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Escherichia coli K-12 Mutants Altered in the Transport of Branched-Chain Amino Acids

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Two mutants of *Escherichia coli* K-12 are described which are resistant to the inhibition that valine exerts on the growth of *E. coli*. These mutants have lesions at two different loci on the chromosome. One of them, *brnP*, is linked to *leu* (87% cotransduction) and is located between *leu* and *azi* represented on the map at 1 min; the other, *brnQ*, is linked to *phoA* (96% cotransduction), probably between *proC* and *phoA* and represented at 10 min. These mutants are resistant to valine inhibition but are sensitive to dipeptides containing valine. Since it is known that dipeptides are taken up by *E. coli* through a transport system(s) different from those used by amino acids, this sensitivity to the peptides suggests an alteration in the active transport of valine. The mutants are resistant to valine only if leucine is present in the growth medium; the uptake of valine is less in both mutants than it is in wild-type *E. coli*, and it is reduced even further if leucine is present. Under these conditions the total uptake of valine is almost completely abolished in the *brnQ* mutant. The *brnP* mutant takes up about 60% as much valine as does the wild type, but no exogenous valine is incorporated into proteins. The apparent K_m and V_{max} of isoleucine, leucine, and valine for the transport system are reported; the *brnP* mutant, when compared to the wild type, has a sevenfold higher K_m for isoleucine and a 17-fold lower K_m for leucine; the V_{max} for the three amino acids is reduced in the *brnQ* mutant, up to 20-fold for valine. The transport of arginine, aspartic acid, glycine, histidine, and threonine is not altered in the *brnQ* mutant under conditions in which that of the branched amino acids is. Evidence is reported that *O*-methyl-threonine enters *E. coli* through the transport system for branched amino acids, and that thiaisoleucine does not.

The growth of *Escherichia coli* K-12 is inhibited by valine, and this inhibition is reversed by isoleucine (21). There is evidence (9) that valine inhibits isoleucine biosynthesis through feedback inhibition of acetolactate synthetase, an enzyme needed for the biosynthesis of isoleucine and valine. Mutants resistant to valine inhibition (Val^r) can easily be obtained (5, 15). These mutants possess either a valine-resistant acetolactate synthetase or an increased rate of isoleucine biosynthesis. Mutants of the second type are regulatory mutants, and operator gene mutants were found in this class (16).

We report here on two previously undescribed Val^r mutants. They are altered in the active transport of valine and are therefore resistant, because this amino acid does not enter the cell. Since the transport of branched-chain amino acids appears to be altered in these mutants, we have chosen for them the symbol *brn* (for branched).

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 (22).

Reagents and media. *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (nitrosoguanidine) was purchased from K & K Laboratories, Inc., Plainview, N.Y. The amino acids used were all L-form of the highest purity available. Dipeptides were purchased from Miles Laboratories, Inc., Elkhart, Ind., or from Nutritional Biochemicals Corp., Cleveland, Ohio. α-Acetolactic acid acetate ethylester was purchased from K & K Laboratories, Inc., Plainview, N.Y. Uniformly labeled ^{14}C-L-amino acids were obtained from New England Nuclear Corp., Boston, Mass. Thiaisoleucine hydrochloride (2-amino-3-methylthiobutyrate) and *O*-methyl-DL-threonine were obtained from Reef Laboratories, Santa Paula, Calif.

"Minimal citrate" medium (25) was used in most experiments, "minimal salts" medium (5) was used during the isolation of mutants, and minimal 121 (23) was used for transduction of *phoA*. Usual supplements, when required, were 0.4% glucose, L-tryptophan (25 μg/ml), L-arginine (100 μg/ml), L-valine (100 μg/ml),

TABLE 1. *Bacterial strains*[a]

Strain	Genotype and origin
RFS54	*thi, araD54*, HfrH; from R. Schleif
X478	*thi, leuB, metE, proC, purE, trp, lysA, ara, xyl, lacZ, azi, str, tonA, tsx*, F⁻; from P. Berg
MI148a	*thi, metE, proC, purE, trp, lysA, ara, xyl, lacZ, str*ʳ, *tonA, tsx*, F⁻; Leu⁺, Azi^s, Ara⁻ transductant of X478
U18	*phoA* (amber near N-terminal); from M. Schlesinger
AB1264	*thi-1, his-4, galK2, lacY1, proA2, str-8, ilvB197*, F'14; (phenotype Ile⁺, Val⁺, Val^r); from E. A. Adelberg
MI183	*thi, araD54, brnP1*, HfrH; from RFS54 by ultraviolet mutagenesis
MI183a	*brnP1, thi, metE, proC, purE, trp, lysA, xyl, lacZ, str, tonA, tsx*, F⁻; Leu⁺, Val^r, Ara⁺, Azi^s transductant of X478
MI174	*brnQ2, thi, leuB, metE, proC, purE, trp, lysA, ara, xyl, lacZ, azi, str, tonA, tsx*, F⁻; from X478 by nitroso guanidine mutagenesis
MI174a	*brnQ2, thi, leuB, metE, purE, trp, lysA, ara, xyl, azi, str, tonA, tsx*, F⁻; Lac⁺, Pro⁺, Val^r transductant of MI174
MI174b	*brnQ2, thi, metE, purE, trp, lysA, ara, xyl, str, tonA*, tsx, F⁻; Lac⁺, Pro⁺, Val^r transductant of MI148a
MI174d	*brnQ2, thi, purE, trp, lysA, ilvC or D, ara, xyl, str*ʳ, *tonA, tsx*, F⁻; Met⁺, Ilv⁻ transductant of MI174b

[a] Symbols for genetic markers are those used by Taylor (22); *brnP* and *brnQ* symbols are the loci defining genes for the transport of the branched-chain amino acids described in this report.

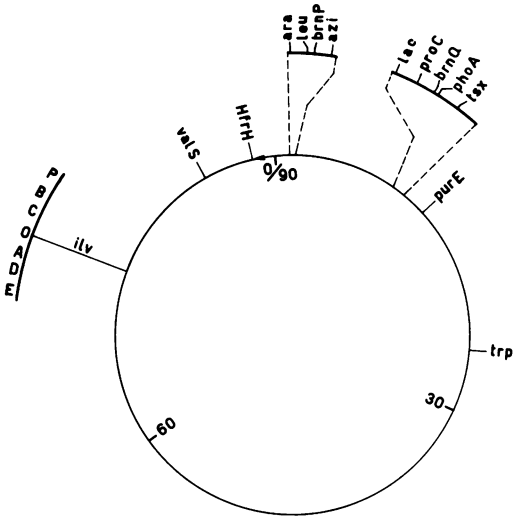

FIG. 1. *Order of markers relevant to this work (22).*

other L-amino acids (50 μg/ml), nucleosides (50 μg/ml), and thiamine (10 μg/ml).

L-Broth was described by Lennox (10). Nutrient broth was made of 5 g of NaCl and 8 g of nutrient broth (Difco) in 1 liter of distilled water.

Transduction. Transductions were performed with P1kc (obtained from C. Yanofsky) prepared by confluent lysis technique as described by Hill et al. (7). Unselected markers were scored by suspending single colonies into a drop of minimal medium; a metal device was used to transfer sets of drops to a tryptone plate which was incubated at 37 C. This plate was then replica-plated on appropriately supplemented minimal plates.

For transduction of *phoA*, selection of the transductants was done by treating bacteria with P1kc phage and mixing with 3 ml of 0.7% agar containing minimal 121 and 0.2 ml of 1 M citrate. The suspension was then poured onto plates containing minimal 121 supplemented with 20 μg of sodium-β-glycerophosphate per ml and the appropriate supplements. From these plates transductants were plated again on minimal 121 plates and then picked and scored for unselected markers.

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 minimal medium.

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

Enzyme assays. Bacteria were grown in the desired conditions until they reached the late exponential growth, harvested by centrifugation at 4 C, washed with 0.15 M NaCl, and then frozen as a pellet at −20 C.

To 50 mg of frozen cells 1 ml of the extraction solution (50 mM potassium phosphate, *p*H 7.4, containing 0.1 mM L-isoleucine, 0.5 mM ethylenediaminetetraacetic acid, and 0.5 mM dithiothreitol) was added, and the suspension was mixed on a Vortex mixer. After disruption (2 min with an MSE sonic oscillator) the suspension was centrifuged for 20 min at 15,000 rev/min with a Sorvall SS34 rotor. The extracts contained between 1 and 5 mg of protein per ml. Proteins were determined by the method of Groves (6) with crystalline bovine plasma albumin as a standard.

Threonine deaminase, the *ilvA* enzyme [L-threonine hydro-lyase (deaminating) EC 4.2.1.16], was assayed by measuring the rate of formation of α-ketobutyrate as described by Iaccarino and Berg (8).

Acetolactate synthetase, the *ilvB* enzyme, was as-

sayed by determining the rate of acetolactate formation (19).

Acetohydroxy acid isomero reductase, the *ilvC* enzyme, was assayed spectrophotometrically by the method of E. A. Adelberg (*personal communication*). The reaction mixture (0.84 ml) contained, per cuvette, 20 mM potassium phosphate (*p*H 7.5), 5 mM $MgCl_2$, 1.8 mM acetolactate, and the appropriate amount of extract. The reaction was started with 30 μliters of 3 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) in 0.1 M potassium phosphate (*p*H 7.5) and was carried out at room temperature. The control cuvette contained no acetolactate. Acetolactate was obtained by saponification of the α-acetolactic acid acetate ethyl-ester with two equivalents of NaOH. The absorbancy at 340 nm (A_{340}) was read at 0.5-min intervals for 3 min and plotted versus time; the slope of the straight line gives ΔA (ΔA_{340}/min).

The specific activity was calculated from the formula: $(\Delta A \cdot V \cdot 10^9)/(\epsilon \cdot \mu\text{liters of extract} \cdot p)$ where V is the volume (in ml) of the reaction mixture, ϵ is the molar absorption of NADPH, and p is the protein concentration of the extract in mg/ml.

The specific activity of the three enzymes is expressed in nanomoles of product formed per minute per milligram of protein.

Inhibition tests. A 0.1-ml amount of a cell suspension grown overnight in minimal citrate medium was diluted in 3 ml of 0.7% minimal citrate agar and layered on supplemented minimal citrate plates; 10 μliters of a 5 mg/ml solution of the substance to be tested was pipetted onto a small circle (6 mm in diameter) of Whatman no. 3 MM paper applied on the agar surface. Inhibition was observed after overnight incubation at 37 C.

Valine uptake in growing cells. Tubes containing ^{14}C-valine and exponentially growing bacteria in minimal citrate medium, for a total volume of 1 ml, were incubated at 37 C for the times indicated; the samples were then quickly filtered through membrane filters (type HA; Millipore Corp., Bedford, Mass.; 0.45-μm pore size) and washed with 5 ml of minimal medium at 37 C. Radioactivity of dried filters was measured in a liquid scintillation analyzer (Nuclear-Chicago or Packard). In each experiment a blank value was obtained with a tube containing no bacteria; this value was usually about 200 counts/min and was subtracted from the sample values. Since washing of the cells could not be extensive and had to be done quickly (14), the presence of the bacteria in the samples probably mechanically trapped some radioactivity on the filter. Therefore, the real background is somewhat higher than the subtracted one (see Fig. 3).

The uptake is expressed in micromoles of amino acid incorporated per gram of cells (dry weight).

Determination of apparent K_m and V_{max} for active transport. Bacteria were grown in supplemented minimal citrate medium, harvested by centrifugation, and washed twice with unsupplemented medium. The determination of apparent K_m and V_{max} was performed by a slight modification of the method described by Piperno and Oxender (14). The washed cells were suspended to give a final concentration of 10 mg/ml in minimal medium containing 300 μg of chloramphenicol per ml and 0.02% glucose. Transport was measured by pipetting 0.15 ml of the warm (37 C) bacterial suspension into a test tube containing 0.85 ml of a ^{14}C-amino acid in minimal medium at 37 C. After 0.5 min, the sample was filtered onto the center of a membrane filter (Millipore, type HA). The cells were immediately washed on the filter with 5 ml of warm (37 C) medium, and radioactivity of the filters was measured as described above.

The K_m and V_{max} values were calculated by plotting c/V against c, where c is the concentration of the substrate expressed in micromoles per liter and V is the micromoles of ^{14}C-amino acid incorporated per 0.5 min per gram of cells, dry weight (an appropriate blank was made and subtracted for each concentration).

RESULTS

Isolation of mutants. MI183 was isolated after ultraviolet mutagenesis from RFS54 by plating on minimal citrate plates containing thiamine, leucine, and 20 μg of valine per ml. MI183 grew on plates supplemented with thiamine, but did not grow if valine was present (20 μg/ml), unless leucine was also added to the plate. The mutation conferring valine resistance (Val^r) to MI183 will be hereafter called *brnP1*.

MI174 was isolated during an attempt to find a strain resistant to a valine concentration not higher than 50 to 100 μg/ml and at the same time derepressed in the isoleucine-valine biosynthetic enzymes. Strain X478 was mutagenized with nitrosoguanidine and was then diluted 10-fold in minimal salts containing supplements and 20 μg of valine per ml; after 24 hr the suspension was fully grown and a portion of it was again diluted into the same medium. A penicillin counterselection was performed in minimal medium containing all supplements and 300 μg of valine per ml in order to eliminate bacteria resistant to this or a higher concentration of valine. The suspension was plated and some Val^r strains were purified, grown in nutrient broth supplemented with glucose, and assayed for the level of the *ilvA* gene product (threonine deaminase). MI174 was chosen for further study, as it was found derepressed for this enzyme. Resistance to valine inhibition is caused by a mutation which has been called *brnQ2*.

Although all the experiments on the effect of a mutation at the *brnQ* locus reported in this paper have been performed on MI174 and its derivatives, strains that are altered in the same gene have been isolated by the following procedure. MI148a was grown in minimal citrate containing the appropriate supplements, diluted to a concentration of about 100 cells/ml, distributed in tubes containing 1-ml portions, grown overnight, and streaked on minimal plates containing, besides

the supplements needed by MI148a to grow, 50 μg of leucine per ml and 20 μg of valine per ml. Leucine was added so as not to select against mutants that were resistant to valine only if leucine was present. Ninety-four independently isolated mutants were purified and checked for their sensitivity to glycyl-valine; 87% (82:94) were inhibited by the dipeptide, and on this basis they were considered permease mutants (see Table 6); since the *brnP* and *brnQ* loci described in this paper are located near *ara* and *proC*, respectively, we decided to check if these mutants are *brnP* or *brnQ* by transduction; 26 of these strains were treated with P1 phage (grown on a wild type), and *ara*$^+$ or *proC*$^+$ transductants were selected. If the mutation that confers resistance to valine is located in the *brnP* or the *brnQ* locus, about 60% of the *ara*$^+$ transductants or of the *proC*$^+$ transductants, respectively, should become Val^s (see Table 2 and 3). For each strain, 16 *ara*$^+$ transductants and 16 *proC*$^+$ transductants were tested and the results obtained were identical for all of them, i.e., no cotransduction when *ara*$^+$ transductants were selected and roughly 50% cotransduction when *proC*$^+$ transductants were selected. All of these strains needed leucine on the plate in order to express fully their resistance to valine inhibition. Thus all of these 26 strains appear to be altered in the *brnQ* gene.

Genetic characterization of MI183. After a preliminary experiment showing that the Val^r mutation of MI183 is linked to *leu* by P1 transduction (87%), the experiment shown in Table 2 was performed. It can be seen that when P1 grown on MI183 was used to transduce X478 and *ara*$^+$ transductants were selected, the frequency of the unselected *leu*$^+$ and *brnP1* markers shows that this mutation is located on the side of *leu* distal to *ara* (Fig. 1). From the known linkage of *azi* to *leu* (22) we infer that *brnP* is located between *leu* and *azi*. No transductants were found resistant to valine in the absence of leucine. An Ara$^+$, Leu$^+$, Val^r transductant (MI183a) was purified and used for further study. It will be shown later that the *brnP* gene codes for a component of the transport system for isoleucine, leucine, and valine, and for this reason it is possible that transduction frequencies were distorted because some of the *leu* recombinants could not grow on leucine. This possibility could be tested if the transduction in Table 2 was repeated using glycyl-leucine in the place of leucine. Since the dipeptide enters the cell via a transport system(s) different from the one(s) for the amino acid (11), all *leu brnP1* transductants should grow. This transduction was performed, and the transductants were scored as in Table 2 by using, when necessary, not only plates supplemented with leucine but also plates supplemented with glycyl-leucine; results were identical on both types of plates and gave transduction frequencies identical to those of Table 2.

Genetic characterization of MI174. A conjugation experiment between an HfrH and MI174 (F$^-$) indicated that the *brnQ2* mutation is linked to *lac* and that the Val^r phenotype needed leucine to be expressed. Appropriate transductions were performed to check cotransduction of this mutation with several markers; no cotransduction was observed with *proB* (0:47) and linkage to *lac* and *proC* was found. Among the selected *lac*$^+$ transductants a Lac$^+$, Pro$^+$, Val^r transductant was purified (MI174a) and P1 phage was grown on it. This phage was used to transduce MI148a with selection of *lac*$^+$ in the presence of leucine; Table 3 shows that the *brnQ2* mutation of MI174a is located on the side of *proC* distal to *lac*. All *brnQ2* transductants needed leucine to exhibit resistance to valine, thus confirming what had been suggested by the conjugation experiment. A Lac$^+$, Pro$^+$, Val^r transductant (MI174b) was purified and used for the study of its phenotype.

Since the transduction of Table 3 suggests that the *brnQ2* mutation of MI174 is located close to *phoA* (Fig. 1), cotransduction with this marker

TABLE 2. *Transduction of X478 with P1 phage grown on MI183 and selection of ara$^+$ transductants*

leu	*brnP1*	Frequency (%)
−	+	27 (44/160)
+	−	62 (99/160)
+	+	9 (14/160)
−	−	2 (3/160)

P1(MI183)	+	+	−
X478	*ara*	*leu*	*brnP1*
	−	−	+

TABLE 3. *Transduction of MI148a with P1 phage grown on MI174a and selection of lac$^+$ transductants*

proC	*brnQ1*	Frequency (%)
−	+	79.5 (127/160)
+	−	12.5 (20/160)
+	+	8 (13/160)
−	−	0 (0/160)

P1(MI174a)	+	+	−
MI148a	*lac*	*proC*	*brnQ2*
	−	−	+

was tried. Strain U18 (*lac*$^+$, *proC*$^+$, *brnQ*$^+$, *phoA*) was transduced with P1 phage grown on MI174 (*lac*, *proC*, *brnQ2*, *phoA*$^+$), and selection was made for Pho$^+$ transductants as described above; 96% of the Pho$^+$ transductants become Val^r, and the frequency of the other unselected markers suggests that the *brnQ* gene is located between *proC* and *phoA* (Fig. 1), although further experiments should substantiate this gene order.

Level of the isoleucine-valine biosynthetic enzymes. The intracellular concentration of the isoleucine-valine biosynthetic enzymes was measured in MI183a and in MI174b. Threonine deaminase activity was taken as a measure of the derepression of the entire *ilvA*, *D*, *E* operon since evidence for coordinate derepression has been shown in this operon (16).

Table 4 shows that MI183a grown in minimal medium plus the required supplements (Table 1) had a wild-type level of the isoleucine-valine biosynthetic enzymes and, although a control experiment with MI148a (Val^s) could not be performed, these enzymes were not dramatically derepressed when valine and leucine were added to the other supplements. Threonine deaminase appeared to be slightly derepressed in the presence of valine and leucine; this is to be attributed to the presence of leucine with no isoleucine and is not a consequence of the *brnP1* mutation. In fact, in agreement with Rogerson and Freundlich (17), we usually observe a two- to threefold derepression of threonine deaminase in a wild-type strain or even in an *ilvO* strain, when leucine is added to a minimal medium. In conclusion, the resistance to valine of this strain cannot be explained with an increased rate of isoleucine biosynthesis.

Table 4 also shows the level of the isoleucine-valine biosynthetic enzymes when MI183a is grown in medium supplemented with isoleucine, leucine, and valine. Repression of the *ilvB* enzyme was not as efficient as in the parental strain.

Table 5 shows that for MI174b also the resistance to valine cannot be explained in terms of increased rate of isoleucine biosynthesis due to derepression of biosynthetic enzymes in minimal medium or minimal medium plus valine and leucine. Here again when richer media were used the level of biosynthetic enzymes was not repressed as efficiently as in the parental strain. The level of the *ilvB* and *ilvC* gene products, when MI174b was grown in minimal medium supplemented with isoleucine, leucine, and valine, was threefold higher than in the parental strain, whereas the level of the *ilvA* and *ilvB* gene products was 18-fold (or more) higher, or fivefold higher, respectively, in MI174b grown in nutrient broth supplemented with glucose than the level in the parental strain. (A high level of the *ilvA* gene product is to be expected in this case because this strain was chosen according to this criterion among the strains resistant to valine which were isolated,)

From the experiments in Table 4 and 5 we conclude that it is unlikely that the *brnP1* and *brnQ2* mutations cause resistance to valine by means of an increased rate of isoleucine biosynthesis.

TABLE 4. *Specific activity of the isoleucine and valine biosynthetic enzymes in MI183a as compared with the parental strain MI148*

Conditions[a]	Gene product		
	ilvA	*ilvB*	*ilvC*
MI148			
Minimal[b]	58[c]	21	6.2
Minimal + isoleucine, leucine, valine	23.9	2.55	7.0
Nutrient broth + glucose	<2.8	2.1	4.2
MI183a			
Minimal	59	36	6.6
Minimal + valine, leucine	95.5	5.3	5.9
Minimal + isoleucine, leucine, valine	34.4	11.2	6.3
Nutrient broth + glucose	<2.8	2.1	4.8

[a] Concentrations of isoleucine, leucine, and valine were 50, 50, and 100 μg/ml, respectively.

[b] This strain requires leucine for growth; therefore leucine was one of the supplements.

[c] Expressed as nanomoles of product formed per minute per milligram of protein.

TABLE 5. *Specific activity of the isoleucine and valine biosynthetic enzymes in MI174b as compared with the parental strain MI148a*

Conditions[a]	Gene product		
	ilvA	*ilvB*	*ilvC*
MI148a			
Minimal	89[b]	12.5	8.7
Minimal + isoleucine, leucine, valine	22	2.6	3.5
Nutrient broth + glucose	>2.8	3.8	3.5
MI174b			
Minimal	58	8.9	6.7
Minimal + valine, leucine	154	12	18.9
Minimal + isoleucine, leucine, valine	13	7.3	12.2
Nutrient broth + glucose	50	19.8	2

[a] Concentrations of L-isoleucine, L-valine, and L-leucine were 50, 100, and 50 μg/ml, respectively.

[b] Expressed as nanomoles of product formed per minute per milligram of protein.

Active transport of the branched-chain amino acids. Two types of Val^r mutants of *E. coli* K-12 are described in the literature: *ilvB* mutants, which have a valine resistant synthetase (9), and *ilvO*, *ilvP* (16), or *valS* mutants (Williams and Freundlich, Bacteriol. Proc., p. 135, 1970), which are derepressed for the isoleucine-valine biosynthetic enzymes. Due to the chromosomal location of the mutation, MI183 and MI174 cannot be altered in any of these loci. Moreover, the resistance to valine does not appear to be a consequence of an increased rate of isoleucine biosynthesis in minimal medium (Table 4 and 5), and therefore a regulatory mutation is unlikely. We decided, therefore, to test whether these mutants were altered in the active transport of valine. This could be tested because dipeptides enter *E. coli* via a transport system(s) different from those for the amino acids (11) and are subsequently cleaved by intracellular peptidases; therefore, while the *ilvB*, *ilvO*, *ilvP*, or *valS* mutants should be resistant both to valine and to a dipeptide containing valine, a transport mutant should be resistant to valine but sensitive to a dipeptide containing this amino acid. Table 6 shows that MI174b and MI183a were resistant to valine (in the presence of leucine) but sensitive to dipeptides containing valine; AB1264, which is resistant to valine because of a mutation causing feedback resistance in the *ilvB* enzyme, was resistant in all cases, whereas a wild-type strain, X478, was sensitive in all cases. Table 6 also shows that leucine was necessary for the expression of the resistance to valine and that it could not be replaced by leucylhistidine. Thus MI174b and MI183a appear to be altered in the transport system for isoleucine, leucine, and valine in such a way that valine uptake in the presence of leucine is not sufficient to inhibit isoleucine biosynthesis.

A single transport system for isoleucine, leucine, and valine in *E. coli* K-12 has been described (14). We studied this system in the two mutants in the following ways: (i) uptake of valine vs. time, (ii) uptake of valine in the presence of leucine with concomitant measurement of incorporation of valine into proteins, and (iii) measurement of the apparent K_m and V_{max} of the branched-chain amino acids for the transport system.

Figure 2 shows that the initial rate of uptake of 2×10^{-5} M valine vs. time was slightly lower in MI183a than it was in the parental strain, whereas it was about 13-fold lower in MI174b. Since the mutant strains were only Val^r when leucine was present, the uptake was also measured in the presence of this amino acid. Figure 3A shows that in the parental strain the uptake of 2×10^{-5} M valine was reduced at 0.5 min about 20-fold by the presence of 4×10^{-4} M leucine (note the different scale of ordinates); a

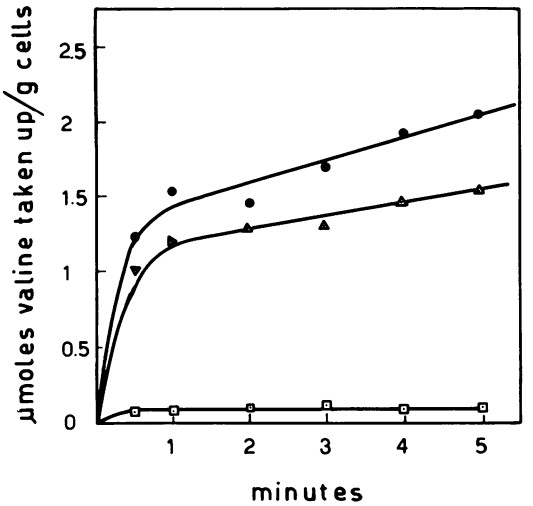

FIG. 2. *Uptake of 2×10^{-5} M ^{14}C-valine, vs. time in MI148a (●), MI183a (△), and MI174b (□). Specific activity was 1.5×10^6 counts per min per μmole (counting efficiency about 80%). For each experimental point 0.81 mg of bacteria (MI148a) or 0.82 mg (MI183a) or 0.9 mg (MI174b) was used.*

TABLE 6. *Effect of valine, leucine, and some dipeptides containing valine or leucine on the growth of several strains*[a]

Strain	Substance tested						
	Valine	Valine and leucine	Valyl-leucine	Leucyl valine	Leucyl-histidine and valine	Leucine, histidine, and valine	Glycyl-valine and leucine
X478	−	−	−	−	−	−	−
AB1264	+	+	+	+	+	+	+
MI174b	±[b]	+	−	−	−	+	−
MI183a	−	+	−	−	−	+	−

[a] Substances were pipetted on paper circles applied on plates. Inhibition (−), no inhibition (+).
[b] Some slow growth is usually observed for MI174b in the absence of leucine.

similar reduction was observed in the two mutants. The most striking alteration in MI183a (Fig. 3B) was not in the total uptake, which was slightly lower than that of the parental strain (similarly to the observations in Fig. 2), but in the incorporation into proteins, which was not detectable even after 5 min of incubation. The consistent incorporation observed in total cells (60% of the parental strain at 1 min of incubation) was all acid soluble. Therefore, MI183a appears to be Val^r, because exogenous valine cannot enter the pool of valine. It is possible that the primary alteration in this mutant is not in transport, but at another level [e.g., regulation of pool(s) size] and that the observed alterations of transport are secondary. Figure 3C shows the uptake of valine by MI174b. As discussed above,

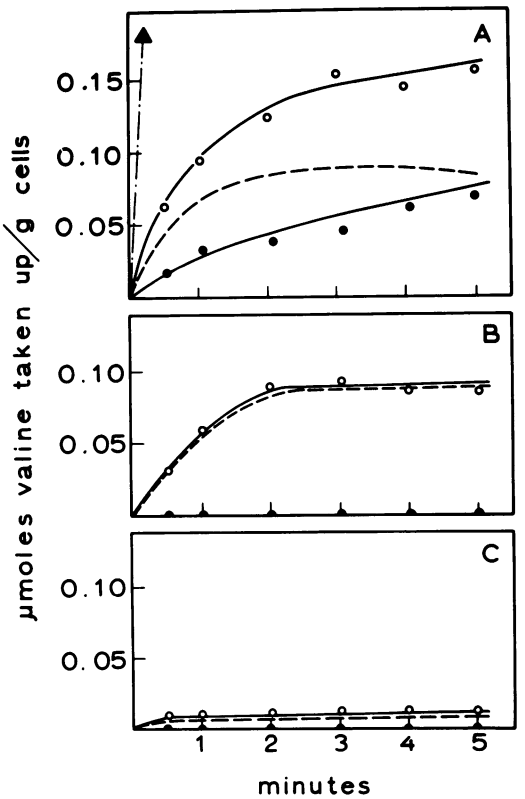

FIG. 3. *Uptake of 2 × 10^{-5} M ^{14}C-valine in the presence of 4 × 10^{-4} leucine in MI148a (A), MI183a (B), and MI174b (C). For comparison, A shows uptake of 2 × 10^{-5} M ^{14}C-valine by MI148a from Fig. 2 (broken line). Specific activity was 1.5 × 10^6 counts per min per μmole (counting efficiency 80%). For each experimental point 0.9 mg of bacteria (MI148a and MI183a) or 1.1 mg (MI174b) was used. Symbols:* (○) *total uptake and* (●) *trichloracetic acid-precipitable counts per min. Broken-line curves are calculated by difference and represent acid-soluble pool.*

the subtracted blank was probably lower than the real one and there was probably no valine uptake under these conditions. From this experiment we conclude that MI183a and MI174b are resistant to valine inhibition because of an alteration in the transport system for isoleucine, leucine, and valine; they need leucine to express the resistance to valine because leucine inhibits valine uptake by competing for the same transport system.

It has been reported (12) that the transport of leucine is repressed by the presence of this amino acid in the medium. It is possible, therefore, that the presence of leucine in the medium was necessary for the expression of valine resistance not only for inhibition of valine uptake, but also for repression of the transport system, which would lower valine uptake even further. We tested this possibility by inoculating MI183a and MI174b in minimal medium or in minimal medium plus 50 μg of leucine per ml; when a cell concentration of about 2 × 10^8 was reached, leucine plus 100 μg of valine per ml, or valine alone when bacteria had grown in leucine, was added. This concentration of valine inhibited the growth rate of the mutants; therefore, if growth in the presence of leucine reduced valine uptake, bacteria pregrown in the presence of leucine should, upon addition of valine, grow faster than those pregrown in the absence of leucine. That this was not true is shown in Fig. 4. Valine inhibits to the same degree the growth rate of MI183a grown in the presence or in the absence of leucine. Inhibition of MI174b was even greater if cells had been previously grown in leucine than if provided leucine at the time of valine addition, owing to the lower concentration of leucine that resulted from the partial consumption of the amino acid during bacterial growth. In fact, when bacteria which had previously grown in leucine were given not only valine but also the amount of leucine that was anticipated to have been consumed no difference in inhibition was seen (Fig. 4).

The *brnP1* and *brnQ2* mutations do not abolish completely the transport of valine (Fig. 2); an indirect way of testing in vivo the transport rate of all branched-chain amino acids is to prepare a strain containing one of the transport mutations together with a mutation causing requirement for isoleucine, valine, or leucine. If the transport rate is severely impaired, these strains should not grow or should grow very slowly. The *leuB* mutation was introduced by cotransduction with *ara*; the *ilvA* or *ilv* mutations were introduced by cotransduction with *metE*. Transductants were isolated on the dipeptides glycyl-leucine, glycyl-isoleucine, and glycyl-valine in place of the corresponding amino acids and then the growth rate was measured on isoleucine (for the

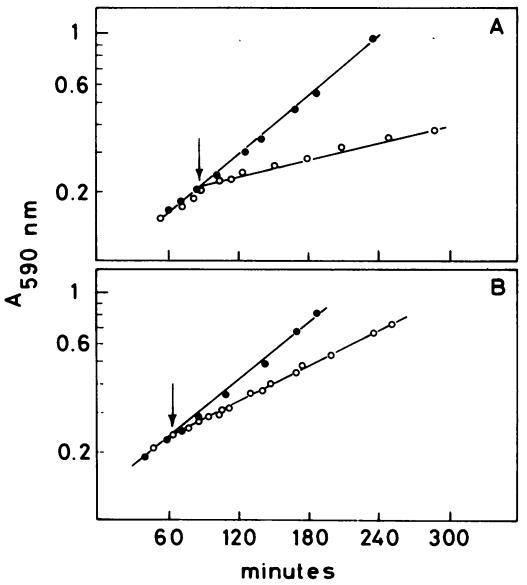

FIG. 4. *Valine inhibition of the growth rate of MI183a (A) and MI174b (B) pre-grown in the presence or absence of leucine. Supplemented minimal medium was inoculated with bacteria with or without 50 μg of leucine per ml (no difference in growth rate was observed and for this reason only one curve is reported), and at the time indicated by the arrow either valine or leucine and valine was added. Symbols, A: (●) MI183a with or without leucine and (○) same with addition of valine (100 μg/ml) to bacteria previously grown in leucine or addition of leucine and valine (50 and 100 μg/ml, respectively) to bacteria previously grown without leucine; B: (●) MI174b with or without leucine and (○) same with addition of leucine (10 μg/ml) and valine (100 μg/ml) to bacteria previously grown in leucine or of leucine (50 μg/ml) and valine (100 μg/ml) to bacteria previously grown without leucine.*

brnP1, *ilvA* and the *brnQ2*, *ilvA* double mutants) or on glycyl-isoleucine and valine (for the *brnP1*, *ilv* and the *brnQ2*, *ilv* double mutants) or on leucine (for the *brnP1*, *leu* double mutant). The *brnQ2* mutation had been originally isolated in a *leuB* strain (MI174), and for this reason the *brnQ2*, *leu* double mutant was not prepared. The growth rate of the *brnQ2*, *ilv* double mutant was normal (about 60-min duplication time, as MI148a) in minimal medium supplemented with glycyl-isoleucine and glycyl-valine (10^{-3} M), but it became slower (120-min duplication time) when glycyl-valine was replaced with an equimolar amount of valine. The growth rate of all other double mutants was normal, thus showing that the transport of isoleucine, valine, and leucine was still active enough to permit a normal growth rate.

The apparent K_m and V_{max} of the branched-chain amino acids was measured in chloramphenicol-treated cells. Under these conditions measurement of the rate of uptake was not complicated by the incorporation of the amino acid into proteins as it was in the experiments represented in Fig. 2 and 3. In Table 7 are reported the K_m and V_{max} values of isoleucine, leucine, and valine for the transport system of the parental strain MI148a and of the mutant strains MI183a and MI174b; values were obtained from plots analogous to the one in Fig. 5. The most striking alteration found in MI183a was in the apparent K_m for leucine transport which was 17-fold lower, whereas the apparent K_m for isoleucine transport was 7-fold higher than that found in the parent strain.

In MI174b the apparent K_m values of the transport system for isoleucine, leucine, and valine were not altered, but the V_{max} values were all lower than the corresponding values of the parental strain, valine being altered maximally.

It must be pointed out that *E. coli* K-12 might possess more than one transport system for branched-chain amino acids (4, 14) and that the variations in K_m and V_{max} values reported in Table 7 might result from a defect in a specific transport system.

As MI183a is altered in the K_m for isoleucine and leucine transport but not in either V_{max} or K_m for valine transport, we assume that this strain is specifically altered in the transport system for isoleucine, leucine, and valine; the same conclusion cannot be drawn from MI174b, and for this reason the uptake of other amino acids was measured. Table 8 shows that the uptake of histidine, threonine, aspartic acid, gly-

TABLE 7. *Apparent K_m and V_{max} of the branched-chain amino acids for the transport system in MI148a, MI183a, and MI174b*

Strain	Amino acid	K_m (micromolar)	V_{max}[a]
MI148a	Isoleucine	4.8	0.9
	Leucine	7	0.5
	Valine	12	1.0
MI183a	Isoleucine	36 (7.5)[b]	1.6 (1.8)
	Leucine	0.4 (0.06)	0.14 (0.3)
	Valine	12.5 (1)	1.1 (1.1)
MI174b	Isoleucine	8 (1.7)	0.14 (0.15)
	Leucine	8 (1.1)	0.10 (0.2)
	Valine	21 (1.4)	0.10 (0.05)

[a] Expressed as micromoles per 0.5 minutes per gram of cells (dry weight).

[b] Values in parentheses represent fold increase (or decrease) over the corresponding values of the parental strain MI148a.

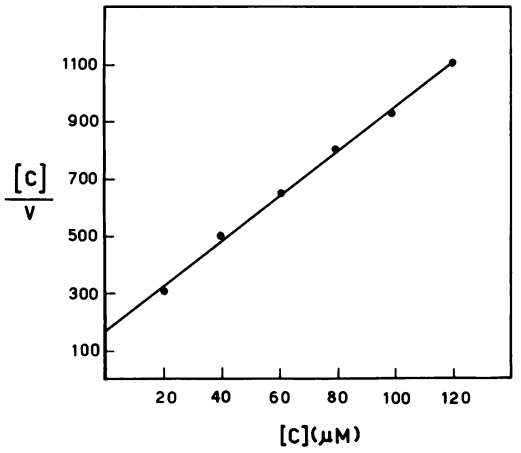

FIG. 5. *Dependence of the rate of uptake in MI174b upon the concentration of valine. Plot of C/V vs. (C), where (C) is the μM concentration of valine and V is the rate of uptake [micromoles per 0.5 minute per gram of cells (dry weight)]. Values are average of two determinations. K_m is 21 μM and V_{max} is 0.1 μmole per 0.5 min per g. Specific activity was 1.5 × 10^6 counts per min per μmole (counting efficiency 80%).*

TABLE 8. *Uptake of several amino acids in MI148a and MI174b*[a]

Amino acid	MI148a	MI174b
Isoleucine	0.6	0.15 (0.25)[b]
Leucine	0.5	0.1 (0.2)
Valine	1.42	0.13 (0.09)
Arginine	0.57	1.2 (2)
Aspartic acid	0.34	0.7 (2)
Glycine	0.1	0.16 (1.6)
Histidine	0.65	0.51 (0.8)
Threonine	2.1	2.3 (1.1)

[a] Uptake (micromoles of ^{14}C-amino acid incorporated per gram of cells) was measured at 5 min in the presence of chloramphenicol; concentration of amino acids was 2 × 10^{-5} M.

[b] Values in parentheses represent the fold increase (or decrease) over the corresponding values of the parental strain, MI148a.

cine, and arginine was not altered in MI174b under conditions in which the uptake of isoleucine, leucine, and valine was altered. A similar control experiment has shown that in MI183a, under the same conditions (under which uptake of isoleucine, leucine, and valine was normal), the uptake of other amino acids was not altered.

Analogues of branched-chain amino acids. Some analogues of the branched-chain amino acids are available, and their effect on the growth of *E. coli* is known. The availability of mutants altered in the transport system for the branched-chain amino acids gave an opportunity to test whether these analogues are concentrated by *E. coli* through this transport system. Results of these experiments are reported in Table 9. *O*-methyl-threonine has been reported to inhibit the growth of *E. coli* 15; this inhibition probably is due to incorporation into proteins, because formation of *O*-methyl-threonyl-transfer ribonucleic acid $(tRNA)^{Ile}$ has been reported in *E. coli* 15 (18) and also in *E. coli* K-12 (24). MI148a was inhibited by *O*-methyl-threonine and this inhibition was overcome by isoleucine or leucine. Whereas isoleucine overcame the inhibition probably because of its interaction with $tRNA^{Ile}$, evidence has been reported that leucine acts at the transport level (18); that this is true for *E. coli* K-12 is shown by the resistance of MI174b to *O*-methyl-threonine inhibition. MI183a was sensitive, which probably means that *O*-methyl-threonine uptake, although slower, was still sufficient to inhibit growth.

Thiaisoleucine inhibits the growth of *E. coli* K-12 and isoleucine overcomes this inhibition (20); MI174b and MI183a are not resistant to thiaisoleucine even in the presence of leucine, suggesting that thiaisoleucine does not enter *E. coli* through the transport system for the branched-chain amino acids. There is evidence that thiaisoleucine, although it behaves as an isoleucine analogue for isoleucyl-tRNA synthetase (20, 24), is on the contrary a threonine analogue for threonine deaminase (F. Cervone and M. Iaccarino, *unpublished data*); therefore, it is possible that thiaisoleucine could enter *E. coli* through the transport system for threonine. In agreement with this possibility is the fact that threonine did prevent thiaisoleucine inhibition and an equimolar amount of glycyl-threonine was less effective.

DISCUSSION

It has been reported (14) that branched-chain amino acids enter *E. coli* through a common transport system which appears to be altered in

TABLE 9. *Effect of O-methyl-threonine and thiaisoleucine on the growth of MI148a, MI174b, and MI183a*[a]

Substance	MI148a	MI174b	MI183a
O-methyl-threonine	−	+	−
O-methyl-threonine and isoleucine	+	+	+
O-methyl-threonine and leucine	+	+	+
Thiaisoleucine	−	−	−
Thiaisoleucine and isoleucine	+	+	+
Thiaisoleucine and leucine	−	−	−
Thiaisoleucine and threonine[b]	+	+	+
Thiaisoleucine and glycyl-threonine	∓	∓	∓

[a] Tests were performed as described in Materials and Methods. Inhibition (−), no inhibition (+).

[b] Threonine (500 μg) or an equimolar amount of glycyl-threonine was applied on the paper disc.

the mutants described in this report because of (i) sensitivity to dipeptides containing valine (Table 6); (ii) altered uptake of valine (Fig. 2 and 3); and (iii) altered kinetics of transport (Table 7). The mutations described do not completely abolish the transport, because (i) leucine is needed for expression of the Val^r phenotype; (ii) an *ilv* mutation or a *leu* mutation can be introduced into strains carrying either the *brnP1* mutation or the *brnQ2* mutation. The behavior of these mutants (no uptake of valine when leucine is present, but unimpaired uptake of dipeptides) suggests that mutations affecting more severely the transport system might be isolated if selection were made for Leu$^-$ Val^r strains which grow on a dipeptide-containing leucine. On the other hand, it is possible that, in addition to the transport system for all branched-chain amino acids, there are other, more specific transport systems for each branched-chain amino acid or for a combination of them (4, 14); the existence of such transport systems would make the suggested isolation procedure impossible and would also explain, at least in part, the "leakiness" of the mutations found.

The active transport of branched-chain amino acids requires the integrity of at least two genes; it is possible that the product of one of these two genes is the leucine-binding protein (2, 12) and that the product of the other gene might be analogous to the *hisP* protein of the histidine transport in *Salmonella typhimurium;* in this case it has been shown that transport requires the integrity of the *hisJ* and *hisP* genes (1); these two genes are located very close on the chromosome, and their activity appears to be regulated by an operator gene, *dhuA*.

The *brnP* gene shows 87% cotransduction with *leuB;* because the gene order (22) is *leuB*, *leuA*, *brnP*, the *brnP* gene might be part of the *leu* operon. The level of leucine-binding protein has been shown to be regulated by the presence of leucine in the medium (12); if the *brnP* gene codes for the leucine-binding protein, leucine might regulate it through regulation of the entire *leu* operon. Experiments to clarify this question are in progress in this laboratory.

It is interesting to note that both *brnP* and *brnQ* genes are located in regions of the bacterial chromosomes which, it has been suggested (22), contain genes related to the structure and function of the cell membrane; *brnP* is close to the *mut T-azi-ace* region, and *brnQ* is close to the *phoA-tsx-lon* region.

Table 4 and 5 show lack of repression of the *ilvB* gene product in MI183a and of the *ilvB* and *ilvC* gene products in MI174b (data for minimal medium supplemented with isoleucine, leucine, and valine); although we do not exclude a possible correlation between transport of branched-chain amino acids and regulation of their biosynthesis, the simplest hypothesis that can be made is that the intracellular concentration of the branched-chain amino acids in the two mutants is lower than that of the parental strain because the transport of these amino acids is altered. Factors other than lack of repression also might be involved; indeed, when an *ilv* mutant was grown in minimal medium containing 50 μg of isoleucine per ml and different concentrations of valine, up to sixfold derepression of the *ilvB* enzyme was observed when the concentration of valine was lowered from 100 μg/ml to 50 μg/ml (M. Iaccarino, *unpublished data*). The lack of repression of *ilvA* and *ilvB* enzymes when MI174b was grown in very rich medium (Table 5) can be interpreted similarly. In this case, there might have been a relative starvation for isoleucine, leucine, and valine owing to the altered transport system and the demand of the higher growth rate.

ADDENDUM IN PROOF

Data of Table 3, together with an experiment in which cotransduction with *phoA* was 96% for *brnQ2*, 21% for *proC*, and 17% for *lac*, support the gene order *lac proC phoA* in our strains. However, a paper recently appeared (E. Yagil, M. Bracha, and N. Silberstein, Mol. Gen. Genet. **109**:18-26, 1971) in which evidence is given for the gene order *lac phoA proC*.

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