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Escherichia coli Transport Mutants Lacking Binding Protein and Other Components of the Branched-Chain Amino Acid Transport Systems

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Further evidence on the role of binding proteins in branched-chain amino acid transport in *Escherichia coli* was obtained by selecting mutants with altered expression of the binding proteins. The mutator phage Mu was used to induce *E. coli* L-valine-resistant mutants defective in branched-chain amino acid transport. By making use of mild selective conditions and strain backgrounds with derepressed high-affinity, binding protein-mediated transport systems, we were able to isolate a new class of transport mutants defective in these systems. Mutant strains AE84084 (*livK*::Mu*cts*) and AE840102 (*livJ*) were found to be defective in leucine-specific and LIV binding proteins, respectively, by transport assay, in vitro binding activity, and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Mutant strain AE4107 (*livH*::Mu), although lacking high-affinity, branched-chain amino acid transport, retained functional binding proteins and therefore evidently codes for an additional component of high-affinity transport. The *livH*, *livJ*, and *livK* mutations were mapped by transduction and shown to be closely linked to each other in the *malT* region (min 74) of the *E. coli* genetic map.

Escherichia coli possesses multiple transport systems for the branched-chain amino acids L-leucine, L-valine, and L-isoleucine (7, 19, 21). The high-affinity systems (LIV-I and leucine specific) are lost upon osmotic shock treatment in parallel with their respective binding proteins. Two regulatory loci on the *E. coli* chromosome have been identified, *livR* and *lstR*, which affect the LIV-I- and leucine-specific systems, respectively (1). In addition, a low-affinity transport system, LIV-II, serves for L-leucine, L-isoleucine, and L-valine and is not sensitive to osmotic shock. Branched-chain amino acid uptake systems with even lower affinities have also been reported, although they have not been well characterized (7, 21). We have been applying genetic approaches to analyze the individual contributions and properties of each system without interference from the others. We previously combined genetic approaches with biochemical approaches to determine the multiplicity of systems serving for leucine entry in *E. coli* and to obtain a mutant strain with a defective leucine-specific binding protein (19). In this report, we describe selection procedures that enabled us to identify a region of the chromosome specifying the expression of the binding proteins and additional elements of the high-affinity, branched-chain amino acid transport systems.

(A preliminary report of portions of this work was presented at the 1976 meeting of the American Society of Biological Chemists, San Francisco.)

MATERIALS AND METHODS

Bacteria and phage. The bacterial strains used were all derivatives of *E. coli* K-12 and are listed in Table 1. Mu*cts*62 and wild-type Mu bacteriophage were gifts of A. I. Bukhari and K. B. Low, respectively. Mu*cts*62 is a thermoinducible phage strain that kills its lysogenic host at 42°C but not at 32°C (11).

Materials. Morpholinopropane sulfonic acid and nutritional supplements were obtained from Sigma Chemical Co., St. Louis, Mo. Reagents for sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis were obtained from Eastman Organic Chemicals, Rochester, N.Y.

Media. Minimal medium for growth of cultures used in transport assays was basal morpholinopropane sulfonic acid-salts medium (16) supplemented with 0.2% glucose and 50 μg of the required amino acids per ml except for L-leucine, which was used at 25 μg/ml. Thymine was present at 50 μg/ml, and various vitamins were present at 1 μg/ml. Luria broth without glucose (15) and supplemented with 50 μg of thymine per ml (LBT broth) was used for routine growth of strains and, when solidified with 1.5% agar, was used for phage titering and lysate preparation. Plates for selecting recombinant colonies contained 1.5% agar, based on half-strength medium 56 (14), and were supplemented with nutri-

ents at the concentrations listed above for liquid media.

Genetic crosses. All transductions were carried out with lysates of phage P1CM*clr*100, as described previously (1). Transport phenotypes were always treated as unselected characters since direct selection was unreliable, as reported previously (1). D-Leucine utilization (Dlu$^+$) was scored as the ability of a *leu* strain to grow on plates supplemented with 100 μg of D-leucine per ml. L-Valine resistance was scored either by radial streak against a sterile filter disk containng 0.5 mg of L-valine or, more commonly, by direct assay for L-valine transport by the rapid transport assay (see below).

Mu phage procedures. Wild type Mu-1 phage (here designated simply as Mu) lysates were prepared on sensitive strains from single plaques generated by phage released spontaneously from Mu-1 lysogens. The soft-agar overlay procedure was used (3). Lysates of the thermoinducible phage Mu*cts*62 were prepared from lysogens by the heat shock method of Howe (11). Titers of both phages averaged 10^{10} plaque-forming units per ml.

Mu mutagenesis. The mutator phage Mu causes mutations in *E. coli* by disrupting the primary sequence of the gene into which the phage deoxyribonucleic acid inserts itself, a process that is apparently random with regard to site and is concomitant with lysogeny (12). We chose Mu phage as a mutagen primarily to increase the probability of detecting binding protein mutants by loss of cross-reacting material (CRM) in our immunochemical assay (see below). Mu insertions have a strongly polar effect (12), which not only would be expected to disrupt the primary sequences of the polypeptide product of the mutated gene (and thus alter immunological cross-reactivity), but also would prevent synthesis of promoter-distal gene products. In this way, some Mu insertions may be expected to alter the expression of the structural genes for the binding proteins. In addition, since transport phenotypes in strains with multiple and overlapping systems are difficult to screen in genetic crosses, in favorable cases the Mu insertion itself can be mapped instead (see Results). Cultures of *E. coli* were mutagenized by growing them to a density of 2×10^8 cells per ml in LBT broth supplemented with 0.01 M $MgSO_4$ and adding Mu or Mu*cts* to a multiplicity of infection of 1.0. After lysis at the appropriate temperature (37°C for Mu; 30°C for Mu*cts*), the cultures were allowed to grow overnight to increase the proportion of lysogens among the survivors (12). For Mu*cts*-infected cultures, over 90% of the survivors were Mu*cts* lysogens as demonstrated by their inability to form colonies at 42°C. The presence of the wild-type Mu in an individual strain was determined by resistance to Mu*cts* when an exponentially growing culture of the unknown was cross-streaked against a Mu*cts* lysate at 42°C. Also, putative Mu and Mu*cts* lysogens were scored for phage release (3) by patching a sample of a colony to be tested on a soft-agar (0.7%) overlay containing 10^8 Mu-sensitive cells and scoring for zones of lysis after overnight incubation at 37°C.

Assay of transport and binding activities. Routine transport assays of the indicated amino acids were carried out as described previously (1). Amino acid binding activities of osmotic shock fluids of cultures were determined by equilibrium dialysis as described previously (1). The rapid transport assay, used to screen large numbers of recombinants for transport characteristics, was a slight modification of that reported previously (1). A mixture of L-

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

Strain	Relevant genotype	Source
Hfr "C" U482	*asd-1*	CGSC[a]
AB2847	*aroB351 mal-354*	CGSC
EO300	Wild type	Rahmanian et al. (19)
EO321	*lstR liv-321*	Rahmanian et al. (19)
AE41	*lstR leu-6 malA1 xyl-7 mtl-2 argG6 his-1 trp-31 str-104 nal*	Anderson et al. (1)
AE4107	*livH*::Mu,[b] otherwise as AE41	This report
AE62	*leu*$^+$ *lstR*$^+$, otherwise as AE41	Anderson et al. (1)
AE84	*lstR*$^+$ *livR pdxC3*, otherwise as AE41	*pdxC livR aroA*$^+$ transductant of AE49 (Anderson et al.) (1)
AE84084	*livK*::Mu*cts*62, otherwise as AE84	This report
AE840102	*livJ* Mu*cts*62 in unknown location, otherwise as AE84	This report
AE89	*livH*::Mu *mal*$^+$, otherwise as AE62	*mal*$^+$ transductant of AE62 from donor AE91
AE90	*livH*$^+$ *mal*$^+$, otherwise as AE62	*mal*$^+$ transductant of AE62 from donor AE91
AE91	*mal*$^+$, otherwise as AE4107	*mal*$^+$ transductant of AE4107 from donor EO300
AE93	*mal*$^+$ *livH*::Mu, otherwise as AE84	*mal*$^+$ transductant of AE84 from donor AE91
AE99	*xyl*$^+$ *malA1 livJ* Mu$^-$, otherwise as AE840102	Mu$^-$ *xyl*$^+$ recombinant of a conjugation between Hfr KL14 and F$^-$ AE840102
AE105	*mal*$^+$, otherwise as AE99	*mal*$^+$ transductant of AE99 from donor EO300
AE116	*aroB351 livH*::Mu, otherwise as AE4107	*mal*$^+$ transductant of AE4107 from donor AB2847

[a] CGSC, *E. coli* Genetic Stock Center.

[b] The symbol :: refers to the locus of a Mu phage (12).

[^{3}H]valine (0.5 μM final concentration) and L-[^{14}C]proline (1 μM final concentration) was used in the assay system. Simultaneous uptake measurements of the noncompeting L-proline served as an internal control for the extent of cell growth of the individual cultures tested and also, where appropriate, as a screen for nonspecific transport mutants. The individual radioisotopes were discriminated in a Packard Tri-Carb scintillation spectrometer, with a standard toluene-based fluor.

Immunochemical test for CRM. Mutant strains were crudely scored for a normal complement of LIV- and leucine-specific binding proteins by visual determination of CRM as follows. Cultures (10 ml each) of cells were grown overnight in morpholinopropane sulfonic acid-salts medium supplemented with 0.2% glucose and other required growth factors. From each culture, 6 ml was rapidly sedimented in an Eppendorf microcentrifuge (model 3200) with standard conical 2-ml centrifuge tubes. The cell pellets were suspended in 0.1 ml of 0.01 M potassium phosphate buffer (pH 7.2), and 0.01 ml of a 1:1 mixture of $CHCl_3$-toluene was added. The samples were sonically oscillated for 5 s at a setting of 5 (sonifier cell disruptor, model W185, Heat Systems-Ultrasonics Inc., Plain View, N.Y.) with a microtip probe. This treatment did not result in obvious lysis of the cells. After 2 min of centrifugation, 0.025-ml amounts of the supernatant liquids were added to the outer radial wells of a conventional Ouchterlony double-diffusion agar plate. The central well contained 0.025 ml of control serum or serum from rabbits immunized against purified LIV binding protein (18). After overnight incubation at 4°C, the plates were inspected for the presence of a precipitin line between antisera and sample wells. A control sample of wild-type *E. coli* grown in the presence of 30 μg of L-leucine per ml (which represses synthesis of the LIV- and leucine-specific binding proteins) showed either no precipitin line or a very faint one. Cells grown without L-leucine showed a single intense precipitin line. Since this antiserum cross-reacts with both LIV- and leucine-specific binding proteins, the test can detect only highly significant changes in the amounts of CRM for each.

SDS-acrylamide gel electrophoresis. Slab gels containing 11% acrylamide and 0.1% SDS were prepared by the method of Laemmli (13). The gels were 1.5 mm thick. Samples of concentrated shock fluids were prepared in 1% SDS by heating at 100°C for 2 min. After electrophoresis, the slabs were fixed, stained and destained by the method of Fairbanks et al. (5), dried on filter paper under vacuum, and photographed.

Selection of L-valine-resistant mutants. We took advantage of the sensitivity of wild-type *E. coli* K-12 strains to L-valine to select transport mutants defective in branched-chain amino acid transport. Similar approaches have been used by other investigators (8, 9). However, to obtain a new class of transport mutants with lesions in the binding proteins, we employed a combination of hypersensitive strains and extremely mild selective conditions. Since this strategy also increases the yield of nontransport mutants identified with valine resistance (6), enrichment and screening procedures were employed to enrich for mutants with specific defects in high-affinity transport.

L-Valine-resistant mutants were selected in two strain backgrounds (Table 1; Fig. 1). Strain AE41 (*leu lstR*) has higher levels of both high- and low-affinity transport systems (1; unpublished data) and is, therefore, more sensitive to L-valine than its parent, strain AE40, or the EO300 strains used in earlier studies (19). Strain AE41 can utilize D-leucine, a property conferred on it by the *lstR* allele since D-leucine transport is derepressed (1). Lysogenic survivors of Mu infection were plated on 0.5 μg of L-valine per ml in the presence of 10 μg of L-leucyl-L-alanine per ml as a source of leucine without competing for L-valine entry (9). Six percent of the valine-resistant colonies recovered had also lost the ability to utilize D-leucine and were defective for the uptake of L-valine and L-leucine. One of these, strain AE4107, was identified as a Mu lysogen and its characteristics are described herein.

A second strain background used in this study was strain AE84 (*livR leu*$^+$), which is also hypersensitive to L-valine since it is derepressed for the LIV-I high-affinity uptake system and related binding protein (1). To minimize the selection pressure and insure the survival of possible transport mutants that are only partially resistant to valine, a plate concentration of 0.05 μg of L-valine per ml was used, which severely retarded colony formation in this strain but not in the isogenic *livR*$^+$ parent. Initial studies showed that Mu*cts*-lysogenized cultures of strain AE84 yielded prodigious numbers of colonies able to grow on low levels of L-valine, and greater than 90% of these showed zones of cross-feeding or otherwise protected microcolonies of nonmutant cells. We assumed that this phenomenon was due to alterations in branched-chain amino acid biosynthesis rather than uptake; hence, we included an enrichment step. Since glycyl-L-valine enters the cell through the dipeptide transport system rather than through the free amino acid uptake systems, a mutant defective only in L-valine transport will still be sensitive to glycyl-L-valine, but mutants resistant to L-valine by virtue of derepressed levels of

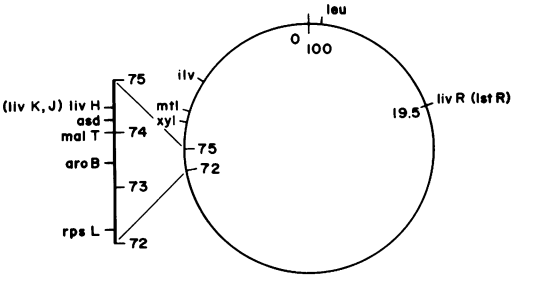

FIG. 1. *Simplified map of the E. coli chromosome showing positions of genes relevant to this study. Genetic symbols are those of Bachmann et al. (2). The positions of livH, livJ, and livK were determined from this study. The locus rpsL has previously been listed as strA (2).*

biosynthetic enzymes will remain resistant to the peptide (9). Accordingly, we grew cultures of the Mucts lysogens of AE84 at 30°C in minimal medium and added glycyl-L-valine (5 μg/ml) to logarithmically growing cultures. After 1 h, during which the growth rate of the culture (monitored by absorbance readings at 420 nm) declined to zero, 20 μg of ampicillin (Wyeth) per ml was added, and incubation continued for 3 h. A crystal of penicillinase was added, and the culture was washed once by centrifugation and then plated on 0.05 μg of L-valine per ml. Such enriched cultures of Mucts lysogens yielded L-valine-resistant colonies of which only 10% were cross-feeders. Altogether, 0.01% of the enriched survivors were resistant to low levels of L-valine. Seventy colonies were cloned and tested for L-valine and L-proline uptake (to monitor specificity of putative transport mutants) by the rapid uptake assay and for CRM by the Ouchterlony plate method described above. Of 50 defective transport mutants, 6 showed abnormal precipitin lines in the CRM test. Two of these are described below: strain AE840102, which had no detectable precipitin line, and strain AE84084, which had a line of reduced intensity.

RESULTS

Transport and binding protein characteristics of *liv* mutants. L-Valine-resistant, Mu-induced mutants obtained from the selections described above were grouped into three classes based on different patterns of binding protein expression, and representative strains were chosen for detailed characterization. We gave different locus designations to the lesions in each strain since their distinct biochemical phenotypes suggest that different genes were affected. Table 2 illustrates the specificities of amino acid uptake in the mutant strains compared with that of the parent strain AE84. Strain AE93 (*livH*::Mu, Table 1) was constructed to bring the *livH*::Mu locus into the same regulatory background (*livR*) as the other strains listed in Table 2. The amino acids L-histidine, L-proline, L-glutamine, and L-phenylalanine each represent separate transport systems; the L-histidine and L-glutamine transport systems require specific binding proteins; and L-proline and glycine are transported by membrane-bound systems (17). No reproducible differences in transport of these five control amino acids were seen between mutant and parent strains, but striking differences were seen in branched-chain amino acid uptake. Strains AE84084 (*livK*::Mucts) and AE93 (*livH*::Mu) sustained four- to sevenfold reductions in L-leucine, L-valine, and L-isoleucine uptake. Strain AE840102 (*livJ*), however, retained disproportionally more L-leucine uptake than the other mutants. Shock fluids were prepared from these strains and tested by equilibrium dialysis for LIV- and leucine-specific binding protein activity. Strain AE93 (*livH*::Mu) retained both wild-type binding activities (Table 3). Shock fluid from strain AE84084 (*livK*::Mucts), however, had no detectable leucine-specific binding activity. Strain AE840102 (*livJ*) lost the majority of LIV binding activity. Therefore, the residual binding activity is mainly specific for leucine. When these shock fluids were analyzed by SDS-acrylamide gel electrophoresis (Fig. 2), strain AE93 (*livH*::Mu) had a binding protein pattern indistinguishable from that of the wild type (not shown), but strain AE84084 (*livK*::Mucts) had lost the band corresponding to the leucine-specific binding protein. Strain AE840102 (*livJ*) had lost the band corresponding to the LIV

TABLE 3. *L-Leucine binding activity in shock fluids of liv mutants*

Strain[a]	L-Leu binding activity (pmol/mg of protein)[b]		Leucine-specific binding[c] (%)
	2.5 μM L-[^{3}H]Leu	2.5 μM L-[^{3}H]Leu + 200 μM L-Ile	
AE84 *livR*, wild-type	823	163	20
AE93 *livR livH*::Mu	1,600	247	15
AE84084 *livR livK*::Mucts	731	0	<5
AE840102 *livR livJ*	349	315	>90

[a] Cells were grown to late log phase in minimal medium plus required supplements at 32°C.
[b] Determined by equilibrium dialysis of crude osmotic shock fluids in (1).
[c] L-Leucine binding resistant to isoleucine inhibition.

TABLE 2. *Specificity of amino acid transport in various liv mutants*

Strain	Amino acid uptake (nmol/min per mg [dry wt] of cells)							
	L-His[a]	L-Pro[b]	L-Phe[a]	Gly[b]	L-Glu[a]	L-Leu[a]	L-Val[a]	L-Ile[a]
AE84 (*livR*, wild-type)	0.85	1.10	0.5	0.5	0.30	7.4	4.0	5.6
AE84084 (*livR livK*::Mucts)	0.74	1.18	0.6	0.8	0.32	0.7	1.0	0.80
AE840102 (*livR livJ*)	0.68	1.35	0.9	0.8	0.38	4.1	0.3	2.0
AE93 (*livR livH*::Mu)	0.60	1.00	0.7	0.7	0.35	1.5	0.6	1.7

[a] Uptake measured at 5 μM amino acid.
[b] Uptake measured at 10 μM amino acid.

binding protein. In the latter two strains, the loss of specific binding protein bands paralleled the loss of the specific transport activities (Table 2) and the binding activities (Table 3). No new bands were observed in the mutant gels.

Since strain AE840102 (*livJ*) retained both the leucine-specific binding protein and a high level of leucine uptake, we tested for the presence of a leucine-specific, high-affinity transport system in this strain. When the uptake of a low (0.75 μM) concentration of L-[^{3}H]leucine was inhibited by unlabeled L-isoleucine, the leucine transport actvity of strain AE840102 (*livJ*) was much more resistant to inhibition than the parent (Fig. 3), supporting the hypothesis that the residual leucine transport in the *livJ* mutant strain is predominantly leucine specific. The residual low-level leucine transport in *livH*::Mu strains was tested similarly, using L-threonine as a specific inhibitor of the LIV-I transport system (19, 21). The isogenic strains listed in Fig. 4 were constructed in a wild-type (*livR*$^+$) background for comparison of the *livH*::Mu mutant with the *livH*$^+$ strain under conditions where the LIV-I system is repressible by growth in exogenous L-leucine (19). The transport of 10 μM leucine was 50% inhibitable by high concentrations of L-threonine in strain AE89 (*livH*$^+$) under nonrepressing conditions (Fig. 4); under conditions where the LIV-I system was repressed by leucine, the residual LIV-II type uptake was resistant to L-threonine inhibition, as reported previously (19). The low level of leucine transport in strain AE90 (*livH*::Mu) was resistant to L-threonine inhibition, however, under both repressing and nonrepressing conditions, supporting the hypothesis that the residual transport has the characteristics of the LIV-II system. Detailed

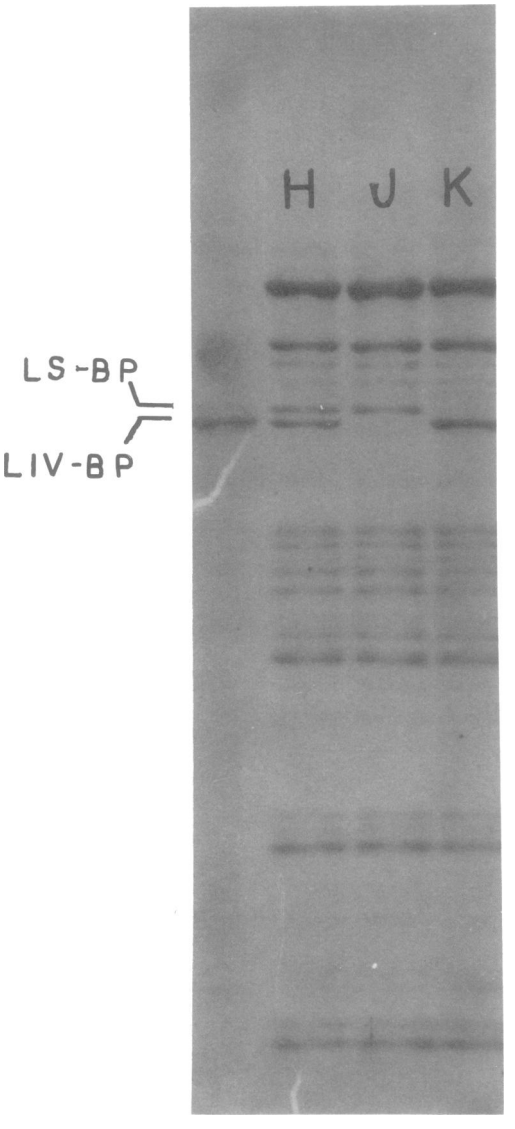

FIG. 2. *SDS-polyacrylamide gel electrophoresis of osmotic shock fluids from strains AE93 (livH::Mu) (H), AE840102 (livJ) (J), and AE84084 (livK::Mucts) (K). A standard containing purified LIV binding protein (LIV-BP) and a trace of leucine-specific binding protein (LS-BP) is shown on the left. The gels are 11% acrylamide, prepared as described in the text, and the direction of migration is from top to bottom.*

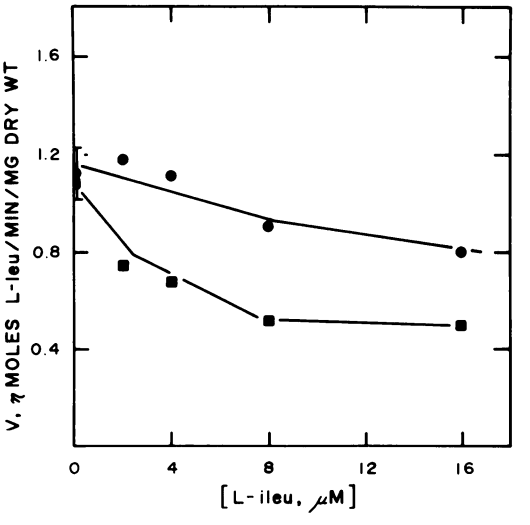

FIG. 3. *Inhibition of the initial velocity of L-[^{3}H]leucine (0.75 μM) uptake by L-isoleucine. Strain AE840102 (livR livJ) (●) and the parent strain AE84 (livR livJ$^+$) (■) were grown in minimal medium without leucine.*

kinetic analysis of the above strains is in progress.

Mapping of *livH, J, K.* Preliminary conjugations were carried out with strain AE4107 (*livH*::Mu) as the F⁻ recipient and various Hfr strains as donors, selecting for recombinants of the various auxotrophic markers in F⁻ (Table 1) and counterselecting either *str* or *nal*. The nonselected *livH*⁺ recombinants were screened among the appropriate selected recombinants by scoring for recovery of D-leucine utilization (specified by the *lstR* allele, which was not involved in the crosses). A gradient of transmission data (results not shown) indicated that *livH* was located in the *malT* region at min 74 of the genetic map (Fig. 1). Table 4 shows the cotransduction frequencies of *livH* for markers in this area. Crosses 1, 2, and 3 show that *livH*⁺ is 27% cotransducible with *malT* but not with *xyl* or *mtl*. A sampling of *mal*⁺ transductants showed that most of the *livH*⁺ cotransductants had acquired Mu sensitivity (and therefore had lost the Mu phage), whereas none of the *livH* transductants had. This indicated close linkage, or identity of the site of a single Mu insertion, with the *livH* locus. One of the *mal*⁺ *livH*::Mu transductants was the donor in a back-cross (data not shown) to the parent AE41 (*malT* Mu*cts*), which had been lysogenized with the thermoinducible Mu*cts* phage in an unknown position. The transduction was carried out at 37°C, and the *mal*⁺ transductants were selected at 42°C. Only *mal*⁺ transductants receiving the wild-type Mu phage of the donor would be expected to survive; all of these had now become *livH* (D-leucine nonutilizers) as would be expected if the wild-type Mu phage were completely linked to *livH*. This information enabled us to determine on which side of *malT livH* was located, since Mu insertions are known to disrupt linkages of cotransducible genes (4). If two normally cotransducible genes are bracketing a Mu insertion (molecular weight, 2.5×10^7, or half the capacity of a P1-transducing particle [12]), their apparent transduction linkage is reduced or eliminated when donor phage is prepared from the Mu lysogen. Linkage would be further reduced if the recipient were not a Mu lysogen already, due to lysis induction of intact Mu transduced in with the bracketing genes. In Table 4, crosses 4 through 7 show that the apparent linkage of *malT* with the bracketing markers *aroB* and *asd* (Fig. 1) is only slightly reduced in the former and not at all with the latter marker; therefore, *livH*::Mu

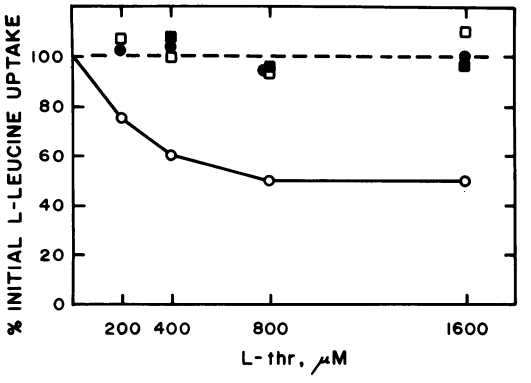

FIG. 4. *Inhibition of the initial velocity of L-[³H]leucine uptake (10 μM) by competing L-threonine in strain AE89 (leu⁺ livR⁺ livH::Mu)* (□) *and its isogenic counterpart AE90 (livH⁺)* (○). *Filled symbols denote cells grown in the presence of L-leucine (30 μg/ml), and open symbols denote growth in its absence.*

TABLE 4. *Transduction frequencies of loci in the malA region*

Cross	Relevant genotype		Selected marker	Unselected mrker	Percent with unselected donor marker
	Donor	Recipient			
1	EO300 wild type	AE4107 *livH*::Mu *mal*	*mal*⁺	*livH*⁺[a]	27.3 (82/300)
2	EO300 wild type	AE4107 *livH*::Mu *xyl*	*xyl*⁺	*livH*⁺	<1 (0/300)
3	EO300 wild type	AE4107 *livH*::Mu *mtl*	*mtl*⁺	*livH*⁺	<1 (0/300)
4	EO300 wild type	AB2847 *aroB malT*	*aroB*⁺	*mal*⁺	53 (53/100)
5	AE91 *livH*::Mu	AB2847 *aroB malT*	*aroB*⁺	*mal*⁺	39 (39/100)
6	AE62 *malT*	Hfr "C" U482 *asd*	*asd*⁺	*malT*	64 (64/100)
7	AE4107 *livH*::Mu *malT*	Hfr "C" U482 *asd*	*asd*⁺	*malT*	64 (64/100)
8	EO300 wild type	AE84084 *malT livK*::Mu*cts*	*mal*⁺	*livK*⁺[b]	20 (8/40)
9	EO300 wild type	AE99 *malT livJ*	*mal*⁺	*livJ*⁺[b]	22 (9/40)
10	EO321 *liv-321*	AE41 *malT*	*mal*⁺	*liv-321*[c]	6.5 (13/200)
11	AE105 *livJ livH*⁺	AE4107 *livJ*⁺ *livH*::Mu *malT*	*mal*⁺	*livJ* or *livH*⁺[d]	34 (68/200)

[a] *livH*⁺ scored as ability to use 100 μg of D-leucine per ml as a L-leucine source.
[b] *livK*⁺ *livJ*⁺ scored by rapid transport assay of L-valine (1 μM) uptake.
[c] *liv-321* scored as loss of ability to use 100 μg of D-leucine per ml as a L-leucine source.
[d] Both *livJ* and *livH*⁺ score as D-leucine utilizers; see text.

cannot lie between *malT* and either *asd* or *aroB*. Table 5, cross 1, shows the results of a four-factor cross analyzing the distantly linked *str* locus, as well as *malT* and *aroB*, when the *livH*::Mu strain is the donor. The results are consistent with the published order *str-malT-asd* (2). Since the linkage of *str* and *malT* (2%, Table 5, cross 1) is similar to the reported frequency (1%) when wild-type cells are the donors (20), *livH*::Mu cannot be distal to *str* and, therefore, must be located clockwise to both *malT* and *asd*. Cross 2, (Table 5) shows that *aroB* is further from *livH* (2.5% cotransduction with *livH*) than is *malT* (44.5%), which is consistent with the gene order *aroB-malT-asd-livH*. The only other reported *malT* cotransducible marker clockwise to *asd* is *gntR*, whose cotransduction frequency with *malT* is 16% (22); therefore, we suggest that *livH*::Mu lies between *asd* and *gntR* (Fig. 5).

The transport mutation of strain AE84084 (*livK*::Mu*cts*) was also found to cotransduce with *malT* (cross 8, Table 4) at a frequency similar to that of *livH*. When the *mal*$^+$ transductants were tested further for temperature resistance (the thermoinducible phage of strain AE84084 kills the cell at 42°C), the *livK*$^+$ cotransductants were temperature resistant and Mu sensitive; therefore, a single Mu*cts* insertion is closely linked or identical with the *livK* mutation. The transport defect of strain AE840102 (*livJ*), which is also linked to *malT* (22%, cross 9, Table 4), was found by conjugational analysis (data not shown) to be unlinked to a Mu*cts* phage elsewhere in the genome. Although *livJ* may be the result of a spontaneous mutation, it is more likely to be a deletion induced by Mu infection although not linked to the phage, a common occurrence in some strains (12). We next wished to determine whether *livJ* and *livH* were on the same side of *malT*. With strain AE4107 (*livJ*$^+$ *livH*::Mu, Dlu$^-$, *malT*) as the recipient and strain AE105 (*livJ livH*$^+$ *mal*$^+$) as the donor, we selected

TABLE 5. *Transductional analysis of the position of livH::Mu*

Cross	Strains (relevant genotype)	Selected marker	Recombinant class	Percentage of total
1	Donor: AE91 *livH*::Mu *str*; recipient: AB2847 *aroB malT str*$^+$	*mal*$^+$	*aro*$^+$ *str*	1.5 (3/200)
			aro$^+$ *str*$^+$	28.5 (57/200)
			aro str	0.5 (1/200)
			aro str$^+$	69.5 (139/200)
		aro$^+$	*mal*$^+$ *str*	2 (4/200)
			mal$^+$ *str*$^+$	46.5 (83/200)
			mal str	16 (32/200)
			mal str$^+$	35.5 (71/200)
2	Donor: AE62 *malT*; recipient: AE116 *aroB livH*::Mu	*aro*$^+$	*mal*$^+$ *livH*$^+$	0.5 (1/200)
			mal$^+$ *livH*	55 (110/200)
			mal livH$^+$[a]	2 (4/200)
			mal livH	42.5 (85/200)

[a] *livH*$^+$ scored as ability to use 100 μg of D-leucine per ml as a source of L-leucine.

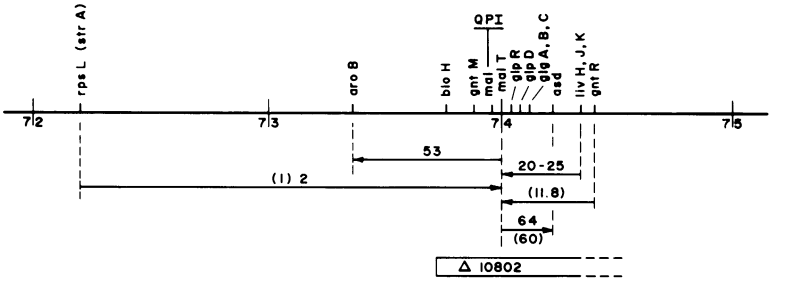

FIG. 5. *Linkage of liv mutations to other loci in the malT region. Positions of all known markers from aroB to gntR are redrawn from Bachmann et al. (2). The numbers below the line are cotransduction frequencies, with the arrowhead designating the selected marker. Numbers in parentheses are data of others: the distances strA-malT and malT-asd are from Schwartz (20); the distance malT-gntR is from Zwaig et al. (22). The bar designated Δ10802 plots the known extent of a deletion spanning bioH to livK (see text).*

mal$^+$ transductants and screened for both D-leucine utilization and L-valine transport. Since *livJ* had been isolated in a *leu*$^+$ background, we had no prior knowledge of what type of D-leucine utilization phenotype to expect when it was introduced into a *leu* background. Cross 11 (Table 4) shows that 34% of the *mal*$^+$ transductants were D-leucine utilizers, as would be expected if the donor were wild type (cross 1, Table 4). However, 20/20 of the Dlu$^+$ transductants were shown by transport assay to have the defective L-valine transport of the *livJ* donor; furthermore, they had become Mu sensitive and had, therefore, lost the Mu insertion associated with the *livH* mutation. We conclude that the apparent leucine-specific system retained in the *livJ* donor replaced *livH* in the recipient and that this system functions for D-leucine uptake as well, confirming our previous suggestion (1). Since *livJ*$^+$ *livH*$^+$ recombinants were not readily recovered among the Dlu$^+$ transductants, we conclude that *livJ* and *livH* are on the same side of *malT* and are closely linked.

An indirect means was employed to determine the relative position of *livK*::Mu*cts*. A *mal*$^+$ *livK*::Mu*cts* strain was constructed and subjected to simultaneous selection for temperature resistance (42°C) and λ phage resistance (*malT*) (10) in the expectation that this would require a deletion that excised both the killing functions of Mu phage and part of *malT*. The region around *malT* from *bioH* to *asd* (Fig. 5) has previously been shown to yield viable deletion mutants (10). When the selection was carried out on LBT plates containing diaminopimelic acid (required by *asd* mutants), temperature-resistant strains were found (frequency of 10^{-9}) which were *mal bio asd glp gnt* (Fig. 5). Thus, selection for a deletion of genetic material between *livK*::Mu*cts* and *malT* yielded mutants lacking the clockwise series of markers; this is understandable only if *livK* is on the same side of *malT* as *livJ* and *livH*.

A fourth mutation, *liv-321*, was described previously (19) in strain EO321 (Table 1). This mutation, which was obtained by penicillin enrichment for the loss of D-leucine utilization, resulted in loss of leucine-specific binding activity but retained chromatographically resolvable material that cross-reacted with antibody directed against the LIV binding protein. Cross 10, Table 4, shows that the Dlu$^-$ character of this donor strain is cotransducible with *malT*, albeit at reduced frequency compared with the other *liv* mutations. Our present linkage data are summarized in Fig. 5; the relative orders of *liv-321*, *livJ*, *livK*, and *livH* are being determined.

DISCUSSION

We identified a cluster of genes involved in branched-chain amino acid transport. The phenotype of several of these mutations was L-valine resistance, thus defining a class of valine resistance mutations not reported previously. The principle difference in our selection process was that we employed L-valine concentrations lower (0.4 to 4 μM) by two (9) to four (6) orders of magnitude than those used in prior investigations. The low L-valine concentrations were employed with strain backgrounds derepressed in the high-affinity uptake systems for branched-chain amino acids to enrich for mutations in these systems without demanding alterations in the lower affinity system(s) such as the LIV-II. Other investigators have employed competitors (L-leucine, L-methionine, L-threonine) or repressors (L-leucine, L-methionine) of the high-affinity uptake systems during L-valine selections to enrich for mutations in the lower affinity systems (9). Mutant loci (*brnR*, *brn-8*) recovered by the latter technique which were defective in high-affinity transport also were defective in the low-affinity transport system; i.e., they were pleiotropic. Mutation *livJ* apparently eliminates expression of the LIV binding protein while retaining a functional leucine-specific binding protein and leucine-specific transport (Table 3, Fig. 2 and 3). This is the first report of mutational separations of the LIV-I and leucine-specific systems. Since this mutation still permits D-leucine utilization in a strain background derepressed in the leucine-specific system (*lstR*), we conclude that the latter system is primarily responsible for the transport of D-leucine. The mutation *livK*::Mu*cts* eliminated the expression of the leucine-specific binding protein without apparently altering the LIV binding protein (Fig. 2 and Table 3). The transport of L-valine, L-isoleucine, and L-leucine in the *livK* mutant is, however, reduced in this strain (Table 2), which would not have been expected a priori if only the leucine-specific system had been altered. This apparent pleiotropic effect might result from the multimeric nature of the transport system where a defective component can affect more than one system. Alternatively, pleiotropic effects might be expected from the highly polar nature of Mu insertions, if common components of both LIV-I- and leucine-specific transport are promoter distal to the site of the *livK*::Mu*cts* insertion. The mutation *livH*::Mu also produces a reduction of leucine, valine, and isoleucine uptake (Table 2) but retains in vitro functional binding proteins with normal mobilities during SDS-acrylamide gel electrophoresis

(Table 3; Fig. 2). This mutation apparently eliminated an additional component(s) of high-affinity uptake distinct from the binding proteins. Again, the polar nature of the Mu insertion may have affected the expression of more than one component. By the same logic, however, the structural gene for the LIV-binding protein cannot be downstream of the *livK*::Mu*cts* insertion since the LIV binding protein is expressed in *livK* mutants. As more information on the genetic structure is forthcoming from the isolation and study of other Mu insertions and point mutants, both the number and polarity of the individual operons in this area may become evident.

We assume that *livJ* and *livK* specify the structural genes of the LIV- and leucine-specific binding proteins, respectively, although the possibility also must be entertained that these genes define positive regulatory elements or processing functions rather than the structural genes themselves. However, the mutation *liv-321*, which changes the binding activity and chromatographic mobility of the leucine-specific binding protein (19) and is, therefore, a candidate for a structural gene, also maps in this region (Table 4, cross 10). We are presently saturating the *malT* region with point mutations to further characterize and map the functions coded there, without the cautions that must acompany Mu insertions.

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