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Mapping of Two Loci Affecting the Regulation of Branched-Chain Amino Acid Transport in *Escherichia coli* K-12

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Two mutant loci resulting in derepression of, respectively, the L-leucine-specific transport system (*lstR*) and both the leucine-specific and the general branched-chain amino acid transport LIV-I systems (*livR*) were mapped by conjugation and transduction. Both *livR* and *lstR* were found to be closely linked to *aroA* at min 20 on the *Escherichia coli* genetic map. The merodiploid *livR*$^+$/*livR* displayed wild-type regulation of L-leucine transport, indicating that the *livR* product is a diffusible, negative controlling element for high-affinity leucine transport systems. Isogenic strains carrying *lstR*, *livR*, and wild-type transport alleles were compared for leucine uptake kinetic parameters and leucine-binding protein levels. The higher levels of leucine transport in the mutant strains under repressing conditions were generally due to increased high-affinity systems, which were accompanied by striking increases in the level of leucine-binding proteins.

The transport of L-leucine, L-isoleucine, and L-valine in *Escherichia coli* K-12 is complex, being mediated by at least three kinetically distinct systems (4, 18, 23). There are two high-affinity systems, the LIV-I and the leucine specific. The LIV-I system is characterized by a low K_m of transport for leucine, isoleucine, and valine and also shows affinity for L-threonine, L-alanine, L-serine, and L-homoserine (23). These high-affinity systems are repressible by leucine, apparently through leucyl-transfer ribonucleic acid (17). The leucine-specific system, which comprises about 20% of the high-affinity transport of leucine in wild-type *E. coli* K-12, can be identified by its resistance to competition by isoleucine. This system is also repressible by leucine and perhaps methionine (23). Both high-affinity systems are lost upon osmotic shock, coinciding with the loss of at least two binding proteins, one identified as the LIV-binding protein (16) and another the leucine-specific binding protein (L-binding protein) (3).

The low-affinity membrane-bound transport system for leucine has a 10-fold higher K_m for all three branched-chain amino acids, is resistant to osmotic shock, and is apparently nonrepressible (18, 23).

Previously this laboratory reported the isolation of two mutant strains of *E. coli* K-12 with derepressed levels of the high-affinity systems (18). Strain EO311 had derepressed levels of the LIV-I system and the leucine-specific system; strain EO318 had derepressed levels of the leucine-specific system. We have designated the mutant locus affecting the regulation of the LIV-I system in strain EO311 as *livR* and the mutant locus affecting the leucine-specific system in strain EO318 as *lstR*. In this paper we report the map positions of these two loci, the characteristics of strains diploid for the *livR* locus, and the transport and binding protein characteristics of isogenic strains carrying the wild-type, *lstR*, and *livR* alleles.

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MATERIALS AND METHODS

Bacterial strains. The strains used were all derivatives of *E. coli* K-12 and are listed in Table 1. The leucine auxotrophs EO311 and EO318 were isolated as D-leucine utilizers as reported previously (18).

Materials. MOPS (morpholinopropane sulfonic acid), tricine [N-tris(hydroxymethyl)methyl glycine], and nutritional supplements were products of Sigma Corp. (St. Louis, Mo.). 5,5,5-Trifluoro-DL-leucine was obtained from Columbia Organic Chemicals Inc., Columbia, S.C., and DL-4-azaleucine was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

Media. Minimal medium for growth of cultures used in transport assay was basal MOPS-salts medium (14) supplemented with 0.2% glucose and 50 μg of required L-amino acids per ml except for L-leucine, which was used at 25 μg/ml. Thymine and uracil were present at 50 μg/ml, and vitamins were present at 1 μg/ml. Luria broth without glucose (11)

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

Strain	Sex	Relevant genotype	Source
EO300	F⁻	Wild-type K-12	Rahmanian et al. (18)
EO311	F⁻	*leu, trp, dlu, livR*	Rahmanian et al. (18)
EO318	F⁻	*leu, trp, dlu, lstR*	Rahmanian et al. (18)
F15/EO311	F-prime	Chromosomal markers as EO311	Unselected sexductant of EO311 from F15/KL110, a K. B. Low strain
Hfr 6-1	Hfr	Point of origin in Low (10)	CGSC[a]
KL208	Hfr	Point of origin in Low (10)	CGSC
KL209	Hfr	Point of origin in Low (10)	CGSC
KL983	Hfr	Point of origin in Low (10)	CGSC
KL99	Hfr	Point of origin in Low (10)	CGSC
MA1079	Hfr	*thy⁺, recA;* point of origin of KL16 (10)	W. Maas strain via K. B. Low
JC1552	F⁻	*argG6, metB1, his-1, leu-6, trp-31, mtl-2, xyl-7, malA1, gal-6, lacY1, or lacZ4, str-104, tonA2, tsx-1,* $\lambda^R \lambda^-$, *supE44*	CGSC
AB1133	F⁻	*ara-14, leu-6*	CGSC
AB2829	F⁺	*aroA354*	CGSC
KL282	Hfr	*serC13, serS16*	CGSC
KL282-1	Hfr	*serC13*	EO300 transductant of KL282 to temperature resistance
KL188	F⁻	*pyrD34*	CGSC
PLS-73	F⁻	*pyrD34*	CGSC
AT3143	F⁻	*pdxC3*	CGSC
AE9	F⁻	*nalA*, otherwise as JC1552	Spontaneous
AE18	Hfr	*leu-6, lstR;* point of origin of KL209 (10)	D-Leucine-utilizing transductant (from strain EO318) of an Hfr recombinant between KL209 and AB1133
AE20	F⁻	*met⁺*, otherwise as AE9	*met⁺* transductant of AE9, from EO300
AE30	F⁻	*leu-6, lstR, trp, his, gal*	*met⁺* recombinant of conjugation of AE18 and AE9
AE40	F⁻	*metB⁺, thyA, araC*, otherwise as in AE9	Sequential spontaneous mutants of AE9
AE41	F⁻	*lstR*, otherwise as in AE40	D-Leucine-utilizing transductant of AE40 from AE30
AE49	F⁻	*aroA354*, otherwise as in AE40	Penicillin-enriched *aroA* transductant of AE40 from AB2829
AE52	F⁻	*livR*, otherwise as AE40	*aro⁺* transductant of AE49 from EO311
KLF3/AE55	F-prime	F103 *his⁺/his, livR*	F103 from KLF3/JC1552; AE55 is an *aro⁺* transductant of AB2829 from EO311, made *his* by P2 eduction (8)
AE59	F⁻	*thyA⁺, recA*, otherwise as in AE49	*thy⁺* recombinant of MA1070 and AE49
AE62	F⁻	*leu⁺*, otherwise as in AE40	*leu⁺* transductant of AE40 from EO300
AE63	F⁻	*leu⁺*, otherwise as in AE41	*leu⁺* transductant of AE41 from EO300
AE64	F⁻	*aroA354, livR, thyA*	*gal⁺* recombinant of conjugation between KLF3/AE55 and AE49
AE65	F⁻	*recA, thy⁺*, otherwise as in AE64	*thy⁺* recombinant of conjugation between MA1079 and AE64
AE66	F⁻	*leu⁺*, otherwise as in AE64	*leu⁺* transductant of AE64 from EO300
AE68	F⁻	*leu⁺*, otherwise as in AE52	*leu⁺* transductant of AE52 from EO300
AE80	F⁻	*pdxC3*	*aroA⁺* transductant of AB2829 from AT3143

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

was used for routine growth of strains and for matings. Recombinant selective plates contained 1.5% agar, based on half-strength medium 56 (9), and were supplemented with nutrients at concentrations listed above for liquid media. Fluoroleucine was used at 0.5 to 5 μg/ml, and azaleucine was used at 50 to 100 μg/ml. Strains with the *aroA* mutation required L-phenylalanine, L-tyrosine, L-tryptophan, and the vitamins *p*-hydroxybenzoate, *p*-aminobenzoate, and 1,4-dihydroxybenzoate. Strains with *serC*

mutations required both L-serine and pyridoxine; strains with *pdxC* required pyridoxine alone. Strains with *pyrD* required uracil.

Genetic crosses. Conjugations were carried out as recommended by Miller (12). Donor Hfr and recipient F^- strains were used at 3×10^8 cells/ml after two doublings in Luria broth and were mixed 10:1 (F^-:Hfr) in a final volume of 4 ml in a 125-ml Erlenmeyer flask, usually unshaken. Matings in this study were not interrupted. Samples were drawn after 60 to 90 min of incubation at 37 C, diluted, and plated in 3 ml of soft agar (0.75%) on selective plates containing streptomycin (100 μg/ml) as counterselective agent. F-prime transfers were done similarly. Transductions were carried out by using phage P1 CM *clr*100 (gift of D. Freidman from L. Rosner), a temperature-inducible phage (20) conferring chloramphenicol resistance upon lysogens. Lysate preparation through heat induction of lysogens was carried out as described (12). For transductions, 0.1 ml of a series of 10-fold dilutions of phage was incubated with 0.1 ml of recipient cells (washed and resuspended in 0.01 M $MgSO_4$-$CaCl_2$) at 37 C for 30 min. Controls of undiluted lysate and cells alone were treated similarly. Killing was reduced by adding 0.2 ml of 1 M sodium citrate (pH 7.0), and the contents of the tubes were plated out directly or in 3 ml of soft minimal agar on selective plates at 37 C. Colonies for analysis were chosen from the highest phage dilution that yielded a 20-fold or better excess of transductants over revertants. Unselected markers were scored by transferring recombinant colonies onto appropriate solid medium with a platinum wire (9) and replicating growing patches to selective plates. In some instances mentioned below, the variable quantity of inocula transferred by the velvet master introduced uncertainty in scoring; in this event, each patch was individually replicated by platinum wire.

Assay of transport and binding activities. Cultures of cells for transport assays were harvested in exponential phase by centrifugation and washed at 4 C three times with unsupplemented MOPS-salts medium. The cells were resuspended in this solution and incubated for 5 min at 37 C. The uptake reaction was started by adding the bacterial suspension to a solution of the radioactive amino acids. Uptake was measured by removing 0.5-ml samples at appropriate time intervals, filtering these through a 24-mm nitrocellulose filter (Millipore HA, Millipore Corp., Bedford, Mass.), and washing immediately with 5 ml of 37 C 10 mM potassium phosphate (pH 7.2) containing 0.1 mM $MgSO_4$. Radioactivity was determined in a Packard liquid scintillation spectrometer with a standard scintillator solution. The rate of uptake was linearly proportional to the dry weight of cells in the range used and remained constant during the time each experiment required. The quantity of cells used was estimated from the absorbance at 420 nm, using a Zeiss PMQ II spectrophotometer. An absorbance of 1.0 equaled 0.16 mg/ml, dry weight.

Experiments to determine the kinetics of transport were made by measuring the transport velocity of leucine at 13 substrate concentrations from 8.5×10^{-8} to 5×10^{-6} M. These data were plotted by the Lineweaver-Burk method, and the slope and intercept of the linear portions were determined by least-squares analyses. When biphasic kinetics were observed, the slope and intercept from the two linear portions of the line were used to calculate two K_m and two V_{max} values, using the method of Neal (13). The four kinetic values obtained were checked by recalculating the expected velocity by the following equation:

$$v_{obs} = \frac{V_1(S)}{K_1 + (S)} + \frac{V_2(S)}{K_2 + (S)}$$

Further refinement of the kinetic parameters was performed by iterative procedures until the calculated values closely approximated the observed.

The isolation and quantitation of protein with leucine-binding activity from osmotically shocked cells was performed as described previously (15).

Rapid assay for transport. For the large-scale assay of L-leucine transport phenotypes among progeny of genetic manipulations, a rapid variant of the standard assay above was devised. MOPS minimal medium was prepared with supplements as required, except that 0.04% glucose was the carbon source and L-leucyl-L-alanine (15 μg/ml) was the source of leucine for both Leu^+ and Leu^- strains. Two-milliliter volumes of this medium were dispensed into test tubes (17 by 125 mm), inoculated with small portions of recombinant colonies, and incubated overnight at 37 C with shaking. Regardless of inoculum size or growth rate, all cultures ceased growth at the same density when glucose was exhausted. Glucose was then added back by a calibrated dropping bottle (Microdels, Microbiological Associates, Rockville, Md.) to 0.2%, and the tubes were incubated for 20 min to allow partial recovery. Chloramphenicol was added similarly (150 μg/ml), and the tubes were placed at 37 C for assay. Standard strains were always included as internal controls. Cell samples (0.5 ml) were then added directly to 0.5 ml of unsupplemented MOPS salts containing L-[^{3}H]leucine at a final concentration of 0.75 μM. After 20 s of incubation, 0.5 ml of the mixture was rapidly filtered, washed, and counted as above. Reproducibility of this test was assayed by using 10 independent tubes for each of the standard wild-type and transport mutants examined in this report. The values obtained for the wild type (strain AE40) were a mean of 204 counts/min, standard error = 4%; for strain AE52 carrying the derepressed LIV-I transport allele *livR*, a mean of 2,668, standard error = 17%; for strain AE41 carrying the derepressed leucine-specific transport allele *lstR*, a mean of 1,821, standard error = 6%. The range of the different transport phenotypes did not overlap under these conditions.

Plate phenotypes of transport mutants. The original strains EO311 and EO318 were found to be multiple mutants (see Results). When strains carrying only the transport mutations were constructed by transduction, the phenotypes changed from those previously reported (18). Strains carrying *livR* were scored in a *leu* strain background by their ability to utilize 200 but not 100 μg of D-leucine per ml as a source of L-leucine. The wild type cannot use D-

leucine at 200 μg/ml. In contrast, strains carrying *lstR* are able to utilize 100 or 200 μg of D-leucine per ml and are easily distinguished on the basis of this property. In *leu*$^+$ strain backgrounds where D-leucine utilization cannot be assayed, strains with either *lstR* and *livR* mutations can be recognized by their sensitivity to 100 μg of DL-azaleucine per ml (the wild-type strain is resistant to this level). Replica platings were used to distinguish wild-type from either *lstR*- or *livR*-carrying strains, although the latter two lesions cannot be distinguished from each other by this technique. They are best distinguished by direct transport assay for leucine uptake (see Rapid uptake assay, above). Strains requiring supplementation with the aromatic amino acids L-tyrosine, L-phenylalanine, and L-tryptophan posed special problems, since these three amino acids together at 50 μg/ml completely prevented D-leucine utilization by otherwise competent strains; reciprocally, the presence of 200 μg of D-leucine per ml prevented use of exogenous aromatic amino acids in *aro* strains. In *leu*$^+$ backgrounds the aromatic amino acids protected sensitive cells from azaleucine, but not trifluoroleucine, inhibition. Accordingly, when *aro* strains were used or screened for transport mutations, trifluoroleucine sensitivity was used to detect the *livR* or *lstR* alleles. Replica plating from velvet masters could not be reliably used, however, since variable inocula obscure sensitivity determinations due to an adaptation of cells to the analogue (19). Instead, each colony was individually streaked on trifluoroleucine-supplemented medium with a platinum wire and scored after overnight incubation. Useful trifluoroleucine concentrations varied from 0.4 to 5 μg/ml, reflecting inherent strain background differences in sensitivity to this analogue.

RESULTS

Direct selection of transport phenotypes. The mutant strains EO311 and EO318 were originally isolated as D-leucine utilizers (Dlu$^+$) (18). The threshold concentrations of D-leucine for growth of the mutant strains on solid media were determined to be 60 and 40 μg/ml, respectively. In contrast, the parental Dlu$^-$ leucine auxotroph cannot utilize 200 μg of D-leucine per ml. Attempts to directly transduce the Dlu$^+$ phenotype associated with increased L-leucine transport using P1 phage grown on the two mutants met with limited success. When the donor was strain EO311, all Dlu$^+$ transductants had wild-type transport activity for L-leucine, which was normally repressible, indicating that a nontransport mutation, *dlu*, existed in strain EO311, which permitted utilization of D-leucine in L-leucine auxotrophs. When the donor was strain EO318, greater than 90% of all directly selected Dlu$^+$ transductants also had normal L-leucine transport regulation and were presumably *dlu* mutants; however, several Dlu$^+$ transductants did show the derepressed transport phenotype. These transductants had a threshold for D-leucine utilization of 80 μg/ml compared with nontransport *dlu* transductants, which had a threshold of 40 μg/ml. Intervening growth of transductants from either donor strain before plating (to overcome phenotypic lag) had no effect on the low efficiency of recovery of D-leucine utilizers with derepressed transport; accordingly, we do not generally use D-leucine utilization to select for derepressed transport alleles.

The leucine prototrophic derivatives of the Dlu$^+$ strains EO311 and EO318 are sensitive to either 100 μg of azaleucine per ml or 1 μg of trifluoroleucine per ml; the Leu$^+$ Dlu$^-$ parental strain is resistant. Attempts to transduce out the transport mutations with phage grown on the transport wild type by selection for analogue resistance were unsuccessful due to high background growth on plates; these findings have forced us to treat the transport phenotypes as unselected characters in the crosses reported below. We do use the phenotypes of D-leucine utilization or analogue sensitivity as screening agents, depending on the genetic background of recipient strains.

Segregation of *dlu* and *livR* from strain EO311. The chromosome of strain EO311 (*dlu livR*) was mobilized by sexduction with the F-prime F15 (Fig. 1) and used as a donor in the cross described in Table 2. Two classes of D-leucine utilizers were recovered from selected *his*$^+$ recombinants: those with a high efficiency of utilization of D-leucine but normally regulated L-leucine transport (*dlu* mutants) and those with low efficiency of utilization but carrying the derepressed leucine transport marker (*livR*). The relative frequency of the unselected *livR*, *dlu*, and *gal* (3/200) alleles among the *his*$^+$ recombinants indicated that *livR* and *dlu* were distal to *trp* but proximal to *gal* with respect to the point of origin of F15 (Table 2). The *dlu* allele evidently maps closer to *his* than does *livR*.

Mapping of *lstR* by conjugation. The *leu* Hfr AE18 (Table 1) was constructed by direct transduction to D-leucine utilization with strain EO318 as donor. The ability to use 80 μg of D-leucine per ml as a source of L-leucine was transduced together with derepressed leucine-specific transport activity (*lstR*). A preliminary uninterrupted mating with a suitably marked *leu* recipient revealed a pattern of distribution of *lstR* (identified both by D-leucine utilization and derepressed leucine transport) among other unselected markers that placed the locus between min 18 and 25 on the genetic map (data not shown). A multiply marked *lstR* F$^-$ recom-

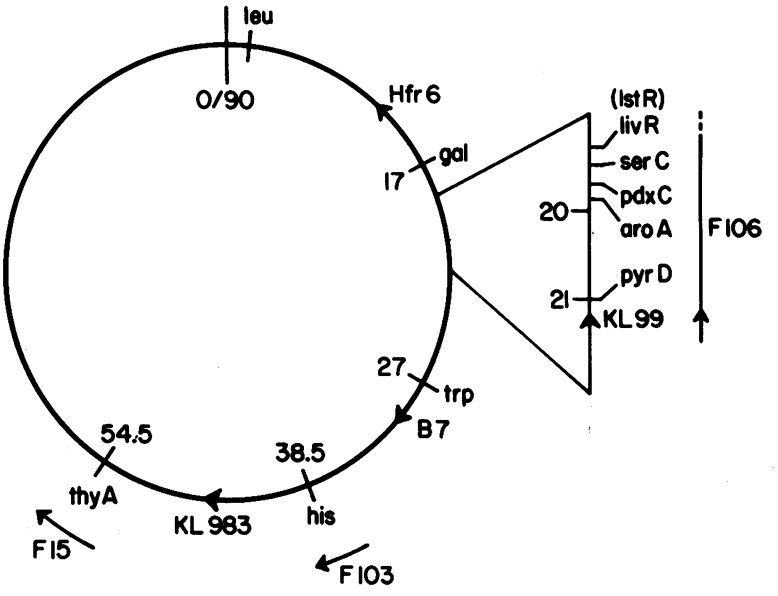

FIG. 1. *Simplified map of the E. coli chromosome showing the positions of relevant genes and points of origin and direction of transfer of Hfr and F-prime strains used in this study (10). Genetic symbols are those of Taylor and Trotter (21), except for lstR and livR in the expanded aroA region. The relative positions of livR, lstR, serC, pdxC, and aroA were determined from this study.*

TABLE 2. *Segregation of two classes of* D-*leucine-utilizing recombinants from the cross F15/EO311 His⁺ Gal⁺ Dlu⁺ Str^S × AE20 F⁻ His⁻ Gal⁻ Dlu⁻ Str^R*

Selected marker (no. scored)	Dlu⁺ recombinants[a]			D-Leucine threshold[c] (μg/ml)
	Type	No. scored	L-Leucine transport[b]	
His⁺ (200)	*dlu*	15	0.05 ± 0.02	40
	livR	9	0.5 ± 0.2	150

[a] Screened for ability to utilize 200 μg of D-leucine per ml.

[b] Uptake of 0.75 μM L-leucine, in nanomoles per minute per milligram of cells. The cells were grown in medium containing 25 μg of L-leucine per ml. Error measurement is standard deviation.

[c] Plate concentration of D-leucine permitting growth of *leu* recombinants.

binant from this cross was used as a recipient in matings with Hfr's that transferred this region early. The gradient of transmission of *lstR⁺* (scored by loss of D-leucine utilization and derepressed leucine transport) with respect to other unselected markers is shown in Fig. 2. No *lstR⁺* recombinants were detected among 400 *trp⁺* selected recombinants when the donor was the Hfr KL99; this indicated that *lstR* was located distal to the point of origin of KL99, between min 20 and 22 on the genetic map (21), in the same general area as *livR*.

Cotransduction of *lstR* and *livR* with loci in the *aroA* region. Two-factor crosses were carried out by using donors grown on the transport mutants (Table 3). Reciprocal crosses were not possible since the transport phenotypes could not be reliably selected. Plate transport phenotypes of transductants were verified by L-leucine transport assay on at least 10 members of each phenotypic class. Crosses 1 to 4 show that *livR* is weakly linked to *pyrD* and closely linked to *aroA* and *serC*. One of the *aro⁺*, *livR* transductants of cross 2 was further transduced to leucine auxotrophy by making use of the linkage of *leu* with *araC*, which is a selectable marker (2). The resulting *leu, livR* transductant showed marginal utilization of D-leucine (200 μg/ml), confirming the identity of the latter character, azaleucine sensitivity, and derepressed L-leucine transport with the *livR* locus.

Crosses 5 and 10 show that *lstR* is also weakly linked to *pyrD;* however, anomalous results were obtained when linkage to *aroA* or *serC* was scored. In crosses 6, 7, and 8, no linkage with *aroA* or *serC* was detected when strain EO318 or AE30 was the donor. Twenty-five *aroA⁺* or *serC⁺* azaleucine-resistant transductants from these crosses were tested for derepressed leucine transport, on the possibility that azaleucine sensitivity and the derepressed transport phenotype might have segregated.

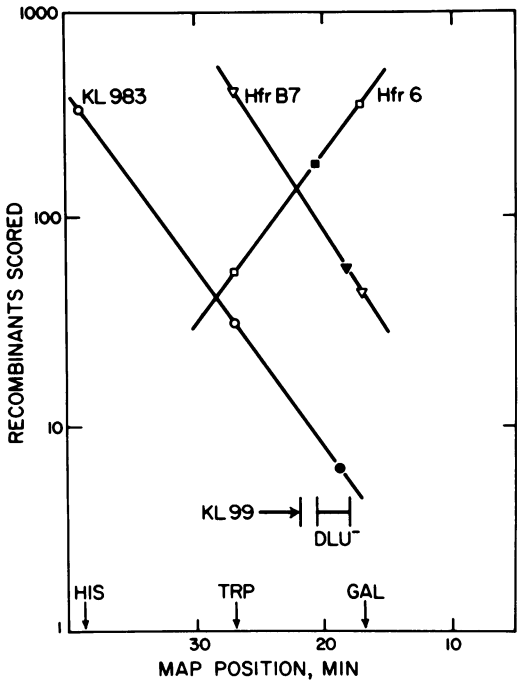

FIG. 2. *Gradient of transmission of unselected markers in crosses of the Hfr strains KL983, B7, Hfr6, and KL99 (all wild type, Str^s, and D-leucine nonutilizers [Dlu$^-$]) with the recipient F$^-$ strain AE30 (leu, trp, gal, his, lstR, [Dlu$^+$], Str^R). In each case an early transferred marker was selected in a 120-min uninterrupted mating: his$^+$ (KL983), trp$^+$ (B7 and KL99), gal$^+$ (Hfr6). Three hundred recombinants (open symbols) from each cross were scored for distal donor genes (open symbols) and loss of D-leucine utilization (closed symbols). The arrowhead indicates the point of origin of strain KL99; no Dlu$^-$ recombinants were scored from this cross.*

However, no *lstR* transductants were detected. Cross 9, however, shows that when strain AE41 was the donor, 50% cotransduction of *lstR* and *aroA* was obtained. Since the biochemical properties (derepressed leucine-specific transport, elevated leucine-specific binding protein) of all three donor strains were similar, we have ruled out the possibility that the *lstR* mutation in strain AE41 arose de novo during direct transduction of this strain to D-leucine utilization. Rather, since strain EO318 was a spontaneous mutant (18), we suspect that the mutational event may have introduced local interference for crossover between closely linked markers in the *aroA* region and that the successive direct transductions that led to the construction of strain AE41 (Table 1) removed this interference. At present we are accepting the linkage relationships of strain AE41 as indicative of the map position of *lstR*. Accordingly, since both *livR* and *lstR* have similar cotransduction frequencies with nearby markers, they must themselves be closely linked.

Three-point crosses. To determine the order of *livR* with respect to the closely linked markers *aroA*, *serC*, *pdxC*, and *pyrD*, three-point crosses were carried out by using the double mutant AE64 (*aroA livR*), constructed by screening *gal*$^+$ recombinants from a conjugation of strains KLF3/AE55 and AE49. This strain was transduced to leucine prototrophy since D-leucine and the aromatic amino acids reciprocally interfere with each other's utilization (see Materials and Methods). This strain was used as both donor and recipient for the crosses listed in Table 4. Cross 1 establishes that the order *livR aroA pyrD* is consistent with the transduction data (Fig. 3). Crosses

TABLE 3. *Cotransduction frequencies of markers in the aroA region*

Cross	Relevant genotype		Selected marker	Percentage with unselected donor marker
	Donor	Recipient		
1	EO311 *livR*	PLS-73	*pyrD*$^+$	3 (12/400)[a]
2	EO311 *livR*	AB2829	*aroA*$^+$	73.5 (294/400)[a]
3	EO311 *livR*	KL282-1	*serC*$^+$	49 (196/400)[a]
4	EO311 *livR*	KL282-1	*serC*$^+$	52 (52/100)[a]
5	EO318 *lstR*	PLS-73	*pyrD*$^+$	2 (8/400)[a]
6	EO318 *lstR*	KL282-1	*serC*$^+$	<1 (0/400)[a]
7	EO318 *lstR*	AE49	*aroA*$^+$	<1 (0/100)[b]
8	AE30 *lstR*	AB2829	*aroA*$^+$	<1 (0/400)[a]
9	AE41 *lstR*	AE49	*aroA*$^+$	50 (37/74)[b]
10	AE41 *lstR*	KL188	*pyrD*$^+$	3.8 (15/400)[a]
11	AT3143 *pdxC*	AB2829	*aroA*$^+$	>95 (196/197)
12	KL282-1 *serC*	AB2829	*aroA*$^+$	73.5 (146/199)

[a] Derepressed transport phenotype scored by sensitivity to 100 μg of DL-azaleucine per ml.
[b] Derepressed transport phenotype scored as ability to utilize D-leucine at 200 μg/ml.

TABLE 4. *Distribution of progeny of three-factor crosses by transduction*[a]

Cross	Strains (relevant genotype)[b]	Selected marker (no. scored)	Recombinant class	Percent total transductants
1	AE64 (*aroA livR*) D	*pyrD*$^+$ (388)	*aroA*$^+$ *livR*$^+$	90.7
	×		*aroA livR*$^+$	3.4
	KL188 (*pyrD*) R		*aroA*$^+$ *livR*	<1 (0/388)
			aroA livR	5.9
2(A)	AE64 (*aroA livR*) D	*pdxC*$^+$ (393)	*aroA*$^+$ *livR*$^+$	3.8
	×		*aroA*$^+$ *livR*	1.8
	AE80 (*pd x C*) R		*aroA livR*$^+$	30.7
			aroA livR	63.6
(B)	AT3143 (*pd x C*) D	*aroA*$^+$ (392)	*pdxC*$^+$ *livR*$^+$	0.5
	×		*pdxC*$^+$ *livR*	3.8
	AE66 (*aroA livR*)R		*pdxC livR*$^+$	71.9
			pdxC livR	23.7
3(A)	AE64 (*aroA livR*) D	*serC*$^+$ (397)	*aroA*$^+$ *livR*$^+$	11.6
	×		*aroA*$^+$ *livR*	5.3
	KL282-1 (*serC*) R		*aroA livR*$^+$	37.3
			aroA livR	45.8
(B)	KL282-1 (*serC*) D	*aroA*$^+$ (296)	*serC*$^+$ *livR*$^+$	2.4
	×		*serC*$^+$ *livR*	22.3
	AE66 (*aroA livR*)R		*serC livR*$^+$	51.0
			serC livR	24.3

[a] Possible orders of the appropriate loci are given in Fig. 3.
[b] Symbols: D, donor; R, recipient.

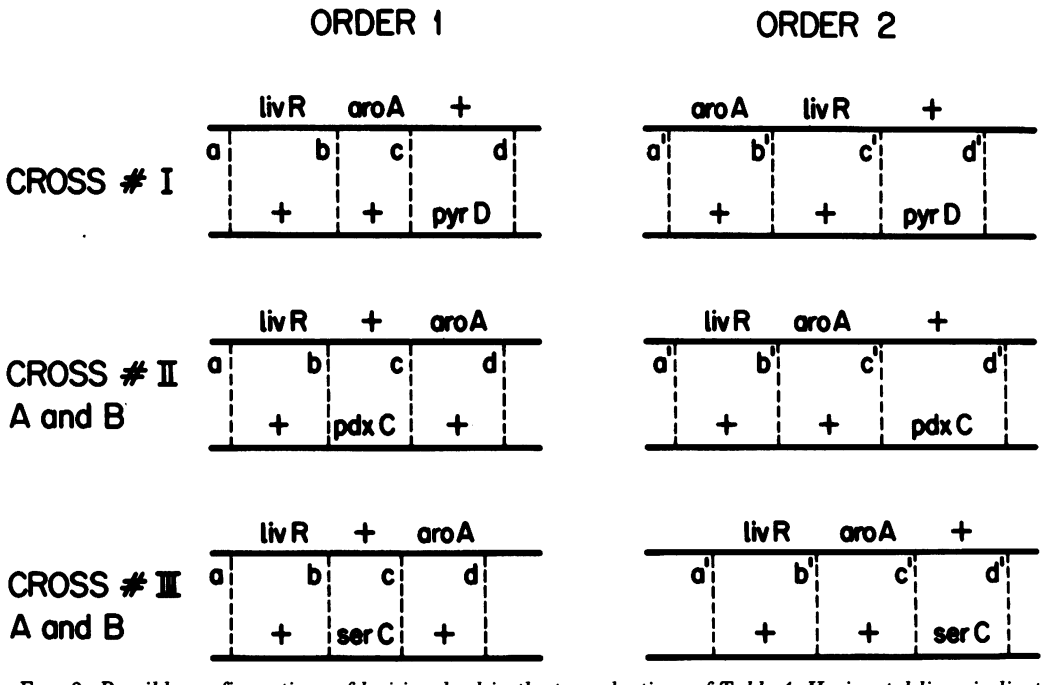

FIG. 3. *Possible configurations of loci involved in the transductions of Table 4. Horizontal lines indicate donor or recipient chromosomes, depending on the direction of the cross. Lower-case letters and dashed lines designate crossover intervals between genes.*

2(A) and 2(B) were reciprocal, treating *livR* as unselected donor marker or unselected recipient marker. The least frequent class of recombinants will be that requiring four crossover events (Fig. 3). The order indicated by the data is *livR pdxC aroA*. Similarly, crosses 3(A) and 3(B) showed a four-crossover class consistent only with the order *livR serC aroA* (Fig. 3). Since both *pdxC* and *serC* were middle markers with respect to *aroA* and *livR*, and *pdxC* was more closely linked to *aroA* than is *serC* (Table 3, crosses 11 and 12), the probable order of markers in this region is *livR serC pdxC aroA pyrD* (Fig. 4). This order does, however, predict a greater frequency of contransduction of *serC* with *livR* than *aroA* with *livR*; the opposite is found (Table 3, crosses 2 to 4), indicating that *livR* might be somewhat deleterious in certain strain backgrounds and be underrepresented in progeny of some crosses.

Linkage of *lstR* and *livR*. Cotransduction data from Table 3 suggested that *lstR* and *livR* were closely linked. A three-point cross was done, using the *aroA livR* mutant as recipient and *lstR* as donor; *aroA*$^+$ transductants were selected and screened for *lstR* and *livR* by their differing capability to utilize D-leucine (Table 5). The data show that both mutant transport phenotypes were recovered, but no wild-type recombinants (D-leucine nonutilizers) were detected; this implies a very close linkage of the two mutant loci or an order (*livR lstR aroA*) that would require four crossovers to generate a wild-type recombinant. Reciprocal crosses are necessary to clarify this point. At present, the position of *lstR* is not uniquely defined (Fig. 4).

Diploid analysis. The F-prime F106, with point of origin between *pyrD* and *pyrC* (9), was found to cover the *aroA* region (Fig. 1). The *recA* derivatives of strains AE49 (*aroA*) and AE64 (*aroA livR*) were used as recipients for KLF6/KL181 in liquid matings, and the resulting *aroA*$^+$ merodiploids were tested for transport phenotype (Table 6). The heterozygous merodiploid *livR*$^+$/*livR* showed wild-type repressed leucine transport; when the episome was lost, the strain regained both derepressed leucine transport and the *aroA* character, thereby demonstrating that the F-prime carried a dominant wild-type *livR*$^+$ allele. Accord-

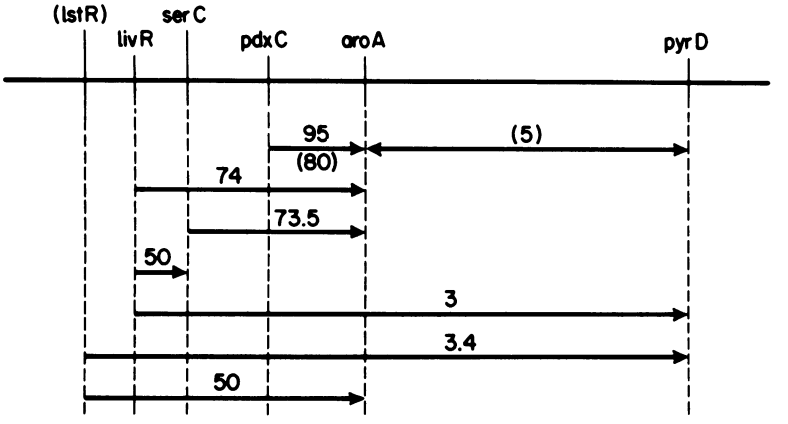

FIG. 4. *Order of loci in the aroA region. Numbers are cotransduction frequencies with the point of each arrow designating the selected marker. Numbers in parentheses are averages of reciprocal crosses reported by Cronan et al. (1). The precise position of lstR with respect to livR and other markers is not established. Not drawn to scale.*

TABLE 5. *Linkage relationships among the markers aroA, lstR, and livR*

Cross (relevant genotype)	Selected marker (no. scored)	Recombinant class[a]	Percentage of total
Donor: AE41 *(lstR)*	*aroA*$^+$ (391)	*lstR livR*$^+$	72
		lstR$^+$ *livR*	28
Recipient: AE64 *(aroA livR)*		*lstR livR*	—[b]
		lstR$^+$ *livR*$^+$	<1 (0/391)

[a] *lstR* scored as ability to utilize 100 μg of D-leucine per ml; *livR* scored as ability to use 200 μg of D-leucine per ml by not 100 μg/ml. The wild type is unable to use D-leucine at 200 μg/ml.

[b] Unknown phenotype.

TABLE 6. *L-Leucine transport activity in haploid and merodiploid strains*

Strain	Relevant genotype	L-Leucine uptake[a]	Percentage of haploid activity
AE59	*aroA livR⁺ recA*	0.068 ± 0.002	
KLF6/AE59	*aroA⁺ livR⁺/aroA livR⁺ recA*	0.056 ± 0.001	82
AE65	*aroA livR recA*	0.663 ± 0.064	
KLF6/AE65	*aroA⁺ livR⁺/aroA livR recA*	0.090 ± 0.006	6
AE59-1[b]	*aroA livR⁺ recA*	0.074 ± 0.002	
AE65-1[b]	*aorA livR recA*	0.460 ± 0.034	

[a] L-Leucine uptake measured at 0.75 μM L-leucine, given as nanomoles per minute per milligram (dry weight) of cells. The cells were grown in minimal medium plus repressing (25 μg/ml) amounts of L-leucine. Error measurement is standard deviation of triplicate samples.

[b] Merodiploids cured of the episome by acridine orange treatment (7).

ingly, *livR* corresponded to a negative controlling element responsible for a diffusible substance regulating expression of the LIV-I system. No gene dose effect was evident in the wild-type merodiploid *livR⁺/livR⁺*; this does not rule out the possibility that structural genes may be present on the episome, since the strain was grown under repressing conditions where the wild type has almost undetectable levels of the LIV-I system.

Characterization of isogenic transport mutants strains. The isogenic strains AE62, 63, and 68 were constructed (Table 1) in order to provide well-marked starting strains for further acquisition of mutations in the branched-chain amino acid transport system and to determine the physiological effects of derepressed transport. The kinetic properties of the *leu⁺* derivatives of these strains are shown in Table 7. The wild-type strain AE62 demonstrated the repression of the low-K_m LIV-I system when the cells were grown with excess branched-chain amino acids; the level of the high-K_m system (LIV-II) was not significantly affected. In comparison with the wild type, both the *livR* and *lstR* strains showed higher V_{max} values for leucine transport and higher binding protein levels regardless of growth conditions. Under repressing conditions, the *lstR* strain AE63 was derepressed in the leucine-specific binding protein and the *livR* strain was derepressed in the LIV-binding protein. When either of these strains was grown under nonrepressing conditions (Table 7), additional increases in the overall V_{max} (V_1 + V_2) of leucine transport and binding protein levels occurred. The relative contributions of LIV-I and LIV-II in the case of AE63 (*lstR*) under repressing conditions could not be determined. A single K_m value was derived from a least-squares analysis of the kinetic data (Table 7). Under nonrepressing conditions, however, strain AE63 (*lstR*) showed biphasic kinetics with an apparent threefold increase in the LIV-II system over the wild-type or strain AE68 (*livR*) level.

TABLE 7. *L-Leucine transport characteristics of isogenic strains with mutant and wild-type transport loci, grown in minimal MOPS medium in the presence and absence of the branched-chain amino acids*

Growth conditions	Kinetic parameters[a]	Strain AE62 (wild type)	Strain AE63 (*lstR*)	Strain AE68 (*livR*)
L-Leucine, L-isoleucine, and L-valine (each at 2.5 μg/ml)	K_1	0.13	0.83[b]	0.2
	K_2	2.60		2.5
	V_1	0.13	2.32[b]	0.7
	V_2	0.80		0.9
	Leucine-binding protein	<0.02	0.72[c]	1.14[d]
No addition	K_1	0.17	0.2	0.4
	K_2	1.7	3.7	5.0
	V_1	0.92	1.5	2.4
	V_2	0.62	1.9	0.7
	Leucine-binding protein	1.11	1.62	1.61

[a] Parameters resolved from biphasic Lineweaver-Burk plots as described in the text. K_1 and K_2 refer, respectively, to the apparent K_m values of the LIV-I and LIV-II leucine uptake systems (micromolar). V_1 and V_2 are the respective V_{max} values in nanomoles of L-leucine per minute per milligram (dry weight) of cells. Leucine-binding protein (nanomoles of L-leucine bound per milligram of crude shock fluid protein) was measured after osmotic shock treatment of logarithmically growing cells as described previously (15).

[b] Only one system detected.

[c] Determined to be 80% leucine-specific binding protein by resistance of L-leucine binding to L-isoleucine inhibition.

[d] Determined to be >90% LIV-binding protein by sensitivity to L-isoleucine inhibition of L-leucine binding.

DISCUSSION

The mutant loci *lstR* and *livR* apparently govern two different patterns of regulation of

branched-chain amino acid transport. Although both show derepressed L-leucine transport as reported previously (18), the pattern of derepression of the *livR* mutant permits growth only on high levels (200 μg/ml) of D-leucine, whereas that of *lstR* permits growth on at least a twofold lower concentration of the D-amino acid. Since the latter lesion leads to a striking increase of the leucine-specific binding protein whereas *livR* derepresses the LIV-binding protein, we tentatively conclude that D-leucine can enter the cell through the leucine-specific system. Although binding studies have not been reported for D-leucine with the leucine-specific binding protein, it is noteworthy that the best inhibitor of the leucine-specific protein is racemic trifluoroleucine (3); it is possible that a considerable portion of the inhibition of L-leucine binding is due to the D-isomer. It is also clear that mutant loci other than those affecting L-leucine transport lead to the Dlu^+ phenotype, since the original mutant strains EO311 and EO318 have been shown to segregate Dlu^+ strains with wild-type transport properties. Our finding that the aromatic amino acids antagonize D-leucine utilization and vice versa suggests an affinity of D-leucine for an aromatic transport system. Harrison et al. (6) have shown that L-azaleucine enters by both the aromatic permease (*aroP*) and by the leucine uptake systems.

Both *lstR* and *livR* map at min 20 on the *E. coli* genetic map (21), a site different from the previously reported mutant locus affecting branched-chain amino acid transport or biosynthesis. Preliminary mapping studies involving crosses between the strains carrying the two mutations indicate that the loci are too closely linked to readily detect crossover between them; this result is complicated by the difference in cotransduction frequencies of *lstR* with *aroA* and *serC*, depending on the source of donor phage carrying *lstR*. The simplest explanation for this phenomenon is that the original mutant EO318 was generated by an event that caused interference with crossover near *aroA*, such as an inversion or insertion. During subsequent sequential transductions of *lstR* by direct selection for the marker this interference was lost, resulting in linkage patterns that do not differ substantially from those of *livR*.

The recent report of Wood (23) on the kinetic parameters of L-leucine transport in a different K-12 strain (strain 7) provides a source of comparison for our own results on mutant and wild-type isogenic strains. We have confined our kinetics to L-leucine concentrations where nonspecific leucine transport would not be seen (less than 5 μM). Since we have not separated leucine-specific from LIV-I transport, (Table 7), we have combined Wood's separate values for the two systems reported in Table 2 of reference 23 into one high-affinity system for purposes of comparison. Our values for the wild-type strain yield 14% high-affinity activity (0.13/0.13 + 0.80; Table 7) and 86% low-affinity (LIV-II) activity in cells under repressing conditions compared with 40 and 60% in Wood (Table 2 in reference 23). For derepressed cells, our values are 60% high-affinity transport (0.92/0.92 + 0.62) and 40% LIV-II versus 80 and 20%, respectively, in Wood (23). Strain differences could easily account for these discrepancies, since we have found that different K-12 strains have varying basal levels of L-leucine transport (S. C. Quay and J. J. Anderson, unpublished data).

The data of Table 7 confirm that as a consequence of *livR* mutation the levels of both LIV-binding protein and the LIV-I transport system are elevated over the wild-type levels during branched-chain amino acid repression, as reported previously for the original *livR* mutant, strain EO312 (18). However, unlike the latter strain, the elevated levels in strain AE68 (*livR*) are derepressed further when the repressing amino acids are withdrawn, suggesting that in this new strain background partial repression can still be observed.

The level of the LIV-II system did not vary appreciably in either wild-type or *livR* strains as a result of branched-chain amino acid supplementation (Table 7). However, in strain AE63 (*lstR*) under nonrepressing conditions, approximately 60% of the increase in leucine transport over wild type was apparently due to an elevated LIV-II system. Whether or not this is also true of strain AE63 (*lstR*) under repressing conditions could not be determined, since the single K_m value obtained for leucine transport is intermediate between the LIV-I and LIV-II systems. Such an intermediate value may represent a mutationally generated new system or simply a fortuitous combination of two or more separate systems whose additive Lineweaver-Burk kinetics are linear within experimental error. The close linkage of *lstR* and *livR*, each governing different patterns of expression of branched-chain amino acid transport, suggests that the associated region is genetically complex.

The finding that *livR* behaves in merodiploids as a recessive character suggests that the wild-type allele $livR^+$ is responsible for the production of a diffusible, negative control element that effects repression of the LIV-I system. Presumably this element is associated with the repressing signal generated by interaction of L-

leucine with the leucyl-transfer ribonucleic acid synthetase (17).

Present work is directed to the acquisition of appropriate mutants to determine the location of structural genes for the binding proteins. Positions of loci determining other elements in branched-chain amino acid transport have been reported (5), but none of these map near the regulatory loci reported here.

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