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Mutations Affecting the Different Transport Systems for Isoleucine, Leucine, and Valine in *Escherichia coli* K-12

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Uptake of isoleucine, leucine, and valine in *Escherichia coli* K-12 is due to several transport processes for which kinetic evidence has been reported elsewhere. A very-high-affinity transport process, a high-affinity transport process, and three different low-affinity transport processes were described. In this paper the existence of these transport processes is confirmed by the isolation and preliminary characterization of mutants altered in one or more of them. The very-high-affinity transport process is missing either in strains carrying the *brnR6*^{am} mutation or in strains carrying the *brn-8* mutation. This appears to be a pleiotropic effect since other transport systems are also missing. Mutant analysis shows that more than one transport system with high affinity is present. One of them, high-affinity 1, which needs the activity of a protein produced by the *brnQ* gene, transports isoleucine, leucine, and valine and is unaffected by threonine. The other, high-affinity 2, which needs the activity of a protein produced by the *brnS* gene, transports isoleucine, leucine, and valine; this uptake is inhibited by threonine which probably is a substrate. Another protein, produced by the *brnR* gene, is required for uptake through both high-affinity 1 and high-affinity 2 transport systems. The two systems therefore appear to work in parallel, *brnR* being a branching point. The *brnQ* gene is located close to *phoA* at 9.5 min on the chromosome of *E. coli*, the *brnR* gene is located close to *lac* at 9.0 min, and the *brnS* gene is close to *pdxA* at 1 min. A mutant lacking the low-affinity transport system for isoleucine was isolated from a strain in which the high-affinity system was missing because of a *brnR* mutation. This strain also required isoleucine for growth because of an *ilvA* mutation. The mutant lacking the low-affinity transport system was unable to grow on isoleucine but could grow on glycylisoleucine. This mutant had lost the low-affinity transport for isoleucine, whereas those for leucine and valine were unaffected. A pleiotropic consequence of this mutation (*brn-8*) was a complete absence of the very-high-affinity transport system due either to the alteration of a common gene product or to any kind of secondary interference which inhibits it. Mutants altered in isoleucine-leucine-valine transport were isolated by taking advantage of the inhibition that valine exerts on the K-12 strain of *E. coli*. Mutants resistant both to valine inhibition (Val^r) and to glycylvaline inhibition are regulatory mutants. Val^r mutants that are sensitive to glycylvaline inhibition are transport mutants. When the very-high-affinity transport process is repressed (for example by methionine) the frequency of transport mutants among Val^r mutants is higher, and it is even higher if the high-affinity transport process is partially inhibited by leucine.

Kinetic evidence for the presence in *Escherichia coli* K-12 of different transport systems for isoleucine, leucine, and valine has been reported (7). These findings make it easier to devise selection procedures for isolation of transport mutants which are necessary to show that the kinetic description is not an artifact.

Mutants in the isoleucine-leucine-valine transport systems were isolated (8) by taking advantage of the growth inhibition that valine exerts on the K-12 strain of *E. coli* (11). When valine reaches a critical intracellular concentration, it inhibits isoleucine biosynthesis and, therefore, stops the growth in minimal medium. Mutants resistant to valine inhibition (Val^r) are either mutants which are altered in the target of

valine action, or are transport mutants which cannot concentrate external valine because of a transport defect. Since *E. coli* K-12 has a transport system for dipeptides (3), which are concentrated and then cleaved inside the cell into the constituent amino acids (13), a mutant altered in valine transport should be resistant to valine but sensitive to valine-containing dipeptides. We have already used (3, 8) this criterion to define a transport mutant.

In this paper we describe the isolation and preliminary characterization of mutants that are altered either in the very-high-affinity transport system for isoleucine, leucine, and valine (7), in the high-affinity transport system for isoleucine, leucine, and valine, or in the low-affinity transport system for isoleucine. We did not try to isolate mutants in which the other two low-affinity transport systems (for leucine and valine, respectively) was altered, but a possible selection procedure is indicated. The results obtained make this system an attractive one for a study on structure and function of specific membrane proteins.

A preliminary account of this work has been published (8) and reported (J. Guardiola, M. De Felice, and M. Iaccarino, Lunteren Lectures on Molecular Genetics, Lunteren, The Netherlands, 1971).

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the strains used, and Fig. 1 indicates the map order of the relevant markers according to Taylor and Trotter (18).

Reagents. Reagents whose source is not mentioned here were of the highest purity commercially available. Amino acids used were all L-form, unless otherwise mentioned. Glycylvaline was obtained from Sigma Chemical Co., St. Louis, Mo. Uniformly ^{14}C-labeled L-amino acids were obtained from New England Nuclear Corp., Boston, Mass. *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (nitrosoguanidine) was purchased from K and K Laboratories, Inc., Plainview, N.Y.

Media. The minimal medium used was minimal citrate (20). Usual supplements, when required, were 0.4% glucose, 25 μg of tryptophan per ml, 100 μg of arginine per ml, 50 μg of other amino acids per ml, 50 μg of nucleosides per ml, and 10 μg thiamine per ml. Rich media were L-broth (12) and nutrient broth (5 g of NaCl and 8 g of nutrient broth [Difco] in 1 liter of distilled water).

Transductions. Transductions were either performed with P1kc obtained from C. Yanofsky, prepared by the confluent lysis technique as described by Hill et al. (9), or with a P1 variant, P1CM, obtained from L. Rosner. The latter, P1CM*clr*100, is a P1 derivative carrying a temperature-sensitive immunity and the genes for resistance to chloramphenicol (15). With this phage, chloramphenicol-resistant, temperature-sensitive lysogens were isolated; clones were grown in rich liquid medium and then induced for 10 min at 42 C, to produce high titers of P1. Transductions were performed on minimal citrate plates as described by Hill et al. (9).

Mutagenesis. For nitrosoguanidine mutagenesis, a culture was grown overnight in minimal medium, centrifuged, washed in unsupplemented minimal medium, resuspended in the same medium at the original concentration, and shaken for 30 min at 37 C in the presence of 30 μg of the mutagen per ml. A 10-fold dilution of this suspension was grown overnight in appropriately supplemented medium.

Ultraviolet mutagenesis was performed by irradiating a bacterial suspension at a dose that kills about 95% of the bacteria.

Isolation of independent Val^r mutants. A suspension of bacteria was diluted to a concentration of about 100 cells per ml in a minimal medium containing all the supplements needed by that strain for growth. The suspension was divided into 1-ml portions which were grown overnight. From each tube, 0.1 ml was spread on plates supplemented with valine and other supplements, as described in the text. From each plate only one colony was purified and retained for further characterization. Isolation procedures different from this one are detailed in the text.

Test for amber mutations. To check whether the phenotype of a mutant was due to an amber mutation, a given strain was lysogenized either with ϕ80psu3 (prepared from strain 594) or with ϕ80, as a control. The lysogens were immune to both ϕ80s but sensitive to ϕ80vir; the ϕ80 lysogens supported the growth of phage T4 but not of phage T4 containing the mutation *amB17* in gene *23* (obtained from A. Coppo and J. F. Pulitzer). The ϕ80psu3 lysogens supported the growth of both phages. The relevant phenotype was checked on the strain to be tested and the corresponding ϕ80psu3 lysogen.

Transport assays. Transport was measured as described previously (7).

RESULTS

Selection of mutants. In the preceding paper (7), we showed that multiple transport processes for branched-chain amino acids are present in *E. coli* K-12, and therefore it should be difficult to isolate transport mutants. On the other hand, mutants which appear to have an altered uptake of the branched-chain amino acids have been described (8). Therefore, we have tried to rationalize the selection of mutants on the basis of the kinetic characterization of the different transport processes for branched-chain amino acids (7) and of the characteristics of the mutants isolated previously (8).

As shown previously (7), a very-high-affinity transport process with a K_m value in the 10^{-8} M range exists in *E. coli* K-12; this transport process takes up isoleucine, leucine, and valine

TABLE 1. *Bacterial strains*[a]

Strains	*brn*	Phenotype on valine[b]	Genotype	Origin
Ca85	+	Val^s	*thi, his, lac^{am}*, HfrH	From J. Beckwith
X478	+	Val^s	*thi, leuB, purE, proC, lysA, trp, metE, ara, lacZ, xyl, azi, str, tonA, tsx*, F	From P. Berg
AT739	+	Val^s	*thr-10, pyrA53, thi-1, HfrH*	From A. L. Taylor
AT2365	+	Val^s	*thr-4, ara-14, pdxA1Mu$^+$, leu-8, proA2, lacY1, galK2*, (λ$^+$), *strA20, xyl-5, mtl-1, thi-1*, F	From A. L. Taylor
594	+		*phoA, trp*, (φ80psu3)	From E. Gallucci (2)
AT2477	+	Val^s	*metB1, rel-1, proB28*, λ$^-$, Hfr	From the Coli Genetic Stock Center
MI2	+		*argH, trpA36, ilvA601*, F	Reference 10
MI148a	+	Val^s	*thi, purE, proC, lysA, trp, metE, ara, lacZ, xyl, str, tonA, tsx*, F$^-$	Leu$^+$, Azi^s, Ara transductant of X478 with a P1 grown on a wild-type strain
MI148d	+	Val^s	*thi, purE, proC, lysA, trp, ara, lacZ, xyl, str, tonA, tsx*, F	Met$^+$ transductant of MI148a with a P1 grown on a wild-type strain
MI174b	*brnQ2*	Val^r (Leu or Thr)	*thi, metE, purE, trp, lysA, ara, xyl, str, tonA, tsx*, F	Reference 8
MI237	*brnQ2,brnR3*	Val^r	*thi, purE, trp, lysA, metE, ara, xyl, str, tonA, tsx*, F	Spontaneous from MI174b
MI237b	*brnQ2,brnR3*		*ilvA601, thi, purE, trp, lysA, ara, xyl, str, tonA, tsx*, F	Met$^+$, Ile$^+$ transductant of MI237 with P1 grown on MI2
MI238	*brnQ4^{am}*	Val^r (Leu or Thr)	*thi, his, lac^{am}*, HfrH	Spontaneous Val^r from Ca85
MI247	*brnR6^{am}*	Val^r	*thi, his, lac^{am}*, HfrH	Spontaneous Val^r from Ca85
MI248	*brnS7^{am}*	Val^r (Leu)	*thi, his, lac^{am}*, HfrH	Spontaneous Val^r from Ca85
MI249	*brnS7^{am},brnQ2*	Val^r	*thi, his*, HfrH	Lac$^+$, Val^r transductant of MI248 with P1 grown on MI174b
MI250	*brnQ2,brnR3,brn-8*		*ilvA601, thi, purE, trp, lysA, ara, xyl, str, tonA, tsx*, F	By nitrosoguanidine mutagenesis from MI237b

[a] Symbols for genetic markers are those used by Taylor and Trotter (18); the *brnQ* symbol has been described in Guardiola and Iaccarino (8); *brnR*, *brnS* symbols are the loci defining genes for the transport of branched-chain amino acids described in this report. The superscript *am* means amber mutation.

[b] Val^r (Leu) or Val^r (Thr) means that the resistance to valine is only expressed if also leucine or threonine are present.

and is both inhibited and repressed by methionine. When the very-high-affinity transport process is present it is more difficult to find transport mutants. This is due to the fact that a single mutation would probably abolish only one transport system and, therefore, the resulting strain would still be sensitive to valine inhibition because of the presence of other transport systems. This conclusion is based on the experiment reported in Table 2, where the phenotype of independently isolated mutants is described. The data show that: (i) when methionine is present before and during selection of Val^r mutants, the frequency of non-transport (i.e., glycylvaline resistant; see De Felice et al. [3]) mutants decreases from 32% to undetectable (<2%) in one case and from 100 to 25% in the other case; (ii) when leucine is present, a lower frequency of non-transport mutants is observed. Most of them require leucine to express the resistance to valine, and most of these Val^r-leucine dependent mutants are still Val^r if leucine is substituted with threonine and, therefore, appear to be mutants altered at the *brnQ* locus (see below).

In conclusion, transport mutants are more

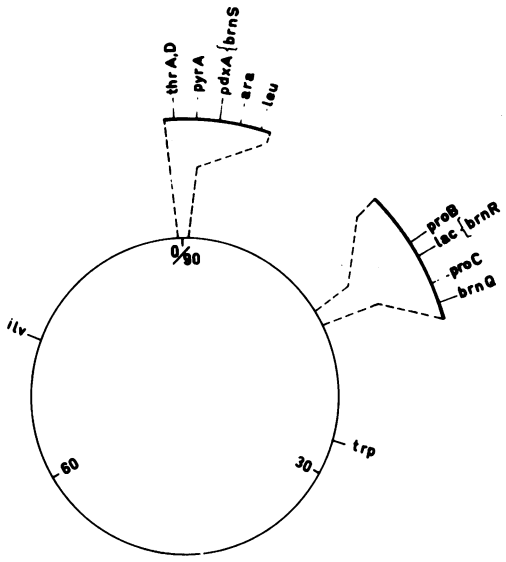

FIG. 1. *Order of markers relevant to this work (18). The brnQ marker has been shown (8) to be located clockwise to proC by a three-point test with P1 transduction.*

TABLE 2. *Effect of the presence of methionine[a] on the recovery of transport mutants*

Mutants isolated on:	Val^r-leucine dependent (%)	Glycylvaline resistant (%)
Valine (20 μg/ml) and leucine (50 μg/ml)[b]	68[d] (33/48)	32 (15/48)
Valine, leucine, and methionine[a, b]	100[e] (48/48)	<2 (0/48)
Valine (40 μg/ml)[c]	ND	100 (48/48)
Valine and methionine[a, c]	ND	25 (7/28)

[a] The different inocula were grown in the presence of methionine (50 μg/ml) before plating on selective plates.

[b] These mutants were isolated from strain MI148d, and the plates contained the other supplements required by this strain for growth.

[c] These mutants were isolated from strain Ca85, and the plates contained the other supplements required by this strain for growth. They were, therefore, all Val^r even in the absence of leucine.

[d] All of these mutants were also resistant to valine if leucine was substituted with threonine.

[e] Forty-six of these forty-eight mutants were also resistant to valine if leucine was present.

easily found if the very-high-affinity transport process is repressed by methionine, and this fact confirms the physiological role of this transport process. Their frequency is even higher if the high-affinity transport process is partially inhibited by either leucine or threonine. The presence of the low-affinity transport processes does not appear to prevent the finding of transport mutants. We believe that this is due either to the low-affinity of the substrates for these transport processes or to their poor contribution to the pool size.

Mutations pleiotropically affecting the very-high-affinity transport process. Two different mutants, whose isolation is described below, show decreased uptake through the very-high-affinity transport process as a consequence of specific mutations. The phenotype, however, is pleiotropic because other transport systems are also affected. One of these mutants, MI250 (*brnQ2, brnR3, brn-8*), shows essentially no uptake through the very-high-affinity transport process for isoleucine, leucine, or valine (see Table 3); the mutation causing this effect was called *brn-8* and affects the low-affinity transport process for isoleucine (see below). The other strain, MI247*brnR6*^{am} (data not reported), shows less than 10% of the uptake through the very-high-affinity transport for branched-chain amino acids shown by an isogenic *brnR*$^+$ strain. The mutation causing this effect, *brnR6*, is an amber mutation and is 93% co-transducible with *lac*.

For both mutants, two alternative explanations are possible: (i) either a gene product common to two different transport systems is nonfunctional or lacking as a consequence of a single mutation; or (ii) because of the mutation, there is a secondary alteration of uptake (due, for example, to altered pool size or amino acid excretion).

Multiplicity of the high-affinity transport systems. The *brnQ2* mutant described previously (8) is resistant to valine inhibition only if leucine is also present in the growth medium, and the action of leucine is most probably related to the inhibition of valine uptake which leucine exerts either on the wild-type strain or the *brnQ2* mutant (see Table 4). In fact, leucylhistidine, which presumably does not inhibit valine uptake, cannot substitute for leucine in

TABLE 3. *Very-high-affinity transport of isoleucine,[a] leucine, and valine in strains MI237b and MI250[b]*

Strain	Isoleucine[c]	Leucine[c]	Valine[c]
MI237b (*brnQ2,brnR3*)	0.155	0.127	0.120
MI250 (*brnQ2,brnR3,brn-8*) ...	<0.010	<0.010	<0.010

[a] [^{14}C]isoleucine, -leucine, or -valine concentrations were 4 × 10^{-8} M. For details see Materials and Methods.

[b] No methionine was present in the growth medium.

[c] Micromoles of amino acid per 0.5 min per g of cells.

the expression of valine resistance. We therefore concluded (8) that the observed leakiness of the phenotype was caused either by a partial alteration of the transport system for branched-chain amino acids or by a more or less complete alteration of one of several transport systems for these amino acids. The finding of several transport processes for branched-chain amino acids (7) makes the second hypothesis more likely and permits one to define conditions in which at least 90% of the rate of amino acid uptake is due to one specific transport process. As a consequence, each transport process can be analyzed separately to check which one is altered. Measurement of the high-affinity transport process in strain MI174b (*brnQ2*) confirmed that the alteration described previously (8) was in the apparent V_{max} value and not in the K_m value; moreover, difficulties in reproducibility led to the finding that the high-affinity transport process is unstable. This instability is greater in strain MI174b than in strain MI148a (*brn*$^+$) (data not reported). Instability takes place under conditions of amino acid starvation even before this affects the growth rate; since the uptake rate is unstable also in chloramphenicol-treated cells, instability is probably due to protein turnover. Reproducible values could be obtained if bacteria were collected in mid-logarithmic phase in which at least 50% of the added amino acids were still present and if uptake was measured exactly 10 min after chloramphenicol addition. Under these conditions only a small alteration in isoleucine, leucine, and valine uptake was found in strain MI174b as compared to the isogenic *brn*$^+$ strain (see Tables 5 and 11). Because of this result, we made two alternative hypotheses: (i) there are two different high-affinity transport systems for branched-chain amino acids with very similar apparent K_m and V_{max}, and only one of them is missing in strain MI174b as a result of the *brnQ2* mutation; (ii) the *brnQ* gene product is still partially active in strain MI174b. The experiments described hereafter demonstrate that the first hypothesis is correct.

If two different high-affinity transport systems do exist, it is likely that, although one of them transports only isoleucine, leucine, and valine, the other might transport some other amino acid(s). Therefore, we examined inhibition by different amino acids also in strain MI174b, and in this way we found that threonine totally inhibits valine uptake in this strain (Table 4). This observation suggests that threonine exerts its action on the fraction of uptake through the high-affinity transport that is left unaffected by the *brnQ2* mutation. The action of threonine is shown in more detail in Table 5—threonine also inhibits isoleucine and leucine uptake, and again the observed inhibition is much stronger in strain MI174b than in strain MI148a (*brn*$^+$). The apparent inhibition constants of threonine inhibition of valine uptake in strains MI148a and MI174b are 9×10^{-4} and 6×10^{-6} M, respectively (Fig. 2). We believe that threonine inhibition of valine uptake in strain MI148a takes place not only with low affinity (K_i 9×10^{-4} M) but also with high-affinity (K_i 6×10^{-6} M), but this is difficult to prove because the high-affinity inhibition affects only a fraction of valine uptake.

The striking inhibition of valine uptake by

TABLE 4. *Inhibition of high-affinity transport of valine in strains MI148a and MI174b by different amino acids*[a]

Inhibitor[b] (2×10^{-4} M)	Valine[c]	
	MI148a	MI174b
None	0.97	0.48
Isoleucine	0.03	0.01
Leucine	0.06	0.01
Threonine	0.70	0.02
Alanine	0.80	0.16
Methionine	0.56	0.25

[a] [^{14}C]valine concentration was 2×10^{-6} M; 50 μg of methionine per ml was present in the growth medium. For details see Materials and Methods.

[b] Arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, lysine, phenylalanine, proline, serine, tyrosine, and tryptophan have no effect on the valine uptake either in MI148a or MI174b.

[c] Micromoles per 0.5 min per g of cells.

TABLE 5. *Inhibition of high-affinity transport by threonine*

Strain	Inhibitor	Substrates		
		Isoleucine[a]	Leucine[a]	Valine[a]
MI148a	None	0.70	0.64	0.71
	2×10^{-4} M threonine	0.53	0.47	0.53
MI174b	None	0.43	0.55	0.45
	2×10^{-4} M threonine	0.01	0.01	0.03

[a] [^{14}C]isoleucine, -leucine, or -valine concentrations were 2×10^{-6} M; 50 μg of methionine per ml was present in the growth medium. For details see Materials and Methods.

threonine has also a physiological consequence, as shown in Fig. 3; this figure demonstrates that, when certain concentrations of valine and leucine are chosen, leucine does not influence at all the expression of valine resistance of strain MI174b; if, however, leucine is substituted with an equimolar amount of threonine, this strain becomes completely resistant to valine. MI174b shows an analogous behavior on agar plates. These data, therefore, are consistent with the hypothesis that two high-affinity transport systems do exist in *E. coli*, one of which transports isoleucine, leucine, and valine and is missing in strain MI174b, and the other transports isoleucine, leucine, and valine and is inhibited by threonine.

Another experiment was performed in which we isolated more mutants altered at the *brnQ* locus with the aim of finding amber mutations (if amber mutations in the *brnQ* locus resulted in the same phenotype as did the *brnQ2* mutation, the leakiness of this mutation would become unlikely). Twenty-seven mutants were independently isolated from strain Ca85 (which contains a *lac* amber mutation) on plates containing (per milliliter): 50 μg of methionine, 20 μg of valine, and 50 μg of threonine. Under these conditions, the very-high-affinity transport system is repressed by methionine, and the fraction of high-affinity transport remaining in the *brnQ2* mutant is inhibited by threonine. Upon further analysis, five of these mutants were found to require the presence of threonine or leucine to express the resistance to valine. All five mutants appeared to be altered in the *brnQ* locus on the basis of co-transduction frequency of the Val^r phenotype with *lac*. In fact, when these five mutants were treated with P1 phage grown on a *brnQ*$^+$, *lac*$^+$ strain and Lac$^+$ transductants were selected, about 12% co-transduction frequency of the Val^r phenotype was observed. These mutants were lysogenized with φ80psu3; Lac$^+$ colonies were purified and their resistance to valine was checked; four of these five mutants had become sensitive to valine. Two of these four Val^s mutants were cured of φ80psu3, and both were found to have become again Lac$^-$ and Val^r.

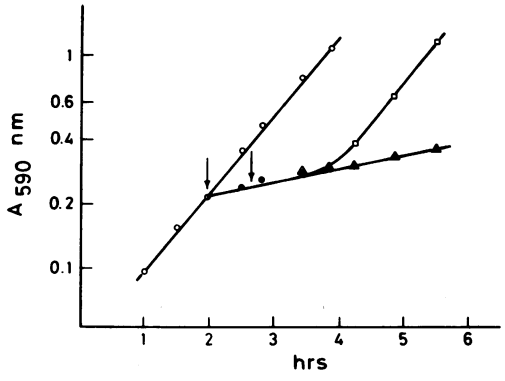

FIG. 3. *Growth curve of strain MI174b (brnQ2) in minimal medium at 37 C in different conditions with the supplements needed by this strain for growth (see Table 1) (○); same plus 2.15 × 10^{-3} M valine (●) added at the time indicated by the left arrow; same plus 2.15 × 10^{-3} M valine and 4.25 × 10^{-4} M (final concentration) leucine (△) (leucine was added at the time indicated by the right arrow); same plus 2.15 × 10^{-3} M μg of valine per ml and 4.25 × 10^{-4} M (final concentration) threonine (□) (threonine was added at the time indicated by the right arrow). Growth was measured as absorbance at 590 nm.*

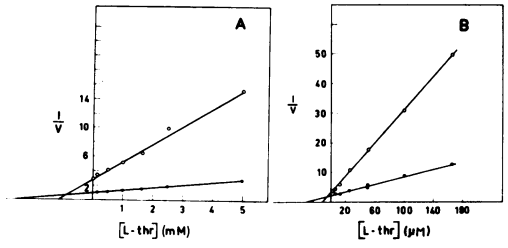

FIG. 2. *(A) Dependence of the rate of uptake of 0.8 μM (○) and 4 μM (●) valine upon threonine concentration in strain MI148a. Plot 1/v versus threonine concentration, where v is the rate of uptake expressed as micromoles per 0.5 min per gram of cells. Values are average of two determinations. K_i is 0.9 mM and was determined by projecting the intersection of the two curves on the abscissae. (B) Dependence of the rate of uptake of 0.8 μM (○) and 4 μM (●) valine upon threonine concentration in strain MI174b (brnQ2). Plot of 1/v versus threonine concentration, where v is the rate of uptake expressed as micromoles per 0.5 min per g of cells. Values are average of two determinations. K_i is 6 μM and was determined by projecting the intersection of the two curves on the abscissae.*

It has been reported (16) that amber mutations are sometimes leaky and that their leakiness decreases when a *strA* mutation is introduced into the strain. We further examined one of the amber Val^r mutants by introducing the *strA120* mutation of strain AT2365 (by P1 transduction) into it. The Val^r phenotype did not change. Thus, it was probably due to the absence of the gene product and not to a low concentration of a normal, or missense, gene product obtained by misreading of the amber triplet. In conclusion, four independently isolated amber mutations in the *brnQ* locus make strain Ca85 resistant to valine (if threonine is also present). In one of these mutants, called

MI238, the high-affinity transport process was analyzed and compared to that of its isogenic *brn*$^+$ strain Ca85. As shown in Table 10, uptake of isoleucine, leucine, and valine is slightly altered as in strain MI174b (Table 5). Threonine abolishes almost completely this uptake (data not reported). Therefore, the phenotype of the strains carrying an amber mutation at the *brnQ* locus and of strain MI174b is not due to a partially active *brnQ* gene product, but to the absence of the activity of the *brnQ* gene product.

We will call hereafter the two high-affinity transport systems high-affinity 1 (the one that requires the *brnQ* gene product) and high-affinity 2 (the one that is inhibited by threonine). Mutants with defects in the latter system were isolated in the following way. The high-affinity transport of one of the branched-chain amino acids is inhibited completely by the other two. Therefore both the high-affinity 1 and the high-affinity 2 systems transport at least isoleucine, leucine, and valine. Moreover, the high-affinity 2 system should transport also threonine (Table 5). Therefore a strain missing the high-affinity 2 system should be Val^r and the resistance should *not* be helped by threonine. On the other hand, when the high-affinity 2 system is missing, the high-affinity 1 system is still active and, therefore, leucine should be required to inhibit the residual valine uptake. Finally, the resistance to valine should be expressed only if the very high-affinity transport system is nonfunctional, for example because of addition of methionine. Mutants altered in the high-affinity 2 transport system were isolated by selecting spontaneous Val^r mutants from strain Ca85 (*brn*$^+$). A flask containing 250 ml of minimal citrate supplemented with thiamine, histidine, 50 μg of methionine per ml, 20 μg of valine per ml, and 50 μg of leucine per ml was inoculated with 2×10^9 bacteria. When full growth occurred (36 h at 37 C with aeration), a penicillin counterselection (6) in a minimal medium containing 50 μg of threonine per ml in place of leucine was performed to decrease the frequency of mutants altered at the *brnQ* locus. The suspension was then plated on tryptone plates, and single colonies were analyzed. Several Val^r mutants that required leucine (but not threonine) to express the resistance to valine were found. In one of them, the Val^r phenotype was shown by lysogenization with ϕ80psu3 to be due to an amber mutation. This strain was called MI248 and the mutation causing resistance to valine was called *brnS7*. The *brnS7* mutation was located on the *E. coli* chromosome by conjugation with the F$^-$ strain X478 (*leuB*, *brnS*$^+$); these experiments showed high linkage with a *leuB* marker. Transduction experiments reported in Tables 6, 7 and 8 show that the *brnS* locus is located between *pyrA* and *ara* (see Fig. 1), very close to *pdxA* (95% co-transduction: 46/48).

The initial rate of valine uptake in strain MI248 was assayed under conditions in which mainly high-affinity transport process is measured (same as those of Table 4). Under these conditions (Table 9), the parental strain, Ca85, gave a value of 0.94 μmol per 0.5 min per g of cells, whereas strain MI248 gave a value of 0.51

TABLE 6. *Transduction of strain AT2365 with P1 grown on strain MI248 and selection of Thr$^+$ transductants*

brnS	*ara*	Frequency (%)
−	+	4 (2/48)
+	−	64 (31/48)
−	−	31 (15/48)
+	+	<2 (0/48)

P1 (MI248)	−	−	+
AT2365	*thr*	*brnS*	*ara*
	−	+	−

TABLE 7. *Transduction of strain AT2365 with P1 grown on strain MI248 and selection of Leu$^+$ transductant*

brnS	*ara*	Frequency (%)
−	+	15 (7/48)
+	−	56 (27/48)
+	+	25 (12/48)
−	−	4 (2/48)

P1 (MI248)	+	+	+
AT2365	*brnS*	*ara*	*leu*
	+	−	−

TABLE 8. *Transduction of strain AT739 with P1 grown on strain MI248 and selection of Thr$^+$ transductants*

pyrA	*brnS*	Frequency (%)
+	−	18 (23/128)
−	+	40.5 (52/128)
+	+	36.5 (47/128)
−	−	5 (6/128)

P1 (MI248)	+	+	−
AT739	*thr*	*pyrA*	*brnS*
	−	−	+

TABLE 9. *Uptake inhibition of isoleucine, leucine, and valine in strains Ca85 (brn+) and MI248 (brn27am) by different amino acids*[a]

Inhibitor (2×10^{-4} M)	Substrates					
	Isoleucine[b]		Leucine[b]		Valine[b]	
	Ca85	MI248	Ca85	MI248	Ca85	MI248
None	0.92	0.70	0.88	0.60	0.94	0.51
Serine.....	0.80	0.60	0.80	0.55	0.80	0.45
Alanine	0.83	0.62	0.72	0.54	0.78	0.43
Threonine .	0.75	0.62	0.72	0.50	0.73	0.44
Isoleucine ..			0.09	0.08	0.10	0.06
Leucine	0.08	0.05			0.08	0.07
Valine	0.07	0.08	0.09	0.08		

[a] The strains were grown in the presence of 50 μg of methionine per ml. For details see Materials and Methods. The figures represent micromoles of amino acid for 0.5 min per g of cells.

[b] [14C]isoleucine, -leucine, or -valine concentrations were 2×10^{-6} M.

μmol per 0.5 min per g of cells. Also, isoleucine and leucine uptake are only slightly reduced, as it should be if, in strain MI248, high-affinity 2 is missing and high-affinity 1 is still functional. As expected (since the resistance to valine is not helped by threonine), threonine gave only very little inhibition. Strains carrying *brnQ* mutations show only partial uptake alterations (see, for example, Table 11); strains carrying *brnS* mutations show also only partial uptake alterations (Table 9); however, when a strain is constructed which carries mutations at both genes *brnQ* and *brnS* the high-affinity transport process is abolished. Such a strain was constructed by treating strain MI248 (*brnS7*, *brnQ*+, *lac*) with P1 grown on strain MI174b (*brnQ2*, *lac*+). Selection was made for Lac+ transductants, and among these about 15 to 20% were found to be Valr even in the absence of leucine. One of these Valr transductants was purified twice by single-colony isolation and named strain MI249 (*brnS7*, *brnQ2*; *lac*+). The *brnQ2* mutation was demonstrated to be present in this strain by rescue with a proper P1 transduction. Uptake of valine in strain MI249 can still be measured if higher substrate concentrations are used. This uptake was shown to be done through the low-affinity transport since it had a K_m value of $0.5 \cdot 10^{-4}$ M (Fig. 4). An experiment similar to that of Fig. 4 (not reported) gave an analogous result for leucine. In Table 10, measurements of the high-affinity transport process of isoleucine, leucine, and valine in the wild-type strain, in a strain missing high-affinity 1 system (MI238), in a strain missing high-affinity 2 system (MI248), and in a strain missing both the high-affinity 1 and high-affinity 2 systems (MI249) are reported. The table shows that, whereas a small alteration in uptake is found in strains MI238 (*brnQ*) and MI248 (*brnS*), almost no uptake is found in strain MI249 (*brnQ*, *brnS*). (The alteration of the other strain of this table, MI247, is described below.)

Mutation affecting both high-affinity transport systems. A single mutation affecting both high-affinity transport systems was found in the course of an attempt to isolate a mutation in the *brnS* gene. This mutation is located at a

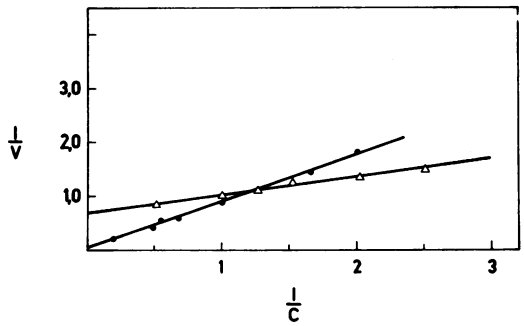

FIG. 4. *Dependence of the rate of uptake in strains MI249 (brnQ2, brnS7) (○) and MI174b (brnQ2) (△) upon the concentration of valine. Plot 1/v versus 1/c, where v is the rate of uptake of valine expressed as micromoles per 0.5 min per g of cells (dry weight) and c is the micromolar concentration of valine. Values are average of two determinations. Specific activity, background values, and counting efficiency are reported in Materials and Methods. The apparent K_m values were 0.5×10^{-4} M for strain MI249 and 2×10^{-6} M for strain MI174b.*

TABLE 10. *High-affinity transport of isoleucine,[a] leucine, and valine in strains with different brn alleles*

Strain	*brn* genotype	Isoleucine	Leucine	Valine
Ca85	*brn*+	0.46[b]	0.46[b]	0.48[b]
MI238	*brnQ4am*	0.36	0.43	0.29
MI248	*brnS7am*	0.45	0.45	0.32
MI249	*brnQ2, brnS7am*	0.04	0.06	0.06
MI247	*brnR6am*	0.06	0.08	0.04

[a] [14C]isoleucine, -leucine, or -valine concentrations were 2×10^{-6} M; 2×10^{-5} M methionine was also present in the assay tubes, and the strains were pregrown in the presence of methionine. Therefore, the very-high-affinity transport is not measured in this experiment. A value of 0.02 for low-affinity transport was found in a separate experiment on strain Ca85 (data not reported), and this figure is subtracted to all the values of this table. For details see Materials and Methods.

[b] Micromoles of amino acid per 0.5 min per g of cells.

locus different from *brnQ* and *brnS*, which we called *brnR*.

Spontaneous mutants resistant to valine in the absence of leucine or threonine, but sensitive to glycylvaline, were selected from strain MI174b (*brnQ2*) because we were looking for a second mutation that abolishes all high-affinity transport process. One of these mutants, MI237, was purified and retained for further study. The new mutation was called *brnR3*. The higher resistance to valine of strain MI237 as compared to the one of strain MI174b suggests that in this mutant there is no high-affinity transport process; this prediction (Fig. 5) turned out to be true. The uptake of valine had a K_m value of 0.5×10^{-4}, i.e., the K_m of low-affinity transport process. Conjugation experiments were performed that suggested a location for the *brnR* locus very close to *lac* and *proC*. The location of markers in this region and co-transduction frequencies of several pairs of markers are summarized in Fig. 6. Data of this figure support the order *proB*, *lac*, *proC*, and *phoA* for these markers although a different order is given by Taylor and Trotter (18). We have not tried to understand the reason for this discrepancy since the purpose of the following experiments was to show that *brnQ* and *brnR* are different markers. Transduction experiments showed that the *brnR3* mutation of strain MI237 is distinct from the *brnQ2* mutation; in fact, when strain MI148a (*lac*$^-$, *proC*$^-$, *brnR*$^+$, *brnQ*$^+$) is treated with P1 grown on strain MI237 (*lac*$^+$, *proC*$^+$, *brnR3*, *brnQ2*), 60% (57/96) of the selected Pro$^+$ transductants and 94% (91/96) of the selected

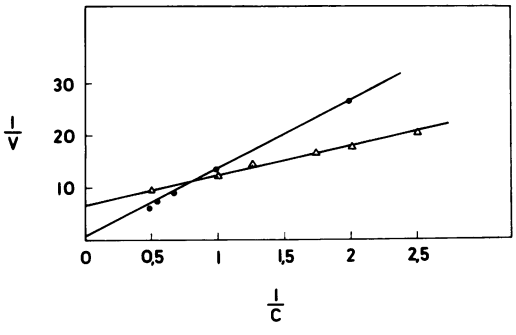

FIG. 5. *Dependence of the rate of uptake in strains MI237 (brnQ2, brnR3) (O) and MI174b (brnQ2) (Δ) upon the concentration of valine. Plot 1/v versus 1/c, where v is the rate of uptake of valine expressed as micromoles per 0.5 min per g of cells (dry weight) and c is the micromolar concentration of valine. Values are average of two determinations. Specific activity, background values, and counting efficiencies are reported in Materials and Methods.*

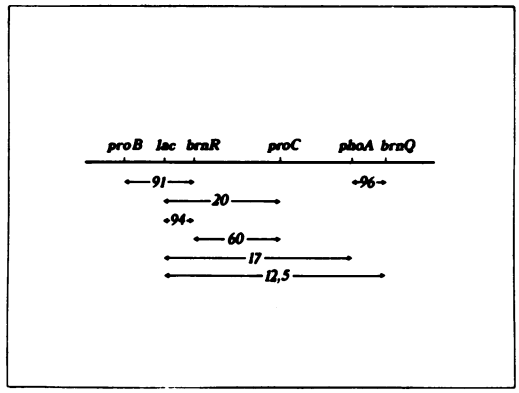

FIG. 6. *Schematic representation of markers located in the lac-phoA region. Numbers represent the frequency of cotransduction of one marker when the other is selected. The position of the proB, lac, proC, and phoA markers is taken from Taylor (17). In the more recent edition of the linkage map of E. coli K-12 (18), the order proB, lac, phoA, and proC is reported. We use the previous order since it is supported by these results. The brnR and brnQ markers are arbitrarily drawn on the right side of lac and phoA, respectively. A three-point test experiment (8) locates brnQ on the right side of proC. The co-transduction frequencies of phoA with brnQ and lac have also been reported (8). Other data are taken from this paper.*

Lac$^+$ transductants are Val^r in the absence of leucine or threonine, and these co-transduction frequencies are different from those reported (8) for *brnQ* with these markers. Moreover, when strain AT2477 (*proB28*, *brnR*$^+$) is treated with P1 grown on MI237 (*proB*$^+$, *brnR3*), 91% (86/95) of the selected Pro$^+$ transductants are Val^r. Since the co-transduction frequency of the *brnQ2* mutation is 12.5% with *lac* (8), the *brnR3* mutation (which shows 94% co-transduction with *lac*) is separated from *brnQ* but co-transducible with it. Moreover, since in the above transduction 94% of the selected Lac$^+$ transductants were Val^r even in the absence of leucine and since only 12.5% of these transductants should contain both *brnR3* and *brnQ2* (see Fig. 6), the phenotype of most of these transductants is due to the *brnR3* mutation alone. Therefore the phenotype of a strain carrying the *brnR3* mutation alone displays a level of resistance to valine equal to that of strain MI237 (*brnQ2*, *brnR3*).

Mutations in the *brnR* gene were also isolated in a single step. Spontaneous, independent mutants resistant to valine in the absence of leucine or threonine were isolated from strain Ca85 pregrown in the presence of methionine (methionine was also present during isolation;

TABLE 11. *Apparent kinetic constants for isoleucine, leucine, and valine transport in the absence or in the presence of competing amino acids*[a]

Strain	Substrate	Inhibitors[b]							
		None		Isoleucine		Leucine		Valine	
		K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
MI148a (*brn*$^+$)	Isoleucine	2.1	0.5			50	1	50	0.5
	Leucine	2.1	0.5	50	0.2			50	0.5
	Valine	2	0.3	50	1	50	1		
MI174b (*brnQ2*)	Isoleucine	2.1	0.14			50	0.33	50	0.5
	Leucine	2.1	0.15	50	0.33			50	0.5
	Valine	2.1	0.1	50	0.21	50	0.2		

[a] K_m, micromolar; V_{max}, micromoles of amino acid per 0.5 min per g of cells.

[b] Concentration of inhibitors was 2×10^{-4} M. Methionine (50 μg/ml) was present in the assay medium. For details see Materials and Methods.

see Table 2). Twenty-one Val^r, but glycylvaline-sensitive, mutants were purified and lysogenized with φ80psu3; 8 of 21 became Val^s and, therefore, were amber Val^r mutants. One of the amber Val^r mutants (MI247 [*brnR6*^{am}]) was treated with P1 grown on strain AT739 (*lac*$^+$, *brnR*$^+$), and Lac$^+$ transductants were isolated; 93% (150/160) of these became Val^s, indicating that strain MI247 is altered at the *brnR* locus and that this locus is very close to *lac*. None of these Val^s transductants was resistant to valine in the presence of threonine, and this shows that strain MI247 is *brnQ*$^+$ (in fact, *brnQ* shows 12.5% co-transduction frequency with *lac*, see Fig. 6).

Transport assays were performed to confirm that the *brnR* mutation alone is able to abolish both high-affinity transport systems. Data of Table 10 show that strain MI247 (*brnR6*) has almost no uptake through the high-affinity transport process of either isoleucine or leucine or valine.

Mutant altered in the low-affinity transport system for isoleucine. It has been shown (7 and Table 11) that three different transport processes specific for either isoleucine or leucine or valine are present in *E. coli*. A mutant in the low-affinity transport system for isoleucine was isolated on the assumption that no other transport systems for isoleucine besides those described in this paper are present. Therefore, an isoleucine auxotroph missing all these transport systems should not be able to utilize isoleucine for growth, but should be able to utilize the isoleucine produced inside the cell from cleavage of a dipeptide containing isoleucine. Strain MI237b (*ilvA*, *brnQ2*, *brnR3*) was mutagenized with nitrosoguanidine, and a penicillin counter selection (6) in the presence of isoleucine (5 μg/ml) and methionine (50 μg/ml) was performed. The strains (2/96) which did not grow on plates supplemented with isoleucine, but grew on plates supplemented with glycylisoleucine, were purified, and one of them (MI250) was retained for further study. The mutation causing absence of growth in the presence of isoleucine was called *brn-8*. (It should be pointed out that, since this mutation has not been localized on the chromosomal map and since it has been obtained with nitrosoguanidine mutagenesis, it is possible that the phenotype of the mutant is due to more than one mutation.) Figure 7 shows the growth yield of strain MI250 grown on increasing concentrations of either isoleucine or glycylisoleucine, in the presence of methionine; no growth can be obtained on isoleucine, whereas a normal growth yield was obtained on glycylisoleucine, which satisfied our general criterion for identifying a transport mutant (3, 8). The rates of uptake through the low-affinity transport of isoleucine, leucine, and valine were measured in strain MI250 and its *brn-8*$^+$ parent MI237b (*brnQ2*, *brnR3*) (Table 12). In this experiment, the uptake through the very-high-affinity transport process is not measured in strain MI250 because a pleiotropic effect of the *brn-8* mutation (Table 3) is an alteration of this transport system. Since the two strains were pregrown and then assayed in the presence of methionine, the very-high-affinity transport process, or at least most of it (7) is absent in the *brn-8*$^+$ strain MI237b also. Furthermore, most of the uptake through the high-affinity transport is not present because of the *brnQ2* and *brnR3* mutations present in these two strains. The rate of isoleucine uptake via the low-affinity transport process was reduced in the mutant to 14% of the

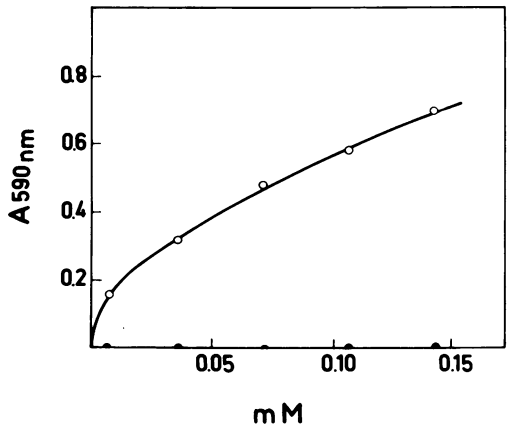

FIG. 7. *Dependence of the growth yield of strain MI250 (ilvA, brnQ2, brnR3, brn-8) upon the concentration of isoleucine (●) or glycylisoleucine (○) in the medium in the presence of 3.3 × 10⁻⁴ M methionine. The growth was determined as the absorbance at 590 nm after 36 h incubation at 37 C.*

value in the parental (*brn-8*⁺) strain (Table 12). Although to a lesser extent, leucine and valine uptake is also altered. This alteration is probably due to the observed pleiotropic effect of the *brn-8* mutation. In fact, the fraction of the uptake through the very-high-affinity transport remaining in strain MI237b after repression might be higher than the fraction remaining in strain MI250. We cannot exclude, however, that the *brn-8* mutation causes an effect at the level of the remaining transport processes or that it causes a secondary uptake alteration due, for example, to excretion of branched chain amino acids. Strain MI250, in fact, grows slowly on glycylisoleucine as compared to strain MI237b, perhaps because the isoleucine produced inside the cell is excreted. If true, this strain might also excrete leucine and valine.

DISCUSSION

The data reported in this paper confirm the necessity of mutant isolation to define a transport system. In fact, what appears from kinetic experiments to be only one transport system, namely the high-affinity one, is resolved into two different ones by mutant isolation. Moreover, the poor affinity and extreme specificity of the low-affinity transport processes made uncertain their existence until we found a mutant in which the isoleucine transport system was altered. However, when a transport mutant is defined, it is important to keep in mind that the transport assay is uptake of radioactive substrate and, therefore, the observed uptake alterations might be due to secondary effects of a mutation which causes an alteration at another level (e.g., regulation of pool size or excretion of amino acids). This is likely to be the case, as we had already suggested (8), of a mutation in another gene, *brnP* (M. De Felice, J. Guardiola, T. Klopotowski and M. Iaccarino, manuscript in preparation). Therefore, we cannot exclude that the same is true for the mutants described in this paper. In particular, we cannot exclude that the pleiotropism shown by the strains bearing the *brnR6*^{am} and the *brn-8* mutations might be due to such a kind of secondary effects, which could have mimicked a genetic alteration of the very-high-affinity transport process.

The *brnQ*, *brnR*, and *brnS* genes described in this paper are different because of their chromosomal locations, and the *brn-8* mutation is probably in a gene different from the other three, because the low-affinity transport process for isoleucine is still present in strains bearing amber mutations in the *brnQ*, *brnR* and *brnS* genes. On the other hand, the different phenotype of two *brnR* mutations suggests the presence of another gene. In fact, in strain MI237b (*brnR3*) the very-high-affinity transport system and the low-affinity transport system for isoleucine are present, whereas in strain MI247 (*brnR6*^{am}) the very-high-affinity transport system is missing, but the low-affinity transport system for isoleucine is present (data not shown). Although other interpretations are possible, a polar effect of the amber mutation *brnR6* on an adjacent gene might be responsible for the phenotype. The location of the *brnS* gene is very similar to the location given by Glover (5) to the Val^{r}-D locus, but it is different from the location of the *ileS* gene (10). Mutations in the latter gene might conceivably show a Val^{r} phenotype, since its gene product, isoleucyl-transfer ribonucleic acid synthetase (L-isoleucine:soluble ribonucleic acid ligase [adenosine monophosphate] EC 6.1.1.5), has been

TABLE 12. *Low-affinity transport of isoleucine,[a] leucine, and valine in strains MI237b and MI250[b]*

Strain	Isoleucine	Leucine	Valine
MI237b (*brnQ2, brnR3*)	0.195[c]	0.190[c]	0.182[c]
MI250 (*brnQ2, brnR3, brn-8*)	0.027 (14%)	0.081 (46%)	0.090 (49%)

[a] [¹⁴C]isoleucine, -leucine, or -valine concentrations were 5 × 10⁻⁵ M. For details see Materials and Methods.
[b] Bacteria were grown in the presence of methionine.
[c] Micromoles of amino acid per 0.5 min per g of cells.

implicated (19) in regulation of isoleucine-valine biosynthesis.

The protein produced from the *brnR* gene appears to be analogous to the *hisP* protein described by Ames and Lever (1). As in that case, our data can be interpreted with the *brnQ* and *brnS* gene products working in parallel and both requiring the *brnR* gene product to permit transport through the membrane. It is not possible to decide whether the *brnR* gene product is more external or more internal as compared to the other two. A schematic representation of the situation is reported in Fig. 8. Another possibility is that the *brnR* gene product is required for expression of the *brnS* and *brnQ* genes.

In Table 13 a summary is reported of the different transport systems for branched-chain amino acids present in *E. coli* K-12. We have suggested in this table the presence of a leucine-specific transport system for which evidence has been reported by Furlong and Weiner (4). They report the isolation and purification of a leucine-specific binding protein from strain K-10 which is a derivative of the HfrC strain of K-12. This protein, used for experiments not reported in this paper, was isolated and purified from the osmotic shock fluid prepared from strain MI148a. The existence of a leucine-specific transport system with high affinity is also suggested by evidence reported previously (7). Three different genetic markers (*brnQ*, *brnR*, and *brnS*) are reported in the table, and it is likely that *brn-8* is a mutation in still another gene. We believe that probably many more genes will be found to be necessary for the function of the different transport systems. We have chosen to look for amber mutations in the

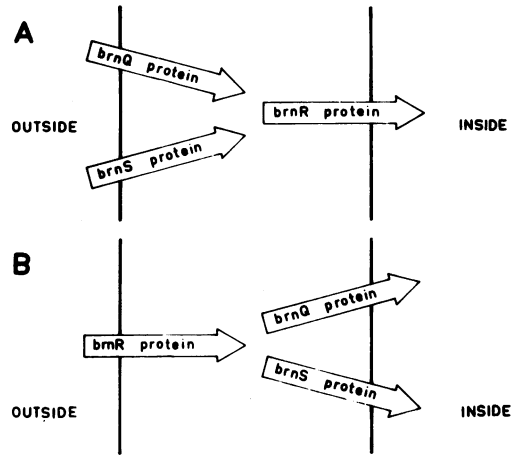

FIG. 8. *Two alternative schematic representations of the flow of transport through the brnQ, brnR and brnS gene products.*

TABLE 13. *Summary of the different transport systems for branched-chain amino acids present in E. coli K-12*

Transport system	Substrates	K_m (M)	V_{max} (μmol/0.5 min/g of cells)	Relevant inhibitors	Genetic markers	Gene products
Very-high-affinity	Isoleucine	1×10^{-8}	0.5	Methionine, threonine, alanine	*brn-8*(?)	Unknown
	Leucine	2×10^{-8}	0.4		*brnR*(?)	Unknown protein
	Valine	1×10^{-7}	2.1			
High-affinity 1[a]	Isoleucine	2×10^{-6}	0.25		*brnQ*,	Unknown protein (LIV-binding protein unlikely)[b]
	Leucine	2×10^{-6}	0.25			
	Valine	2×10^{-6}	0.15			
					brnR	Unknown protein
High-affinity 2[a]	Isoleucine	2×10^{-6}	0.25	Threonine	*brnS*,	Unknown protein (possibly the LIV-binding protein)[b]
	Leucine	2×10^{-6}	0.25			
	Valine	2×10^{-6}	0.15			
					brnR	Unknown protein
High-affinity ? (leucine)	Leucine (?)					Leucine-binding protein (?)
Low-affinity-isoleucine	Isoleucine	0.5×10^{-4}	0.75		*brn-8*	Unknown
Low-affinity-leucine	Leucine	0.5×10^{-4}	0.35		Unknown	Unknown
Low-affinity-valine	Valine	0.5×10^{-4}	1		Unknown	Unknown

[a] Apparent K_m values are those for strain MI148a from Table 11, and V_{max} values are 50% of those for strain MI148a in the same table.

[b] The leucine-isoleucine-valine (LIV)-binding protein described by Penrose et al. (14).

brnQ, *brnR*, and *brnS* genes because the phenotype of an altered membrane gene product is likely to be complex, whereas the phenotype caused by an amber mutation is usually due to the absence of the gene product. Moreover, with these mutations the gene product can be identified as a protein. It is clear, however, that now it is possible to isolate mutations of different type (e.g., temperature sensitive or deletions) at the same loci.

Column five in Table 13 is helpful for characterization of the different transport systems. These compounds inhibit the uptake of a radioactive substrate and might also be substrates of the specific transport system that they inhibit. We examined the uptake of [^{14}C]threonine (experiment not reported) in strains MI148a (*brn*$^+$) and MI174b (*brnQ2*). Isoleucine, leucine and valine gave no inhibition of the uptake, and this shows that either threonine is only an inhibitor and not a substrate of the high-affinity 2 transport system or that there is a multiplicity of transport systems also for threonine.

A column for gene products has been added in Table 13 only to point out that some gene products are proteins and that genes have yet to be found for the leucine-isoleucine-valine-binding protein (14) and for the leucine-binding protein (4).

The differential sensitivity to valine and glycylvaline has proved very useful for isolation of the transport mutants described in this paper. Valine acts through the inhibition of isoleucine biosynthesis, and Val^r mutants are of two types—either regulatory or transport mutants. Valine is also used for protein synthesis, and therefore when low concentrations of valine are used a fraction of it is consumed and, if the fraction is big enough, inhibition is relieved. For this reason we never use a concentration of valine lower than 20 μg/ml, but we add 50 μg of leucine per ml to decrease the intracellular concentration of valine. Val^r mutants which need leucine to express the resistance to valine are more often transport mutants, but they can also be regulatory mutants. In this case, leucine acts at the transport level by decreasing valine uptake and can usually be substituted by threonine.

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