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Genetic and Biochemical Studies of Transport Systems for Branched-Chain Amino Acids in *Escherichia coli* K-12: Isolation and Properties of Mutants Defective in Leucine-Repressible Transport Activities

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The characteristics of a mutant (*hrbA*) of *Escherichia coli* K-12 that is defective in a leucine-nonrepressible transport system, the LIV-3 system, for branched-chain amino acids were described previously (I. Yamato et al., J. Bacteriol **138**:24-32, 1979). New mutants requiring a high concentration of isoleucine for growth were isolated from strain B763 (*hrbA ileA*) after mutagenesis with ethyl methane sulfonate. These mutants had a defect of the leucine-repressible transport activities for branched-chain amino acids of the parental strain. One of these mutants, strain B7634, had defects of two independent genetic loci (*hrbBC* and *hrbD*). The genes *hrbBC* were mapped at min 76 near *malT*, and the gene *hrbD* mapped at min 77 near *xyl* on the *E. coli* genetic map. The substrate specificity, kinetic properties, and source of coupling energy of the transport system coded for by each of these genes were studied using cytoplasmic membrane vesicles and intact cells. The results identified three transport systems with characteristic features other than the LIV-3 system. The *hrbB* and *hrbC* systems are responsible for the uptake activities of the LIV-2 system, with a high K_m value, and the LIV-1 system, with a low K_m value, respectively. Both activities are repressed by leucine and inhibited by threonine and the *b*(−) isomer of 2-aminobicycloheptyl-2-carboxylic acid. They both utilize adenosine 5′-triphosphate as coupling energy and are not detected in cytoplasmic membrane vesicles. The *hrbD* system is responsible for the LIV-4 system, with a high K_m value. Its activity is repressed by leucine and partially inhibited by threonine. It is detected in cytoplasmic membrane vesicles with a proton motive force as the driving energy.

The molecular organization of the multiple components of the active transport systems for branched-chain amino acids in *Escherichia coli* K-12 has been discussed in detail in terms of the mutual functional correlation between transport carriers in the cytoplasmic membrane and binding proteins in the periplasmic space (6). Although genetic studies on the transport systems, which have been made by several groups of investigators utilizing different phenotypic characters for isolating mutants, showed the existence of many genes that affect the apparent uptake activities in intact cells (3-5, 10, 11, 23), the basic functions of the gene products have not been clarified biochemically, except for those of *hrbA* (23) and *livJ* and *livK* (3, 5, 17, 18).

Since a periplasmic binding protein itself has no ability to translocate a substrate across the cytoplasmic membrane (6, 21), it is of fundamental importance to study the functions of carriers more specifically, using appropriate in vitro assay systems. Previously, we reported (23) the properties of the gene *hrbA*, which determines a transport carrier activity detectable in the cytoplasmic membrane vesicles: the product of the gene $hrbA^+$ is an essential component of the LIV-3 system for branched-chain amino acid transport, and the activity of this system is not repressed by leucine (23). During the course of this work we noticed that a mutant (*hrbA* Ilv^- Hrb^-) grown without added leucine had normal, leucine-repressible transport activity, detectable in cytoplasmic membrane vesicles (23), and that when it was made an auxotroph for isoleucine only, the resulting mutant (*hrbA ileA*), strain B763, could grow well in the presence of a low concentration of isoleucine. On the basis of these distinct phenotypic characteristics, we attempted to isolate new types of mutants that would be suitable for studying the properties of leucine-repressible transport systems for branched-chain amino acids.

This paper reports the isolation from strain B763 of mutants that require a high concentra-

tion of isoleucine for growth. One of these mutants, with simultaneous mutations of two independent genetic loci, had almost completely lost leucine-repressible branched-chain amino acid transport activities. These genes were mapped, and strains with each of these genes were prepared. The substrate specificity and other biochemical properties of the transport systems coded for by each of these genes are described.

MATERIALS AND METHODS

Bacterial strains, growth medium, and genetic studies. The strains used in this study are listed in Table 1. Cells were grown in Davis minimal salts medium (9) supplemented with 0.5% glucose and 500 μg of isoleucine per ml unless otherwise noted. Conjugational and transductional crosses were performed as described previously (16).

Isolation of mutants requiring a high concentration of isoleucine. Mutants showing the phenotype of requirement of a high concentration of isoleucine for growth (Hrb$^-$) were obtained as described previously (16, 23). Parental strain B763 (*hrbA ileA*) has a defect in the leucine-nonrepressible transport system, the LIV-3 system (23). Therefore, it cannot grow with a low concentration of isoleucine (20 μg/ml) in the presence of leucine (90 μg/ml), but grows in the absence of leucine. After mutagenesis of strain B763 with ethyl methane sulfonate (15) and screening with penicillin twice, colonies that grew on a plate supplemented with 500 μg of isoleucine per ml were replica-plated on a plate supplemented with 10 μg of isoleucine per ml. Ten of about 5,000 colonies were unable to grow well on the plate with this low concentration of isoleucine, and one of them was named strain B7634. We also obtained this kind of mutant from other batches of mutagenized cultures. The properties of strain B7634 were examined further genetically and biochemically.

TABLE 1. *E. coli K-12 strains*

Strain	Relevant genotype[a]	Source
B761	*ilv rpsL tsx*	Our collection (23)
B762	*ilv hrbA rpsL tsx*	Our collection (23)
B765	*ileA*[b]	A Val$^+$ Ile$^-$ transductant of strain B761 with P1 *kc* phage grown on strain 113-4
B763	*ileA hrbA*[b]	A Val$^+$ Ile$^-$ transductant of strain B762 with P1 *kc* phage grown on strain 113-4
B7634	*ileA hrbA hrbB hrbC hrbD*	This study
B7634R	*ileA hrbA hrbB hrbD*	This study
B7634M	*ileA hrbA hrbC hrbD*	This study
B3410	*ileA hrbA hrbB hrbC*	This study
B7634L	*ileA hrbB hrbC hrbD*	This study[c]
B7634RU	*hrbA hrbB hrbD uncA*	This study[d]
B7634MU	*hrbA hrbC hrbD uncA*	This study[d]
W3110		Our collection (16)
AB313	Hfr *thi lac leu thr rpsL*	Our collection (16)
AB312	Hfr *thi lac leu thr rpsL*	Our collection (16)
1761/F'$_8$	F'$_8$ *malA1 thi hrbA ilv*	Our collection (23)
176/F'$_{13\text{-}1}$	F'$_{13\text{-}1}$ *leu malA1 thi hrbA ilv*	Our collection (23)
1761/F'$_{128}$	F'$_{128}$ *malA1 thi hrbA ilv*	Our collection (23)
E5014	F'$_{128}$ *mal-24 spc-12 thi-1*	Our collection (23)
113-4	*ileA*	Our collection (23)

[a] *hrb* means that requirement for high concentrations of branched-chain amino acids to feed the existing auxotrophy in the strain's background. The phenotype of the *hrbA* mutant is described in the previous report (23).

[b] Other genotypes of strains B765 and B763 are *ara lac tsx gal mal-1 xyl mtl rpsL*.

[c] A transductant of strain B7634 with P1 *vir* phage grown on strain W3110. The transductant can grow on 10 μg of isoleucine per ml in the presence of 90 μg of leucine per ml. The gene location was ascertained with its linkage with the *tsx* gene.

[d] An Ile$^+$ Unc$^-$ transductant of strain B7634R or B7634M with P1 *vir* phage grown on strain NR-70 (13).

Preparation of cytoplasmic membrane vesicles. Cells were grown in Davis minimal salts medium as mentioned above. Cytoplasmic membrane vesicles were prepared by disrupting spheroplasts in a French press as described elsewhere (22). Protein was determined by the method of Lowry et al. (14), using bovine serum albumin as a standard.

Assay of amino acid uptake by intact cells and cytoplasmic membrane vesicles. Cells were starved by the method of Kobayashi et al. (13). Amino acid uptake activities of intact cells were measured as described elsewhere (13) with 10 mM glucose as an exogenous energy source unless otherwise noted. D-Lactate-driven uptake activity of the cytoplasmic membrane vesicles was assayed as described previously (21). The substrate was added at a concentration of 2 μM.

Osmotic shock and assay of isoleucine-binding activity. Intact cells were subjected to cold osmotic shock (7), and the isoleucine-binding activity of the concentrated shock fluid was assayed as described previously (1).

Reagents. L-[^{14}C]proline (163 mCi/mmol), L-[^{14}C]isoleucine (342 mCi/mmol), and L-[^{14}C]leucine (298 mCi/mmol) were purchased from Daiichi Chemicals, Tokyo. D-Lactate (Li$^+$ salt) was obtained from Calbiochem, Inc. The amino acids used were all in the L-form. The *b*(−) isomer of the norbornane amino acid, 2-aminobicycloheptyl-2-carboxylic acid [*b*(−)-BCH] (20) and DL-trifluoroleucine were kindly supplied by H. N. Christensen, University of Michigan, and T. Hoshino, Mitsubishi Kasei Institute of Life Sciences, respectively. Other reagents used were commercial products of analytical grade.

RESULTS

Properties of a mutant requiring a high concentration of isoleucine for growth and mapping of the mutant loci. Figure 1 shows growth curves of the mutant, strain B7634. The mutant requires 400 μg of isoleucine per ml of medium to grow as well as the wild-type parent, strain B763, which grows well in medium supplemented with only 2 μg of isoleucine per ml.

The mutant loci were mapped by conjugational and transductional crosses. Results of con-

jugational crosses of the mutant with strains AB312, AB313, 176/F'$_{13-1}$, 1761/F'$_8$, 1761/F'$_{128}$, and E5014 showed that the mutation(s) responsible for the phenotype requiring a high concentration of isoleucine for growth were located in the region between *malT* and *xyl*. After uninterrupted conjugations of strain B7634 with Hfr strains AB312 and AB313 and selection of Mal$^+$, Xyl$^+$, Gal$^+$, Ara$^+$, or Lac$^+$ recombinants, the phenotype of Hrb was examined. The results suggested that the mutations except *hrbA* gene localized only near *malT*. F'-mediated conjugations with 1761/F'$_8$, 176/F'$_{13-1}$, 1761/F'$_{128}$, or E5014 also suggested the linkage of mutations other than *hrbA* with *malT* (data not shown).

Some of the results of the conjugation with AB312 or with AB313 are shown in Table 2. After conjugation with AB312, the Mal$^+$ exconjugants were selected. We found that 88 and 275 of 353 Mal$^+$ recombinants were Xyl$^+$ and Hrb$^+$, respectively, and 92% of the Xyl$^+$ Mal$^+$ recombinants were Hrb$^+$. Of the 275 Xyl$^+$ exconjugants with AB313, 58 and 118 were Mal$^+$ and Hrb$^+$, respectively, and 95% of the Mal$^+$ Xyl$^+$ recombinants were Hrb$^+$ (Table 2). These results suggested that the mutation(s) are located between *malT* and *xyl*. Uptake activities of isoleucine in intact cells of the Xyl$^+$ Mal$^-$ Hrb$^+$ exconjugants with AB313 were examined. The kinetic properties of the activities of the seven recombinants examined were the same as those of the parental strain B763, which has threonine-sensitive transport activities with low and high affinities (see Table 3).

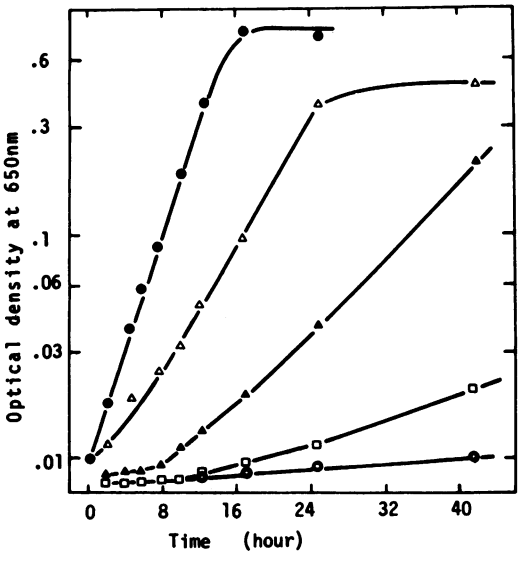

FIG. 1. *Growth curves of strain B7634. Cells were grown at 37°C in the medium described in the test, supplemented with 400 (●), 100 (△), 40 (▲), 10 (□), or 2 (○) μg of isoleucine per ml.*

TABLE 2. *Mapping of the hrb genes by uninterrupted conjugations*

Selected marker	Counterselected marker	Phenotype tested	Frequency (%)
Mal$^+$ [a]	Leu$^+$, Thr$^+$	Xyl$^+$ Hrb$^+$	23
		Xyl$^+$ Hrb$^-$	2
		Xyl$^-$ Hrb$^+$	55
		Xyl$^-$ Hrb$^-$	20
Xyl$^+$ [b]	Leu$^+$, Thr$^+$	Mal$^+$ Hrb$^+$	20
		Mal$^+$ Hrb$^-$	1
		Mal$^-$ Hrb$^+$	23
		Mal$^-$ Hrb$^-$	56

[a] The recipient was strain B7634, and the donor was strain AB312.

[b] The recipient was strain B7634, and the donor was strain AB313.

Results of transductional crosses are summarized in Fig. 2. Strain B7634 was transduced with the P1 *vir* phage grown on strain W3110, and Xyl$^+$ or Mal$^+$ transductants were selected. We found that 8 and 24 of 181 Xyl$^+$ transductants were Hrb$^+$ and Mtl$^+$, respectively. None of the Hrb$^+$ transductants was Mtl$^+$. Therefore, the *hrbD* gene was located at min 77 on the genetic map of *E. coli* (8). We named one of the Xyl$^+$ Hrb$^+$ recombinant strains strain B3410. Although the conjugational crosses (Table 2) suggested that the genes responsible for the Hrb$^+$ phenotype should localize very close to *malT*, the map position of the gene *hrbD* was not so close to it. This unexpected result will be discussed later.

We also found that 12 of 96 Mal$^+$ transductants were Hrb$^+$; one of these was named strain B7634M. Strain B7634 was transduced with P1 *vir* phage grown on a Xyl$^+$ revertant of strain B7634M, and Xyl$^+$ transductants were selected. None of 177 transductants was Hrb$^+$, suggesting that the *hrbB* gene is different from the *hrbD* gene (Fig. 2). In addition, strain B763 was transduced with the P1 *vir* phage grown on an Xyl$^+$ or Mal$^+$ revertant of strain B7634, and Xyl$^+$ or Mal$^+$ transductants were selected. None of the transductants was Hrb$^-$, and this was consistent with the observation that either *hrbB*$^+$ or *hrbD*$^+$ alone allows growth on low concentration of isoleucine. Combining these results with those of the uptake studies of exconjugants and transductants (see Table 3) and the results of Anderson and Oxender (3, 4), it appears that the *hrbB* gene maps at min 76 (Fig. 2).

Hrb$^+$ Ile$^-$ revertants of strain B7634 were isolated at a frequency of about 10^{-7}. One of the revertants, strain B7634R, was transduced with the P1 *vir* phage grown on a Mal$^+$ or Xyl$^+$

TABLE 3. *Kinetic parameters of isoleucine uptake of various mutants*[a]

Strain	Genotype	K_m (μM)	V_{max} (nmol/min per mg of protein)
B765	Wild type	{0.3 4	1.6 4
B763	*hrbA hrbB*⁺ *hrbC*⁺ *hrbD*⁺	{0.2 3	1.2 4
B7634	*hrbA hrbB hrbC hrbD*	—[b]	<0.1
B7634R	*hrbA hrbB hrbC*⁺ *hrbD*	0.3	0.35
B7634M	*hrbA hrbB*⁺ *hrbC hrbD*	2.5	3.1
B7634L	*hrbA*⁺ *hrbB hrbC hrbD*	6.5	4.2
B3410	*hrbA hrbB hrbC hrbD*⁺	1.3	0.50

[a] For assay of uptake activities, concentrations of 0.1 to 10 μM isoleucine were used. Cells were grown in minimal medium supplemented with 0.5% glucose and 400 μg of isoleucine per ml.

[b] Could not be determined.

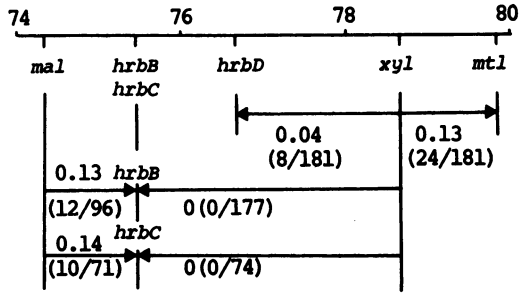

FIG. 2. *Mapping of the genes hrbB, hrbC, and hrbD. Cotransductional frequencies of hrbB, hrbC, and hrbD with malT and xyl are shown. Arrows indicate unselected markers. Distances are not drawn to scale. For details of transductional properties, see the text.*

revertant of strain B7634, and Mal⁺ or Xyl⁺ transductants were isolated. We found that 10 of 71 Mal⁺ transductants of strain B7634R were Hrb⁻, and that none of 74 Xyl⁺ transductants was Hrb⁻ (Fig. 2). We could obtain the Hrb⁻ Mal⁺ transductants with a similar frequency when P1 *vir* phage grown on an Mal⁺ revertant of strain B3410 was used. Thus, this mutation of the locus which we call *hrbC* also appears to the map at min 76. These results suggest that the *hrbC* locus is very close to or at the same locus as *hrbB*, but that it is different from the *hrbD* locus.

The uptake studies of Hrb⁺ exconjugants suggested that genes responsible for the high- and low-affinity transport systems should be located between *malT* and *xyl*. However, all of 10 Mal⁺ Hrb⁺ transductants (*hrbB*⁺) of strain B7634 examined had only the low-affinity transport activity, as has strain B7634M (Table 3). All of the five Hrb⁺ revertants (*hrbC*⁺) of strain B7634 examined had only the high-affinity transport activity, as has strain B7634R (Table 3). Thus, although it is probable that the *hrbC*⁺ is a reversion of the *hrbB* gene with a different affinity in its transport system, the results of uptake studies suggest that the *hrbC* gene is another gene responsible for the high-affinity transport system. No further genetic studies were made on these genes.

We prepared concentrated shock fluid from strain B7634 and measured isoleucine-binding activity. We did not detect a significant difference from the activity of the wild-type parent. This suggests that the mutant (*hrbA hrbB hrbC hrbD*) has normal activity of the leucine-isoleucine-valine-threonine (LIVT)-binding protein (1).

From these results we conclude (i) that at least three independent genetic loci for branched-chain amino acid transport are present in *E. coli* K-12, (ii) that these genes do not affect the activity of the LIVT-binding protein, and (iii) that the gene *hrbA* is nonrepressible by leucine, whereas the genes *hrbBC* and *hrbD* are repressible by leucine. The gene *hrbA* determines the LIV-3 system as reported previously (23).

Isoleucine uptake activities by cytoplasmic membrane vesicles. As we reported previously (23), the *hrbA* mutant has activity for uptake of isoleucine that is detectable in cytoplasmic membrane vesicles of cells grown in the absence of leucine. To decide which gene is responsible for this uptake activity, we measured the activities for isoleucine uptake by cytoplasmic membrane vesicles from strains B765 (wild type), B763 (*hrbA*), B7634 (*hrbA hrbB hrbC hrbD*), B7634R (*hrbA hrbB hrbC*⁺ *hrbD*), B7634M (*hrbA hrbB*⁺ *hrbC hrbD*), and B3410 (*hrbA hrbB hrbC hrbD*⁺) (Table 4). We also examined the uptake activity of strain B7634L, an *hrbA*⁺ transductant of strain B7634.

Cytoplasmic membrane vesicles from all the strains had normal activities for uptake of proline. The vesicles from strain B3410 showed activity for uptake of isoleucine, whereas the vesicles from strains B7634, B7634R, and B7634M showed no isoleucine uptake activity. These results clearly indicate that the gene *hrbD* is responsible for the uptake activity detectable in the cytoplasmic membrane vesicles. Thus, we named this new transport system the LIV-4 system.

The cytoplasmic membrane vesicles from strain B7634L (*hrbA*⁺), which has the LIV-3 system, showed activity for uptake of 0.087 nmol of isoleucine per min per mg of protein, which is four times higher than the activity of the wild-type strain B765 (Table 4). We cannot explain this observation at present.

Assignment of *hrb* genes to transport systems. Previously, we described the properties of three independent transport systems for branched-chain amino acids (23). Considering the transport properties detected in newly isolated mutants in which the uptake activities are coded for by each of *hrb* genes (Table 3), we further attempted to assign the genes *hrbB*, *hrbC*, and *hrbD* to transport systems. The Lineweaver-Burk plot of the activity of strain B763 showed a biphasic curve, and two apparent K_m values were obtained as described previously (23). The mutant B7634 had a very low activity for uptake of isoleucine. Strain B7634R (*hrbC*$^+$) showed a K_m value of 0.3 μM, which was as low as the K_m value of the high-affinity transport component, the LIV-1 system (23). However, unlike previous biochemical studies (23), the repressibility of the activity of a high-affinity transport system, such as that in strain B7634R, was established genetically. Therefore, we redefine the LIV-1 system as a system with a high affinity for substrate, whose activity is repressible by leucine.

Strain B7634M (*hrbB*$^+$) showed a K_m value of 2.5 μM, which is about 10 times that of the LIV-1 system. We named this transport system the LIV-2 system, since its biochemical properties are essentially the same as those of the LIV-1 system, except for its affinity for substrate (see below). These results indicated that the functions of the gene products of *hrbC*$^+$ and *hrbB*$^+$ are not identical kinetically.

Strain B3410 (*hrbD*$^+$) showed a K_m value of 1.3 μM for isoleucine, and the V_{max} value of the system, the LIV-4 system, was found to be smaller than that of the LIV-2 system. We confirmed kinetically that all the uptake activities of isoleucine of strains B7634R, B7634M, and B3410 were completely repressed when the cells were grown in the presence of 90 μg of leucine per ml of medium (data not shown). Strain B7634L (*hrbA*$^+$) had a K_m value of 6.5 μM, and its activity was not repressed by leucine as reported previously (23).

Substrate specificities and sources of coupling energy. The substrate specificities of the four transport systems were examined, using intact cells (Table 5). *b*(−)-BCH (20) strongly inhibited all four transport systems. DL-Trifluoroleucine and norleucine also inhibited the activities. Essentially the same inhibitory effects of *b*(−)-BCH and DL-trifluoroleucine were observed when the uptake activities were determined with [^{14}C]leucine as a substrate. Thus, judging from the extents of inhibition by these inhibitors, we concluded that the substrate specificities of the four transport carriers in the cytoplasmic membrane are essentially the same.

TABLE 4. *Isoleucine and proline uptake activities of cytoplasmic membrane vesicles from various mutants*[a]

Strain	Genotype	Uptake activities (nmol/min per mg of protein) of:	
		Proline	Isoleucine
B765	Wild type	0.41	0.023
B763	*hrbA hrbB*$^+$ *hrbC*$^+$ *hrbD*$^+$	0.43	0.018
B7634	*hrbA hrbB hrbC hrbD*	0.38	<0.002
B7634R	*hrbA hrbB hrbC*$^+$ *hrbD*	0.42	<0.002
B7634M	*hrbA hrbB*$^+$ *hrbC hrbD*	0.37	<0.002
B7634L	*hrbA*$^+$ *hrbB hrbC hrbD*	0.35	0.087
B3410	*hrbA hrbB hrbC hrbD*$^+$	0.47	0.006

[a] D-Lactate-dependent uptake was measured as described in the text. Cells were grown in minimal medium supplemented with 0.5% glucose and 400 μg of isoleucine per ml.

TABLE 5. *Inhibition of isoleucine uptake by various compounds*[a]

Strain	Inhibition (%)			
	b(−)-BCH	DL-Trifluoroleucine	Threonine	Norleucine
B7634R	92	64	95	50
B7634M	96	50	92	45
B7634L	96	50	5	68
B3410	88	40	70	43

[a] Percent inhibition of control activity with 1 μM of [^{14}C]isoleucine as substrate was measured in the presence of each inhibitor at 200 μM.

The inhibitory effect of threonine on the transport systems is important, although it is rather complicated. Threonine inhibits the activities of strains B7634R and B7634M almost completely (Table 5). Its inhibitory effect is competitive, and we determined the K_i values of threonine for isoleucine uptake to be about 30 μM. These K_i values are about the same as the K_d value of the LIVT-binding protein for threonine (1). Furthermore, practically no isoleucine uptake was observed when intact cells of strains B7634R and B7634M were subjected to osmotic shock, indicating that the activities of the LIV-1 and LIV-2 systems are obligatorily coupled with a function of the periplasmic LIVT-binding protein (6).

On the other hand, threonine does not inhibit the activity of strain B7634L even in the presence of the LIVT-binding protein, and partially inhibits the activity of strain B3410 (Table 5). These results are consistent with the previous observation (21) that the isoleucine uptake activity detectable in cytoplasmic membrane vesicles from an *hrbA* mutant is not inhibited by threonine, but is stimulated by addition of the

LIVT-binding protein. Thus, we conclude that the activity of the LIV-3 system is not affected by the LIVT-binding protein, whereas that of the LIV-4 system may be stimulated by the LIVT-binding protein.

As mentioned above, the coupling energy driving the LIV-3 and LIV-4 systems is proton motive force, as evidenced by the fact that these activities are measurable in cytoplasmic membrane vesicles with D-lactate as a respiratory substrate. The sources of energy driving the LIV-1 and LIV-2 systems were examined using strains B7634RU and B7634MU, the *uncA* derivatives of strains B7634R and B7634M, respectively. Figure 3 shows that the isoleucine uptake activities were both stimulated by glucose even in the presence of 10 mM KCN and were inhibited almost completely by 10 mM arsenate. These results indicate that the source of energy that drives the LIV-1 and LIV-2 systems is ATP, not a proton motive force, as discussed previously (13).

DISCUSSION

Although the multiplicity of the active transport systems for branched-chain amino acids of *E. coli* K-12 has been studied genetically and biochemically, it is still difficult to explain some of the results obtained by different groups of investigators (6). Aiming to find an adequate explanation, we isolated a novel class of mutants with transport activities that is repressible by leucine in the medium. We obtained strain B7634 as a mutant with the new phenotype of requiring a high concentration of isoleucine for growth and studied its genetic and biochemical properties, together with those of various transductants derived from it. Fortunately, this mutant was found to have two independent mutation loci on the chromosome, *hrbBC* and *hrbD*, which make the phenotype very advantageous for use in obtaining the leucine-repressible branched-chain amino acid transport mutants selectively.

On the basis of our present results, we now classified the active transport systems for branched-chain amino acids into four subgroups (Table 6), with a slight revision of our previous conclusion (23). We named the leucine-repressible transport system coded for by the new gene *hrbD* the LIV-4 system instead of LIV-2 (23). This activity is resistant to osmotic shock and is detectable in cytoplasmic membrane vesicles. Threonine inhibits the activity partially, coinciding with the previous finding that the LIVT-binding protein stimulates the transport activity detectable in the cytoplasmic membrane vesicles (21).

We confirmed the characteristics of the LIV-3 system described previously (23). Very high uptake activity of isoleucine was observed in the cytoplasmic membrane vesicles from strain B7634L, as compared to the parental strain B763 (Table 4). This may be due to the LIV-3 system functioning mainly in the process of entry into intact cells, or to the membrane vesicles being tight. In contrast, the parental LIV-4 system would function in the process of exit from intact cells or would be leaky in membrane vesicles. It

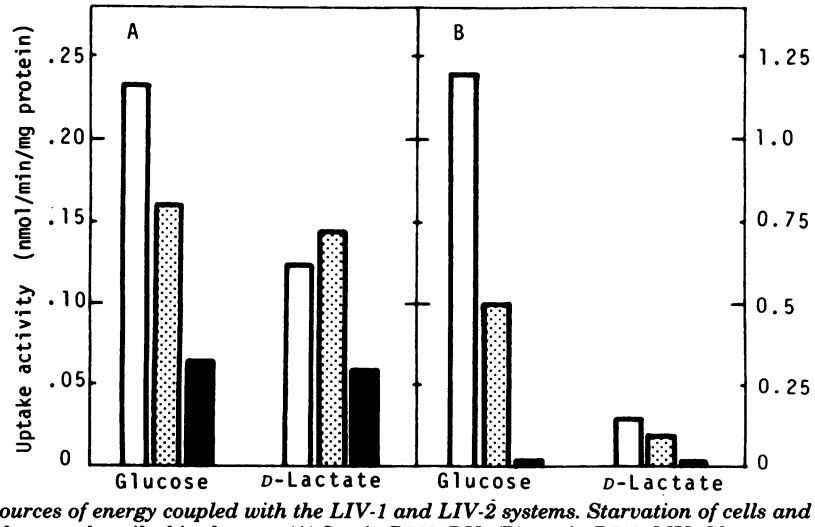

FIG. 3. *Sources of energy coupled with the LIV-1 and LIV-2 systems. Starvation of cells and uptake assay were carried out as described in the text. (A) Strain B7634RU; (B) strain B7634MU. Glucose or* D*-lactate was added at a concentration of 10 mM. Open bars indicate results with no inhibitor; dotted bars, 10 mM KCN; solid bars, 10 mM arsenate.*

TABLE 6. *Summary of active transport systems for branched-chain amino acids of E. coli K-12*

Transport system	K_m[a] (μM)	Energy	Osmotic shock	Inhibition by threonine	Repression by leucine	Gene
LIV-1	0.3	ATP	Sensitive	Inhibited	Repressed	*hrbC*
LIV-2	2.5	ATP	Sensitive	Inhibited	Repressed	*hrbB*
LIV-3	6.5	Proton motive force	Resistant	Not inhibited	Not repressed	*hrbA*
LIV-4	1.3	Proton motive force	Resistant	Partially inhibited	Repressed	*hrbD*

[a] The values cited are from Table 3.

should be noted that the activity of the LIV-3 system is not inhibited at all by threonine (Table 5). These two transport systems utilize a proton motive force as energy (Table 6).

Anderson and Oxender (4) have shown that another gene(s) (*livP*, *liv-12*, or both) may be related to the activity of the leucine-nonrepressible, osmotic shock-resistant, low-affinity LIV-II system in their classification, but the gene loci seem to be different from the *hrbD* or *hrbA* gene, though the transport properties show some similarities to the LIV-3 system.

As we mentioned in Results, the map position of the *hrbD* gene seems to be inconsistent with the results of conjugational crosses. All of the Hrb^+ exconjugants of strain B7634 with AB313, whose uptake activities for isoleucine we measured (see Table 3), seemed to have reflected the transport systems determined by the $hrbB^+C^+$ genes. Also we could not detect Hrb^+ exconjugants that were composed of the $hrbD^+$ gene only, although we expected to obtain such ones, judging from the location of the *hrbD* gene. This can be explained by poor growth of the $hrbD^+$ *hrbBC* strains on the selection plates in contrast to the growth of the $hrbB^+C^+$ strains, which caused miscounting of some of the $hrbD^+$ *hrbBC* strains as the Hrb^- phenotype. The $hrbD^+$ gene alone can support the growth of cells on a low concentration of isoleucine, but the growth rate might be slow in concentrations of isoleucine much lower than 2 μg/ml, which would occur in the growth on plates. This poor growth is conceivable, considering the transport properties of the LIV-4 system with the low affinity and velocity. We at present do not know any specific phenotype of the $hrbD^+$ gene to distinguish it from other hrb^+ genes on plates.

Both the LIV-1 and LIV-2 systems are defined as systems whose activities are sensitive to osmotic shock, utilizing ATP as energy, and being completely inhibited by threonine (Table 6). The competitive inhibition of these activities by threonine strongly suggests that the LIVT-binding protein is an obligatory component of the transport carrier complexes of these systems, as discussed previously (6). We think that the LIV-1 system is the same as the LIV-I system reported by Rahmanian et al. (19), although they stated that it shares both threonine and alanine as substrates.

As we described in Results, the *hrbC* gene is very close to or the same as the *hrbB* gene. We did not examine the genes further by genetic means, but the uptake activity of Mal^- Xyl^+ Hrb^+ exconjugants of strain B7634 with AB313 had the same properties as the parental strain, showing the biphasic curve of the Lineweaver-Burk plot, and the $hrbB^+$ transductants had only an uptake activity, corresponding to the low-affinity transport system (see Table 3). Considering these observations, any one of the following three explanations is conceivable.

The first explanation would invoke identity of the $hrbB^+$ gene with the $hrbC^+$ gene which, in turn, would be a reversion from the *hrbB* gene with a different affinity for isoleucine. In this case, there should exist a gene(s) responsible for the high-affinity transport system (LIV-1 system) in the *malT-xyl* region. This gene(s) should be different from the *livJKHG* gene cluster of Anderson and Oxender (4). The complexities invoked render this possibility unlikely. The second explanation is that the *hrbC* gene is identical with the *hrbB* gene and that this gene is responsible for both the LIV-1 and LIV-2 systems. If this were the case, the $hrbC^+$ revertant could be explained as a pseudorevertant of the *hrbB* gene which complements the other mutation(s) in the *malT-xyl* region, responsible for the LIV-1 transport system. Considering that this additional mutation was necessary to obtain a strain B7634 that is completely defective in transport activity, this possibility also seems improbable. The third explanation would invoke distinct genes for *hrbC* and *hrbB*, encoding for the components of the LIV-1 and LIV-2 systems, respectively. Even if this were the case, the $hrbC^+$ revertant would be a pseudorevertant (see above). At present, we think this possibility is the most likely one.

On the basis of the results of Anderson and Oxender (4), we assumed that the *hrbC* gene is one of the genes of *livH* and *livG*, and that the *hrbB* gene is another gene responsible for the low-affinity transport system, not previously reported by these authors (3, 4). To analyze all

these possibilities is worth doing for further interpretation. Notable differences in transport of threonine and its inhibition by serine in strains B7634R and B7634M will be described elsewhere (T. Fujimura, I. Yamato, and Y. Anraku, manuscript in preparation). Consequently, we think that these four mutants are good starting strains to investigate the genes responsible for each activity as well as mutual interactions of the gene products.

We will not discuss the uptake activity of the leucine-specific transport system (19) here, because all the mutants studied in this work lack this system, judging from the complete inhibition of their activities by *b*(−)-BCH (Table 5 and reference 20). Our interpretation of the apparent substrate specificity of this system has already been reported (6). Tager and Christensen (20) demonstrated that the four isomers of BCH are useful probes of the stereochemical specificities of the transport systems for branched-chain amino acids in Ehrlich cells and in *E. coli* cells, and that *b*(−)-BCH, the levorotatory isomer of the compound, is the most potent inhibitor for these transport activities. Here we should point out that *b*(−)-BCH is a strong inhibitor that is useful in determining the substrate specificity of transport carriers for branched-chain amino acids in the cytoplasmic membrane.

Guardiola et al. (10, 11) isolated the *brnQ*, *brnR*, and *brnS* transport mutants for branched-chain amino acids, having the phenotype of valine resistance. With our screening procedures for isolating transport mutants, no mutant with genotypes such as *brnS* and *brnR8'* (10, 11) was obtained. It remains to be determined what products are coded for by these genes. We also could not obtain mutants defective in activity of the LIVT-binding protein by our screening methods. This indicates that the phenotypes used in this work and the others (16, 23) are useful in obtaining the mutants that are defective in the activities of transport carriers in the cytoplasmic membrane. Two genes coding for binding proteins for branched-chain amino acids are known in *E. coli* K-12 (5), and their functions in transport and in intracellular homeostasis of the pool amino acids have been discussed in detail (2, 6).

The activities of the LIV-1 and LIV-2 systems depend on the intracellular concentration of ATP (Fig. 3), although the mechanism of energy coupling is still unknown. Strains B7634R and B7634M and their *uncA* derivatives should be suitable sources of genetic material for investigating the mechanisms of ATP-driven active transport systems composed of multiple components including binding protein in studies such as those of Hofnung et al. (12), who established a genetic collection of mutants for the maltose transport system of *E. coli* K-12.

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