pheP* Δ*mtr* Δ*tna* Δ*tyrP* background could be dependent on a system that transports branched-chain amino acids.

Identification of the LIV-I/LS system as the third phenylalanine transporter in *E. coli*. Branched-chain amino acids are transported into *E. coli* cells by an osmotic-shock-sensitive system designated LIV-I/LS (1–3, 18, 24, 28, 29, 32, 37, 38, 45, 50) and by an osmotic-shock-resistant system, BrnQ (15, 16, 31, 45, 53, 54), formerly called LIV-II (3, 31, 37, 38, 45, 50). Whereas transport by the BrnQ system is mediated by a single membrane protein (38, 45, 50), uptake by the LIV-I/LS system depends on two substrate-binding proteins (BP), LIV-BP and LS-BP, located in the periplasm (2, 14, 24, 33, 38, 45, 50). Previous studies involving purified BPs showed that LIV-BP, encoded by the *livJ* gene, binds isoleucine, leucine, and valine with K_d values of 10^{-6} to 10^{-7} M and threonine, serine, and alanine with lower affinity and that LS-BP, encoded by the *livK* gene, binds leucine with a K_d value of approximately 10^{-6} M (24). To enable the ATP-hydrolysis-coupled transport of their substrates into the cytoplasm, LIV-BP and LS-BP interact with the common inner-membrane components LivHMGF, which constitute the LIV-I and LS systems, respectively (1, 28, 29, 45, 50). These six *liv* genes are clustered at 77 min on the chromosome (45) and divided into two transcription units, one for *livJ* and the other for *livKHMGF* (1, 18, 45). In the region between *livJ* and *livK* there is the *yhhK* gene; the deletion of this region results in pantothenate auxotrophy (1).

To determine whether BrnQ or LIV-I/LS carries out the uptake of phenylalanine, a series of *E. coli* strains expressing individual transport systems was constructed and assayed for transport: AroP-expressing strain YG109 (Δ*brnQ* Δ*livHMGF* Δ*pheP*), BrnQ-expressing strain YG74 (Δ*aroP* Δ*livHMGF* Δ*pheP*), LIV-I/LS-expressing strain YG106 (Δ*aroP* Δ*brnQ* Δ*pheP*), and PheP-expressing strain YG108 (Δ*aroP* Δ*brnQ* Δ*livHMGF*). The transport activity was measured in the presence of 1 μM labeled phenylalanine and compared to that of wild-type strain MG1655 and strain YG201 lacking portions of the *aroP*, *brnQ*, *livHMGF*, and *pheP* genes.

As shown in Fig. 2, neither BrnQ-expressing strain YG74 (Δ*aroP* Δ*livHMGF* Δ*pheP*) nor strain YG201 (Δ*aroP* Δ*brnQ* Δ*livHMGF* Δ*pheP*) could accumulate phenylalanine. A sodium gradient made by adding NaCl (final concentration, 1 mM) to the assay mixture did not have any effect on the uptake activity of these strains. On the other hand, LIV-I/LS-expressing strain YG106 (Δ*aroP* Δ*brnQ* Δ*pheP*) was able to accumulate phenylalanine, demonstrating the involvement of the LIV-I/LS system, but not BrnQ, in phenylalanine transport, although the initial rate and the steady-state level were considerably lower than those in the strains expressing AroP and PheP. It seemed likely that the small inhibition halo observed around the disk impregnated with threonine shown in Fig. 1A reflected the substrate preference of LIV-BP (24). Despite the low phenylalanine transport activity, the LIV-I/LS system alone could support the growth of Phe$^-$ strain YG210 (Δ*aroP* Δ*brnQ* Δ*pheP pheA18*::Tn*10*) in minimal medium supplemented with 10 μM phenylalanine, indicating the participation of the LIV-I/LS system in the accumulation of phenylalanine. The K_m value for phenylalanine in the LIV-I/LS system was determined to be 30 μM, which is considerably higher than those for AroP (0.4 μM) and PheP (2 μM) (36).

AroP-expressing strain YG109 (Δ*brnQ* Δ*livHMGF* Δ*pheP*) exhibited the highest uptake activity, and its activity was essentially equal to that of wild-type strain MG1655, suggesting that the AroP protein ordinarily acts as the major phenylalanine transport system in wild-type cells. As for PheP-expressing strain YG108 (Δ*aroP* Δ*brnQ* Δ*livHMGF*), more than 40 pmol of phenylalanine/mg (dry weight of cells) was accumulated in the cells, which was comparable to the steady-state level in the case of the AroP system, although the initial rate of uptake was significantly lower than that for AroP.

Next, we tested which binding protein, LIV-BP or LS-BP, participates in the transport of phenylalanine. For this end, LIV-BP and LS-BP were expressed in *E. coli* cells individually in the presence of membrane machinery components LivHMGF. Strain YG228 [Δ*aroP* Δ*brnQ* Δ(*livJ-yhhK-livKHMGF*) Δ*pheP*] was transformed with two compatible plasmids; one was a pSC101-derived vector carrying the genes for membrane components LivHMGF (pYG218), and the other was a Mini-F-derived plasmid carrying either the *livJ* gene (LIV-I) (pYG237; Fig. 3A) or the *livK* gene (LS) (pYG239; Fig. 3B). These strains were used for uptake assay in the presence of 10 to 300 μM phenylalanine (Fig. 3A and B). The results clearly show that both BPs are capable of effecting transport of the substrate. The amounts of phenylalanine accumulated in the cells significantly differed between them, but we cannot comment about this difference because the organization of the *liv* genes on plasmids was different from that on the chromosome. In the absence of BP, no accumulation was observed in the cells (Fig. 3A and B). Considering that the disruption of *livHMGF*, the

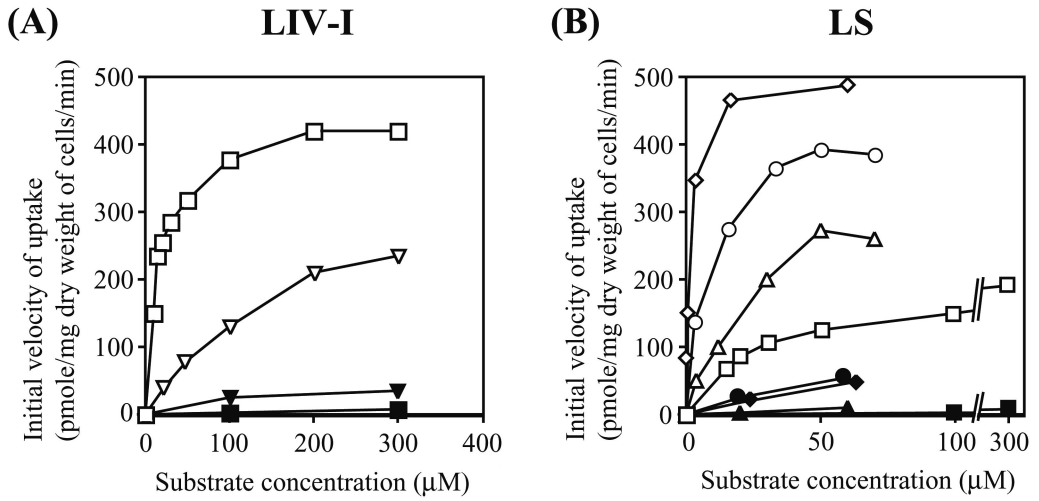

FIG. 3. Uptake studies of various amino acids with *E. coli* cells expressing the LIV-I (A) and LS (B) systems. (A) Strain YG228 [Δ*aroP* Δ*brnQ* Δ(*livJ-yhhK-livKHMGF*) Δ*pheP*] carrying pYG218 (pSC101 replicon *bla*$^+$ *livH*$^+$*M*$^+$*G*$^+$*F*$^+$) (membrane components) was transformed with either pYG237 (Mini-F replicon *kan*$^+$ *livJ*$^+$) (LIV-I; open symbols) or pYG249 (Mini-F replicon *kan*$^+$) (control; filled symbols). For the tyrosine transport assay, YG256 [Δ*aroP* Δ*brnQ* Δ(*livJ-yhhK-livKHMGF*) Δ*pheP* Δ*tyrP*] was used instead of YG228. Cell suspensions were incubated in the presence of 10 to 300 μM L-phenylalanine (□, ■) and 25 to 300 μM L-tyrosine (▽, ▼). (B) Strain YG228 carrying pYG218 was transformed with either pYG239 (Mini-F replicon *kan*$^+$ *livK*$^+$) (LS; open symbols) or pYG249 (control; filled symbols). Cell suspensions were incubated in the presence of 10 to 300 μM L-phenylalanine (□, ■), 0.4 to 70 μM L-leucine (◇, ◆), L-isoleucine (○, ●), or L-valine (△, ▲). All experiments were repeated three times with essentially the same results; the data for a representative experiment are shown.

genes encoding the membrane components, completely abolished phenylalanine transport (Fig. 2), it can be concluded that both BPs interact only with LivHMGF.

Recently, using NMR with fluorine-labeled LS-BP, Salopek-Sondi and Luck revealed that LS-BP binds phenylalanine in addition to leucine and that the phenylalanine-binding ability is specific for LS-BP but not for LIV-BP (39). Our transport studies corroborated the ability of LS-BP to bind phenylalanine but contradicted the results obtained for LIV-BP.

Taken together, these results led us to the conclusion that in *E. coli*, there are three phenylalanine uptake systems, AroP, PheP, and LIV-I/LS, all of which may allow phenylalanine accumulation.

Kinetic studies of the LIV-I and LS systems. To further characterize the LIV-I and LS systems, kinetic constants for both systems were determined by monitoring phenylalanine uptake in the absence or presence of probable competitive inhibitors, branched amino acids. The K_m values for phenylalanine in the LIV-I and LS systems were determined to be 19 and 30 μM, respectively, by double-reciprocal plotting of the data in Fig. 3 (Table 2). In inhibition assays, as expected from the substrate specificity of LIV-BP, phenylalanine uptake by the LIV-I system was found to be decreased in a concentration-dependent manner upon the addition of isoleucine, leucine, and valine (data not shown), the K_i values for them having been determined to be 2.3, 1.7, and 1.5 μM, respectively (Table 2). These K_i values were comparable to the respective K_m values determined by means of transport assays with LIV-I-expressing cells incubated in the presence of 0.4 to 70 μM labeled branched-chain amino acids (Table 2). The K_i values for valine and phenylalanine inhibition of leucine uptake were also determined by incubating cells under conditions of 0.4 to 20 μM labeled leucine in the presence of cold valine (0.5 to 20 μM) and phenylalanine (15 to 50 μM). The values obtained (K_i = 1.4 μM for valine and 30 μM for phenylalanine) were in good agreement with the K_m values (2.4 μM for valine and 19 μM for phenylalanine) (Table 2).

The finding that phenylalanine is a good substrate prompted

TABLE 2. Kinetic constants (K_m and K_i) for various substrates in the LIV-I and LS systems as determined by in vivo uptake assays

System[a] and substrate	K_m[b] (μM)	K_i[b] for inhibition of Phe uptake (μM)	K_i[b] for inhibition of Leu uptake (μM)
LIV-I (LivJ)			
Ile	8.0	2.3	ND[d]
Leu	2.3	1.7	
Val	2.4	1.5	1.4
Phe	19		30
Tyr[c]	230	120	200
LS (LivK)			
Ile	5.0	6.6	ND
Leu	2.3	2.1	
Val	9.2	2.7	8.3
Phe	30		74

[a] *E. coli* strain YG228 [λ$^-$ *rph-1* Δ*aroP* Δ*brnQ* Δ(*livJ-yhhK-livKHMGF*) Δ*pheP*] carrying pYG218 (pSC101 replicon *bla*$^+$ *livH*$^+$ *M*$^+$ *G*$^+$ *F*$^+$) (membrane component) was transformed with either pYG237 (Mini-F replicon *kan*$^+$ *livJ*$^+$) (LIV-BP) or pYG239 (Mini-F replicon *kan*$^+$ *livK*$^+$) (LS-BP) and then used for assaying.

[b] The apparent K_m and K_i values were determined by double-reciprocal plotting of the data. For determination of K_m, assays were carried out in the presence of 0.4 to 70 μM Ile (L-isoleucine), Leu (L-leucine), and Val (L-valine), 10 to 300 μM Phe (L-phenyalanine), and 25 to 300 μM Tyr (L-tyrosine). Values are the averages of three independent results. For K_i determination, the phenylalanine and leucine concentrations were varied (from 10 to 200 μM and 0.4 to 20 μM, respectively) in the presence of cold isoleucine, leucine, valine (0.5 to 20 μM each), phenylalanine (15 to 50 μM), or tyrosine (100 to 500 μM). Values are the averages of two independent experiments.

[c] *tyrP*-disruptant YG228 [λ$^-$ *rph-1* Δ*aroP* Δ*brnQ* Δ(*livJ-yhhK-livKHMGF*) Δ*pheP* Δ*tyrP*] (YG256) transformed with pYG218 and pYG237 was used for assaying.

[d] ND, not determined.

us to examine the possibility of other aromatic amino acids being transported by the LIV-I system. We found that the uptake of phenylalanine by the LIV-I system was inhibited in the presence of tyrosine with a K_i value of 120 μM (Table 2), suggesting the ability of the system to transport tyrosine. Δ*tyrP* YG228 was constructed (YG256), similarly transformed with pYG218 (*livHMGF*) and pYG237 (*livJ*), and then examined for transport. Low-level accumulation of labeled tyrosine was observed (Fig. 3A) but not in the strain carrying the empty vector. The K_m value for tyrosine (230 μM) in the LIV-I system was comparable to the K_i value (200 μM) determined by its inhibition of leucine uptake. Although the accumulation of tyrosine was appreciable, the presence of the LIV-I/LS system alone could not support the growth of a tyrosine-auxotrophic strain (Δ*aroP* Δ*brnQ* Δ*mtr* Δ*pheP* Δ*tna* Δ*tyrP* Δ*tyrA*::*kan*$^+$) in minimal medium even in the presence of 100 μM tyrosine, maybe due to the low affinity for tyrosine. Therefore, it seems likely that the LIV-I/LS system is not a physiologically important tyrosine transporter in *E. coli*. As for tryptophan, neither inhibition of leucine uptake nor accumulation in the cells by the LIV-I system was observed (10 to 300 μM) (data not shown). Alanine, serine, and threonine acted as inhibitors of phenylalanine transport by the LIV-I system (data not shown), as expected from the results obtained in binding studies with LIV-BP by Rahmanian et al. (38).

Similar experiments were performed with LS-expressing cells, and not only leucine but also isoleucine and valine were found to inhibit phenylalanine uptake. This was surprising, because it has been shown that purified LS-BP preferentially binds leucine (0.4 μM) but not isoleucine or valine (>1 mM each) (24). We carried out transport assays with labeled substrates (Fig. 3B) and found that the LS system was able to transport isoleucine and valine in addition to leucine. The DNA sequence of the *livK* gene on pYG239 was again analyzed, but no difference was found from the results reported by Blattner et al. (4). The K_m values for isoleucine, leucine, and valine in the LS system were determined to be 5.0, 2.3, and 9.2 μM, respectively. There are apparent contradictions between the results of binding studies (14, 33, 40) and transport studies; is an auxiliary protein involved in the recognition of substrates by LS-BP or does the presence of membrane components LivHMGF alter the substrate specificity of LS-BP? In vitro uptake studies with the LS system reconstituted in liposomes are necessary to explain this discrepancy.

Of the aromatic amino acids tested (10 to 300 μM), only phenylalanine acted as a substrate for the LS system. Phenylalanine inhibited leucine uptake with a K_i value of 74 μM, which was comparable to the K_m value of 30 μM. Likewise, the K_i values estimated for isoleucine (6.6 μM), leucine (2.1 μM), and valine (2.7 μM) in inhibition assays of phenylalanine uptake were in good accordance with the K_m values obtained for them (5.0, 2.3, and 9.2 μM, respectively). The presence of alanine, serine, and threonine (each at 100 μM) did not affect phenylalanine uptake by the LS system at the saturating concentration.

Thus, consistent results were obtained in our transport studies, which revealed new aspects of the substrate specificity of the LIV-I and LS systems. The neutral amino acid ATP-binding cassette-type transport system (Nat) of *Synechocystis* sp. strain PCC 6803 has been identified by means of insertional mutagenesis, and it was shown that the strain inactivated for NatB, a periplasmic binding protein, leaked significant amounts of amino acids alanine, isoleucine, leucine, valine, and phenylalanine into the medium (27), indicating a role of the Nat system in the recapture of these amino acids. Although the LivJ (LIV-BP) and LivK (LS-BP) proteins of *E. coli* exhibit low levels (16%) of identity with NatB with respect to amino acid sequences, a similar substrate specificity was suggested, which may help us understand the mechanism underlying the substrate recognition by these proteins.

Functional distinction among the three phenylalanine uptake systems AroP, PheP, and LIV-I/LS. To obtain a better understanding of the LIV-I/LS system as the phenylalanine transporter, the physiological significance of the AroP, PheP, and LIV-I/LS systems was evaluated. A strain expressing one of the three transport systems was made Phe$^-$ (*pheA18*::Tn*10*) by P1 transduction and then streaked onto an M63-glucose minimal medium plate containing phenylalanine (MMF) and onto an MMF plate including isoleucine, tryptophan, or tyrosine (MMF+I, MMF+W, or MMF+Y) (Fig. 4A). In parallel, a Phe$^-$ strain possessing either all or none of the phenylalanine transporters was constructed and streaked onto similar plates. A phenylalanine transport-deficient Phe$^-$ strain was obtained by spreading the transductants [YG201 and P1(NK6024)] on LB plates containing 1 mM phenylalanine. Since phenylalanine accumulation could not be detected in the YG74 (Δ*aroP* Δ*livHMGF* Δ*pheP*) cells, even in the presence of 100 μM phenylalanine (data not shown), it seems likely that nonspecific diffusion of phenylalanine at the high concentration (1 mM) can support the growth of the strain.

As shown in Fig. 4A, growth of the Phe$^-$ strain in the presence of phenylalanine was dependent on the presence of any one of the AroP, PheP, or LIV-I/LS systems and no growth was observed for the Phe$^-$ strain lacking them. The same results were obtained regardless of the presence or absence of the BrnQ system (data not shown). The growth rates of the strains carrying the respective phenylalanine transporters did not differ significantly. These results confirm the physiological importance of these three transporters in phenylalanine accumulation.

The addition of isoleucine, which is a good substrate for both the LIV-I and LS systems, to the MMF medium severely inhibited the growth of LIV-I/LS-expressing Phe$^-$ strain YG210 (Δ*aroP* Δ*brnQ* Δ*pheP pheA18*::Tn*10*), whereas the growth of the AroP- and PheP-expressing Phe$^-$ strains was not affected. Likewise, the presence of tryptophan or tyrosine, either of which acts as a competitor for phenylalanine transport in the AroP system, caused significant retardation of the growth of AroP-expressing Phe$^-$ strain YG212 (Δ*brnQ* Δ*livHMGF* Δ*pheP pheA18*::Tn*10*) on the MMF+W or +Y medium. The inhibitory effect was greater for tryptophan than for tyrosine. The same results were obtained for five independently constructed strains. This was surprising, because the expression of the *aroP* gene is known to be strongly repressed by tyrosine but not by tryptophan (9, 22, 36, 46–48, 55) and the AroP system is known to exhibit almost equal affinity for the three aromatic amino acids (5, 36). At present, the reason for this phenomenon is unclear. PheP-expressing Phe$^-$ strain YG211 (Δ*aroP* Δ*brnQ* Δ*livHMGF pheA18*::Tn*10*) grew well under all conditions tested (PheP). Although the PheP and LIV-I systems are

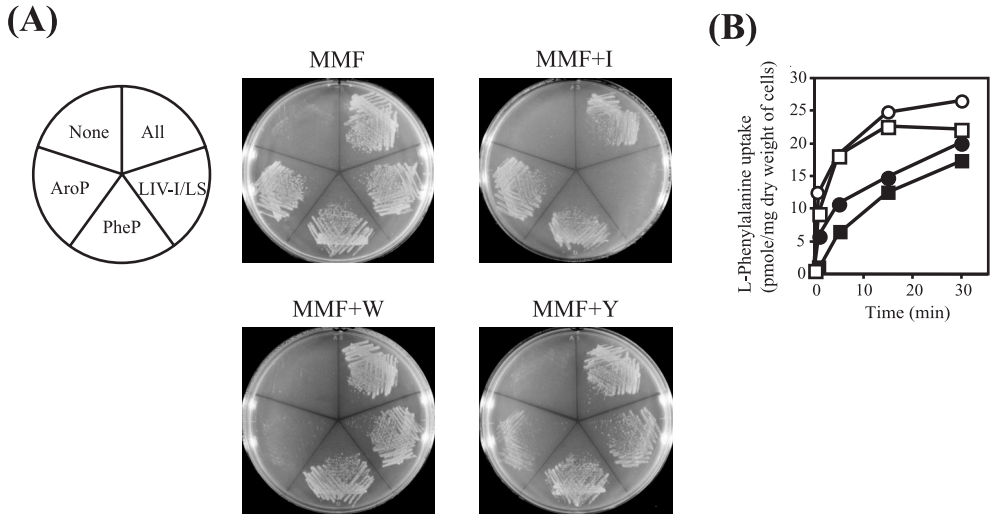

FIG. 4. Physiological role of each phenylalanine transport system in *E. coli* cells grown under various conditions. (A) AroP-, BrnQ-, LIV-I/LS-, and PheP-expressing Phe$^-$ strain YG208 (All), LIV-I/LS-expressing Phe$^-$ strain YG210 (LIV-I/LS), PheP-expressing Phe$^-$ strain YG211 (PheP), AroP-expressing Phe$^-$ strain YG212 (AroP), and Phe$^-$ strain YG213 not carrying any of these transport systems (None) were streaked on M63-glucose minimal medium plates containing 100 μM phenylalanine (MMF) and on MMF supplemented with 1 mM isoleucine (MMF+I), tryptophan (MMF+W), or tyrosine (MMF+Y). (B) Accumulation of phenylalanine in *E. coli* cells with various phenylalanine transport systems in the presence of 1 mM tyrosine. Strains MG1655 (□), YG106 (LIV-I/LS) (○), YG108 (PheP) (●), and YG109 (AroP) (■) were grown in MMF+Y, and after the optical density at 600 nm had reached 0.5, the cells were harvested and suspended in MMF+Y containing 60 μg of chloramphenicol/ml. Cell suspensions were incubated in the presence of 100 μM labeled phenylalanine, and samples were withdrawn at the times indicated. The experiments were repeated three times with essentially the same results; the data for a representative experiment are shown.

capable of transporting tyrosine, no inhibitory effect was observed in the presence of tyrosine (MMF+Y), reflecting the high K_m values for tyrosine compared to those for phenylalanine in these systems.

Transport studies were carried out using these cells grown under the same conditions, and the results were consistent with the growth behavior shown in Fig. 4A. It is notable that when cells expressing the individual phenylalanine transport systems were grown in MMF+Y and then assayed for transport (Fig. 4B), the LIV-I/LS-expressing cells exhibited the highest phenylalanine uptake activity, which was almost the same as that of wild-type strain MG1655. Similar results were obtained when these cells were grown in MMF+W (data not shown). Thus, in the presence of tryptophan or tyrosine, the LIV-I/LS system plays a major role in phenylalanine accumulation in *E. coli* cells.

In conclusion, the LIV-I/LS system was identified as the third phenylalanine transporter in *E. coli*, which plays a significant role in the accumulation of phenylalanine in cells, especially when grown in the presence of tryptophan or tyrosine. The substrate specificities of the LIV-I and LS systems revealed by transport studies contradicted those found previously in binding studies; the reason for this contradiction remains to be elucidated.

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