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Identification of *livG*, a Membrane-Associated Component of the Branched-Chain Amino Acid Transport in *Escherichia coli*

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Branched-chain amino acids are transported into *Escherichia coli* by two osmotic shock-sensitive systems (leucine-isoleucine-valine and leucine-specific transport systems). These high-affinity systems consist of separate periplasmic binding protein components and at least three common membrane-bound components. In this study, one of the membrane-bound components, *livG*, was identified. A toxic analog of leucine, azaleucine, was used to isolate a large number of azaleucine-resistant mutants which were defective in branched-chain amino acid transport. Genetic complementation studies established that two classes of transport mutants with similar phenotypes, *livH* and *livG*, were obtained which were defective in one of the membrane-associated transport components. Since the previously cloned plasmid, pOX1, genetically complemented both *livH* and *livG* mutants, we were able to verify the physical location of the *livG* gene on this plasmid. Recombinant plasmids which carried different portions of the pOX1 plasmid were constructed and subjected to complementation analysis. These results established that *livG* was located downstream from *livH* with about 1 kilobase of DNA in between. The expression of these plasmids was studied in minicells; these studies indicate that *livG* appears to be membrane bound and to have a molecular weight of 22,000. These results establish that *livG* is a membrane-associated component of the branched-chain amino acid transport system in *E. coli*.

Active transport in gram-negative bacteria is mediated mainly by two classes of transport systems: osmotic shock-insensitive systems and osmotic shock-sensitive systems (12, 20). Osmotic shock-insensitive systems appear to utilize only one membrane-bound component (12, 30). Osmotic shock-sensitive systems appear to be more complex and require several membrane components along with a periplasmic binding protein (1, 9, 12, 26). The periplasmic components are soluble proteins with binding activities for a specific substrate or set of substrates (12, 14, 21). It has been proposed that their major role in transport is to deliver the substrate to the membrane components by direct interaction of the binding protein substrate complex with at least one membrane protein (3, 26). This interaction may activate conformational changes of one or more of the membrane proteins, which results in the delivery of free substrate inside the cell (1, 3, 26). Most shock-sensitive transport systems have more than two membrane-associated components which are present in much smaller quantities than the binding proteins (2, 7, 9, 10, 12, 27, 28).

We have found that the transport of the branched-chain amino acids in *Escherichia coli* is carried out by two periplasmic binding protein-dependent, high-affinity transport systems designated the leucine-isoleucine-valine (LIV-I) and the leucine-specific (LS) transport systems (4, 13, 22, 24). In addition, a membrane-bound low-affinity system designated LIV-II is present (5). The structural genes for the LIV-binding protein (*livJ*), the leucine-specific binding protein (*livK*), and one of the membrane-associated components (*livH*) were initially identified by using genetic approaches involving the mutator phage, Mu, to isolate transport mutants (4). Mutants have also been identified for the LIV-II transport system (*livP*) (5). The genes for the shock-sensitive transport systems (LIV-I and LS) contained in a 13-kilobase (kb) *Eco*RI DNA fragment have been cloned into the pACYC184 plasmid vector, yielding the pOX1 plasmid (20). By using subcloning strategies combined with transposon insertion mutagenesis and DNA sequencing, the *livJ*, *livK*, and *livH* genes have been identified on the pOX1 plasmid (13, 21; R. Landick, Ph.D. thesis, The University of Michigan, Ann Arbor, Mich., 1983).

In this paper, we report the identification of an additional membrane-associated component, *livG*, which is required for both of the high-affinity periplasmic transport systems. For this study, we first isolated ethyl methanesulfonate (EMS)-induced mutations in various components of the high-affinity transport systems and then subjected them to genetic complementation studies. In a second approach, a series of derivative plasmids were constructed from pOX1, carrying various portions of the *liv* regulon which allowed us to both physically and functionally identify the *livG* gene. The *livG* gene product has been tentatively identified as a membrane-associated protein with an apparent molecular weight of 22,000, by using a minicell expression system.

MATERIALS AND METHODS

Bacterial strains and phages. The bacterial strains used for these studies were all derivatives of *E. coli* K-12 and are listed in Table 1. Bacteriophage P1CM*clr*100 was used for transductions and was a gift of D. Friedman and L. Rosner.

Media and chemicals. Cells for transport assays and osmotic shock treatment were grown in MOPS (morpholinopropanesulfonic acid) minimal medium (17) or Vogel-Bonner medium (29) supplemented with 0.2% glucose and 50 μg of each of the required amino acids per ml except for leucine, which was present at 25 μg/ml. Thymine was present at 50 μg/ml, and pyridoxine-hydrochloride was present at 1 μg/ml when required. Luria broth without glucose (18) was supplemented with 50 μg of thymine LBT per ml and used for

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TABLE 1. Strains used in this study

Strain	Relevant genotype	Source
AE84	*argG6 hisG1 trp-31 thyA746 malA1 rpsL104 mtl-2 araC601 tonA2 lacY1 supE44 gal-6 gyrA260 xyl-7 pdxC3 livR*	Anderson[a]
AE840201	*livG*; otherwise as AE84	This study
AE840203	*livK*; otherwise as AE84	This study
AE840212	*livH*; otherwise as AE84	This study
AE114	*recA lstR livH::Mu thyA*$^+$; otherwise as AE84	Anderson
AE126	*mal*$^+$; otherwise as AE84	By transduction
AE179	*recA* Tet^r; otherwise as AE84	By transduction
AE300	*mal*$^+$ *glpD*; otherwise as AE840203	By transduction
AE301	*mal*$^+$ *glpD*; otherwise as AE840212	Transductant from line 8 *glpD malT*$^+$
AE302	*mal*$^+$ *glpD*; otherwise as AE840201	Transductant from line 8 *glpD malT*$^+$
AE305	*snl::*Tn*10*; otherwise as AE300	From K230 by transduction
AE306	*snl::*Tn*10*; otherwise as AE301	From K230 by transduction
AE307	*snl::*Tn*10*; otherwise as AE302	From K230 by transduction
AE404	*recA thy*$^+$; otherwise as AE301	By mating
AE405	*recA thy*$^+$; otherwise as AE302	By mating
KL16-99	*recA1 relA1 thi-1 deoB1 3* HFr	From CGSC[b]
JM101	*lacI*^{q}, ZM15 *traD1*, F′ Δ(*lac-pro*)	From Bethesda Research Laboratories
F′104-5/86	F′ *mal*$^+$ *lin*$^+$	From F′140

[a] Reference 4.
[b] CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

routine growth of strains. Plates for selecting mutants, transductants, and sexductants contained 1.5% agar and were based on Vogel-Bonner medium supplemented with the same concentrations of nutrients as described above. Ampicillin was used at 25 μg/ml. EMS was purchased from Eastman Organic Chemicals, Rochester, N.Y. ^{14}C- and ^{3}H-amino acids were from New England Nuclear Corp., Boston, Mass. L-[^{35}S]methionine was from Amersham Corp., Arlington Heights, Ill. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside and isopropyl-β-D-pyranothiogalactoside were from Bethesda Research Laboratories, Inc., Gaithersburg, Md.

Genetic techniques. All phage transductions were carried out with lysates of phage P1CM*clr* 100, as described previously (6). Matings were performed by the replica mating technique by the procedure of Miller (16). Homogenotes of several of the mutants were made by the following procedure. Mutant *malT* strains were mated with F′ 140-5/86, containing the *malT* locus which is closely linked to the *liv* mutations (4). Sexductants were grown overnight in maltose minimal medium to ensure retention of the F′. Strains carrying the *liv* mutation on both the F′ and the chromosome were selected by plating on minimal plates containing either maltose or glycerol and 0.1 μg of L-valine per ml. Valine-resistant colonies were plate mated with a *recA* F$^-$ strain to recover the recombinant F′. Resultant sexductants were purified and used for complementation studies.

Transport and binding assays. Routine transport assays of the indicated amino acids were carried out as described previously (4, 6). We also applied the following version of the rapid transport assay. Cells were grown overnight in 0.04% glucose–MOPS minimal medium to arrest growth at a constant, low cell density. The cells were harvested by centrifugation, washed four times with MOPS minimal medium, and suspended in MOPS with 0.2% glucose. The transport activity of the cells was determined by measuring the uptake of 0.5 μM L-[^{3}H]valine or 0.1 μM L-[^{3}H]leucine. Amino acid-binding activities of osmotic shock fluids were determined by equilibrium dialysis with 0.1 μM L-[^{3}H]leucine or L-[^{3}H]isoleucine as previously described (23). Preparation of osmotic shock fluids was performed by the procedure of Neu and Heppel (19).

Isolation of EMS-induced mutants. The *liv* transport mutants were isolated from strain AE84 by mutagenesis with EMS and selection on plates for growth in the presence of the toxic analog azaleucine. EMS mutagenesis was carried out by the procedure of Miller (16). Azaleucine-resistant mutants were selected by plating dilutions of mutagenized cells on plates containing a concentration gradient of azaleucine. Mutants were also selected by plating untreated cells on minimal plates containing 100 μg of D,L-azaleucine per ml with a disk containing EMS in the center of the plate. In all cases, small colonies barely discernible above the background growth were picked, purified, and assayed for [^{3}H]leucine and [^{14}C]proline transport by the rapid transport assay. Decreases in [^{3}H]leucine uptake relative to [^{14}C]proline uptake indicate specific transport mutants for the LIV-I system.

Fifty-four azaleucine-resistant mutants were screened by the rapid transport assay. Roughly half (40%) had less than 50% of parental leucine transport while retaining normal proline transport. These mutants were subjected to osmotic shock treatment, and the periplasmic proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis. Two classes of mutants were obtained, based upon the presence or absence of the binding proteins. One class had both LIV- and LS-binding proteins present (class I). The other class of mutants was missing the LS binding protein (class II).

DNA manipulations. Restriction endonucleases, T4 ligase, and DNA polymerase Klenow fragment were obtained from Bethesda Research Laboratories and from New England Nuclear Corp. Restriction endonuclease digestions were performed as described in the instructions of the suppliers. DNA ligation, transformation, and filling-in reactions were performed as described by Maniatis et al. (15). Procedures for plasmid DNA isolation have been described previously (8). Plasmid DNA fragments were analyzed by electrophoresis on horizontal 0.8% agarose gels or on 5% polyacrylamide gels as described previously (25).

Isolation of minicells. Minicells were purified from transformed *E. coli* minicell-producing strain X1411 grown in MOPS-rich medium as described previously (20). Purified minicells (200 to 400 μl; optical density at 420 nm, 1.0) were centrifuged, suspended in 100 μl of MOPS complete medium without L-methionine, incubated for 20 min to decrease background mRNA levels, and then labeled with L-[^{35}S]methionine for 45 min. Labeled minicells were washed, suspended in 50 μl of sample buffer (11), and subjected to SDS-polyacrylamide gel electrophoresis by the Laemmli procedure (11). Fluorography enhancement reagents were obtained from New England Nuclear Corp.

For the minicell fractionation, labeled minicells were treated with 50 mM Tris (pH 8) containing 25 mM EDTA and 500 μg of lysozyme per ml for 15 min on ice and disrupted by

sonication. Minicell membrane preparations were separated from the cytoplasmic-periplasmic fraction by centrifugation at 100,000 × *g* for 16 h.

RESULTS

Phenotypes of EMS-induced *liv* transport mutants. In a wild-type *E. coli* K-12 strain with normal regulation of leucine transport, the kinetics of leucine transport yield biphasic reciprocal plots due to the presence of low-affinity (LIV-II) and high-affinity (LS and LIV-I) transport systems (24). In *livR*$^-$ strains, however, derepression of the LIV-I and LS systems increases high-affinity transport and largely masks the biphasic nature of the kinetic plots, so that the LIV-II contribution to the total leucine transport is essentially negligible at low leucine concentrations (6). LIV-I transport mutants show kinetics characteristic of having only the low-affinity LIV-II transport present. Two classes of LIV-I transport mutants resistant to azaleucine were selected as described in the Materials and Methods section. When the kinetics of uptake in the azaleucine-resistant mutant strains AE840201 and AE840212 of class I and AE840203 of class II were examined, we found only the LIV-II, or low-affinity, system present. Values for the kinetic parameters, K_m and V_{max}, derived from Lineweaver-Burke plots of leucine transport of the various mutant classes are summarized in Table 2. The lower K_m value for strain *liv302* may result from a partially defective membrane component. Also shown in Table 2 is the measurement of the binding activity in osmotic shock fluids from the *mal*$^+$ derivatives of azaleucine-resistant mutant strains AE840201, AE840203, and AE840212 and the parent strain AE84. The results show that the shock fluid from the mutant strain AE300 (AE840203 *mal*$^+$) exhibits leucine-binding activity which can be completely inhibited by isoleucine, indicating a lack of functional LS binding protein. In addition, the shock fluids from mutant strains AE301 and AE302 of class I appear to have normal leucine-binding activity, indicating lesions in the nonbinding protein components of the transport system. These conclusions were further supported by examining the presence of the binding proteins from the shock fluids of the above strains by polyacrylamide gel electrophoresis (data not shown).

It appears that these EMS-induced azaleucine-resistant mutants show phenotypes similar to some of the Mu phage induced mutations in LIV-I isolated previously in this laboratory (4). Azaleucine-resistant mutant strain AE300 gives a LivK phenotype, while strains AE301 and AE302 have phenotypes similar to that reported for *livH* (4). The mutations in these strains were referred to initially as *liv301* and *liv302*.

TABLE 2. Summary of kinetic parameters in LIV mutants

Transport genotype	V_{max} (nmol/min per mg)[a]	K_m(μM)[a]	L-leucine binding activity[b] (nmol/mg of protein)	
			Without isoleucine	Plus isoleucine
liv$^+$	8.80	0.5	0.91	0.18
livK300	2.66	8.4	0.71	—[c]
livH301	2.19	6.8	0.60	0.06
livG302	1.14	2.5	0.70	0.20

[a] Uptake was measured with 0.1 μM L-[^{3}H]leucine in strains AE84, AE840203, AE840212, and AE84201, respectively.
[b] LS binding protein activity was determined by equilibrium dialysis of crude osmotic shock fluids in 2.5 μM L-[^{3}H]leucine plus 200 μM L-isoleucine. LIV-binding activity was calculated as the activity in 2.5 μM L-[^{3}H]leucine minus LS-binding protein activity. These results were measured with strains AE126, AE300, AE301, and AE302.
[c] —, Not determined.

TABLE 3. Complementation analysis of *liv* mutations[a]

Recipient strains	Mutations on F140-5/86 (nmol/min per mg)				Uptake of haploids (nmol/min per mg)
	*livK*300	*livH301*	*livH302*	*liv*$^+$	
AE306 *livH301*	0.03	0.05	1.03	0.52	0.08
AE307 *livH302*	0.07	1.00	0.02	0.44	0.05
AE305 *livK300*	0.04	0.05	0.05	1.14	0.08
AE114 *livH*::Mu	0.07	0.10	0.26	0.76	0.16
AE84 *liv*$^+$	—[b]	—	—	—	1.1

[a] Assayed by uptake of 0.5 μM L-[^{3}H]valine.
[b] —, Not determined.

The EMS-induced mutants were located by F′ mapping by using F′ 140-5/86, which covers the *liv* region near *malT* at min 74 on the *E. coli* chromosome, a region in which the previous Mu-induced leucine transport mutants were found to map (4). Complementation data (Table 3) indicate that all of the mutations are complemented by F′ 140-5/86.

Complementation studies of the *liv* transport mutants. To determine the number of the genes that can be assigned to branched-chain amino acid transport from the EMS-induced mutations, genetic complementation studies were performed. Homogenotes from the mutant strains AE300 (*livK300*), AE301 (*livH301*), and AE302 (*livH302*) were isolated as described in Materials and Methods and used for mating with *recA* derivatives of each of the mutant strains. The complementation properties of the resultant sexductants were examined by measuring L-valine transport activity, and the results are presented in Table 3. Mutations *livH301* and *livH302*, both from class I, were found to complement one another. Although not conclusive, these results suggest that mutants originally designated as *livH301* and *livH302* represent two distinct genes, which were tentatively named *livH* and *livG*, respectively. There was the possibility, however, that intracistronic complementation occurred within the *livH* gene. To confirm the existence of the *livG* gene, we took advantage of the availability of the pOX1 plasmid which contained all the genes for LIV-I and LS transport systems (20). We constructed a number of recombinant plasmids containing a subset of pOX1 DNA fragments representing different sequences downstream from the *livK* gene and attempted to map functionally and physically the *livG* gene as described below.

We also found that the *livK300* mutation failed to complement either the *livH301* or the *livG302* mutation and that the *livH*::Mu mutation was not complemented by the *livH301* mutation and only weakly complemented by the *livG302* mutation. These complementation patterns can be attributed to polar effects due to a point mutation in *livK* or to the insertion of the Mu element in *livH*, respectively, leading to the conclusion that the *livK*, *livH*, and *livG* genes are part of the same transcriptional unit (see Discussion).

Identification and cloning of *livG*. Previous work reported from this laboratory has shown that the 13-kilobase (kb) *Eco*RI DNA fragment contained in the pOX1 plasmid carries the genetic region for the branched-chain amino acid transport (20). Moreover, the exact locations of the *livJ*, *livK*, and *livH* genes (Fig. 1) have been defined by using subcloning strategies combined with DNA sequence analysis (13, 21;

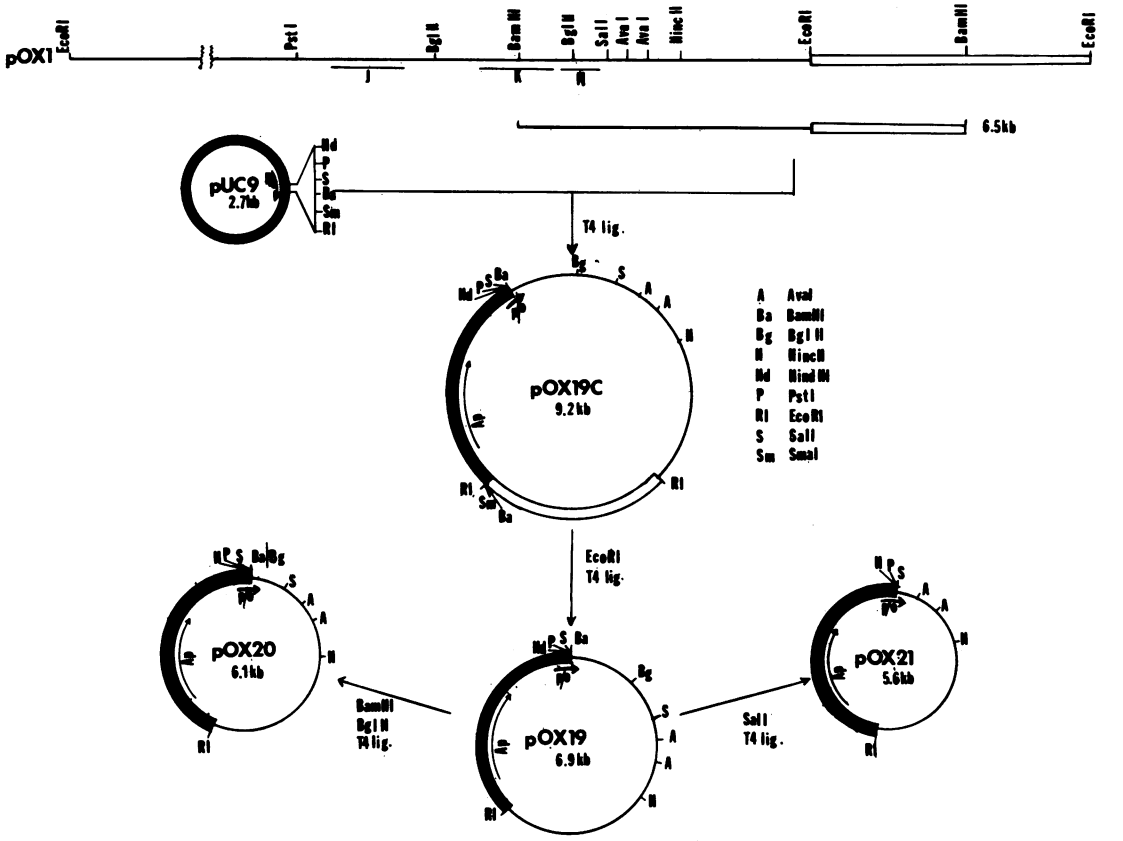

FIG. 1. Construction scheme for plasmids pOX19, pOX20, and pOX21. Plasmid pOX19C was derived by inserting the 6.5-kb *Bam*HI DNA fragment from pOX1 into the single *Bam*HI site of the pUC9 plasmid vector. Plasmid pOX19C was digested with *Eco*RI and religated, which deleted the smaller *Eco*RI fragment and gave plasmid pOX19. Digestion of pOX19 with *Sal*I and subsequent religation produced pOX21. In addition, pOX19 was digested with *Bam*HI and *Bgl*II and religated to produce plasmid pOX20. The double open line represents the portion of the pACYC184 vector of plasmid pOX1, the double solid line represents the plasmid vector pUC9, and the single line indicates *E. coli* chromosomal DNA.

Landick, Ph.D. thesis). Because of the physical map positions of *livK* and *livH* and the potential polar effects of *livK300*, *livH301*, and *livH114*::Mu mutations, we assumed that the *livG* gene should map downstream from the *livH* gene. We could eliminate the region on pOX1 upstream from the *livJ* gene as a potential location of the *livG* gene since a new operon, *htpR*, has been recently shown to map immediately upstream from the *livJ* gene (18).

To locate the *livG* gene physically in the *liv* regulon, we constructed recombinant plasmids pOX19, pOX20, and pOX21 from pOX1, with variable start points within or downstream from the *livH* gene, by applying the strategy shown in Fig. 1. DNA samples were isolated from plasmids pOX19C and pOX19C2, which carried the 6.55-kb *Bam*HI DNA fragment cloned into pUC9 vector in both orientations, with respect to the *lac* promoter. Plasmid pOX19C, which was shown by restriction enzyme analysis (data not shown) to carry the *livH* and *livG* genes in the correct orientation, was used for further constructions. We were able to eliminate the pACYC184 DNA sequences (vector of pOX1) in plasmid pOX19C by digesting it with *Eco*RI and religating the mixture at a low DNA concentration to produce the pOX19 plasmid (Fig. 1). Plasmid pOX19 carries the 4.2-kb *Bam*HI-*Eco*RI DNA fragment from pOX1 cloned into the pUC9 vector. Moreover, both the *Bam*HI and *Sma*I sites of the pUC9 plasmid were deleted when the 2.3-kb *Eco*RI fragment was removed. The presence of a single *Bam*HI site, a *Bgl*II site (in the *liv* locus), and two *Sal*I sites (one in the vector linker and one in the *liv* locus) allowed us to perform the following constructions. The pOX19 plasmid DNA was further digested with *Sal*I and religated to eliminate the *livH* gene contained within the 1.3-kb *Sal*I fragment, yielding the pOX21 plasmid. In an alternative approach to the preparation of plasmid pOX20, the pOX19 plasmid was cut with *Bam*HI and *Bgl*II and then religated to destroy the 5′ end of the *livH* gene by eliminating a 0.85-kb *Bam*HI-*Bgl*II fragment, yielding the pOX20 plasmid. The 1.3-kb *Bam*HI-*Sal*I DNA fragment from pOX1, which carries the *livH* gene, was cloned into pBR322 cut with *Bam*HI and *Sal*I and gave the pOX14 plasmid (manuscript in preparation).

The transport gene components contained in the pOX14, pOX19, pOX20, and pOX21 plasmids were determined by transforming the AE404 *livH301 recA* and AE405 *livG302 recA* transport-defective strains with these plasmids and by testing their ability to restore L-leucine transport activity. The results obtained from these complementation studies, along with a restriction map of the *Bam*HI-*Eco*RI restriction fragment from pOX1, are summarized in Fig. 2. As shown in the figure, plasmid pOX14 was able to complement the *livH301* mutation (strain AE404) but failed to complement the *livG302* mutation (strain AE405). Alternatively, plasmids pOX20 and pOX21, which do not contain the *livH* gene, were able to complement the *livG302* mutation but failed to complement the *livH301* mutation. These results, combined

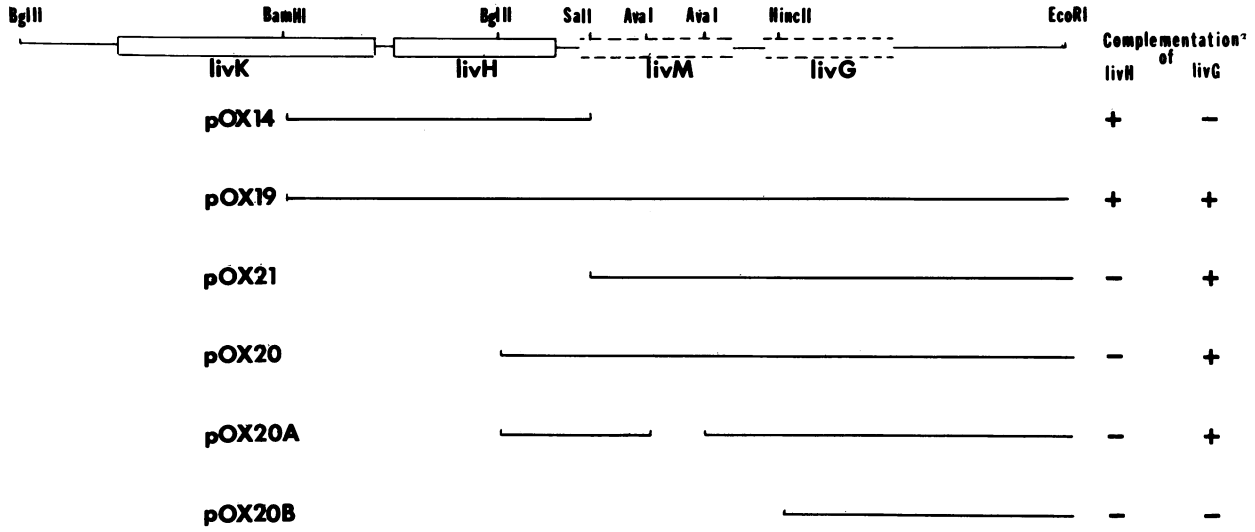

FIG. 2. Complementation analysis of *livH* and *livG* genes by various pOX plasmids. The solid lines indicate the portion of the *Bgl*II-*Eco*RI fragment shown at the top that is contained in the various plasmids listed on the left. On the right, the positive or negative results of the complementation analysis with these plasmids and the *livH* and *livG* strains are shown. Complementation was determined by measuring transport of 0.1 μM L-[^{3}H]leucine in plasmid-transformed *livH* mutant strain AE404 and *livG* mutant strain AE405.

with the known physical location of the *livH* gene on pOX1 derived from DNA sequence information, strongly suggest that the *livG302* mutation represents a new gene which we have named *livG* and which is located downstream from the previously identified *livH* gene.

To localize the *livG* gene more accurately within the cloned 2.9-kb *Sal*I-*Eco*RI DNA fragment, we constructed a number of internal deletions in the pOX20 plasmid and examined the ability of these deletion plasmids to complement the AE405 *livG* mutant strain. To make these plasmids, the pOX20 plasmid was digested with *Ava*I restriction endonuclease, treated with the Klenow enzyme to create blunt ends, and religated to produce the pOX20A plasmid which carries a 300-bp *Ava*I deletion approximately 300 bp downstream from the *livH* gene (Fig. 2). In addition, we took advantage of the presence of two *Hinc*II restriction sites in the pOX20 plasmid (Fig. 1), one in the pUC9 polylinker region and a second one about 1.1 kb downstream from the *livH* gene, to produce another deletion plasmid. The pOX20 plasmid was cut with *Hinc*II restriction endonuclease and religated to give the pOX20B plasmid which carries a 1,000-base-pair (bp) deletion ending approximately 1 kb downstream from the *livH* gene. These plasmids were used to transform the AE405 (*livG*) mutant strain and were tested for their ability to restore L-leucine high-affinity transport activity. Plasmid pOX20A complemented the *livG302* mutation whereas plasmid pOX20B failed to complement it (Fig. 2). From these results, we concluded that the *livG* gene begins 600 to 1,000 bp downstream from the *livH* gene, within the *Ava*I-*Hinc*II DNA fragment. We currently are determining the DNA sequence of this region. We also attempted to identify the *livG* gene product by examining the pOX19-, pOX20-, and pOX21-encoded proteins in minicells harboring these plasmids. The polypeptides were labeled with L-[^{35}S]methionine, separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. Minicells containing the plasmid pOX19 produced two proteins with molecular weights of approximately 27,000 and 22,000 which were absent from the pUC9-containing minicell background. The 27,000-molecular-weight protein (27K protein) was also synthesized by the pOX20 plasmid containing minicells (data

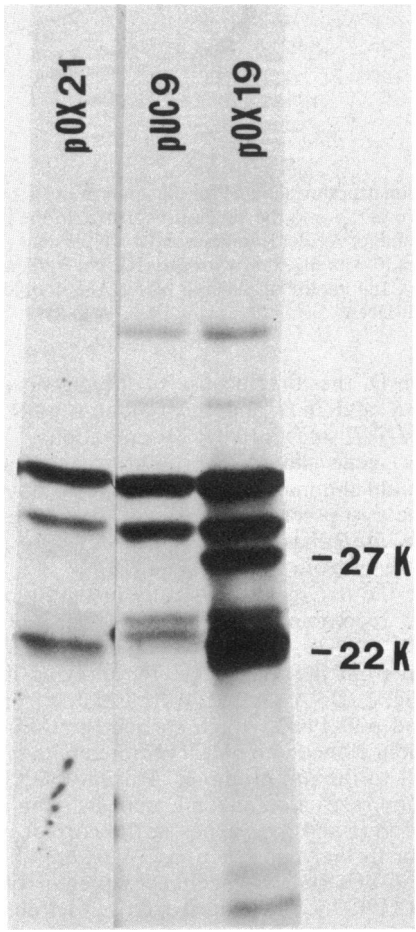

FIG. 3. Fluorogram of 12.5% SDS–polyacrylamide gel electrophoresis, illustrating L-[^{35}S]methionine-labeled proteins synthesized in minicells carrying the indicated plasmids. The unlabeled molecular weight markers (not shown) included bovine serum albumin (M_r, 67,000), ovalbumin (45,000), pepsin (34,700), trypsinogen (24,000), and lysozyme (14,300).

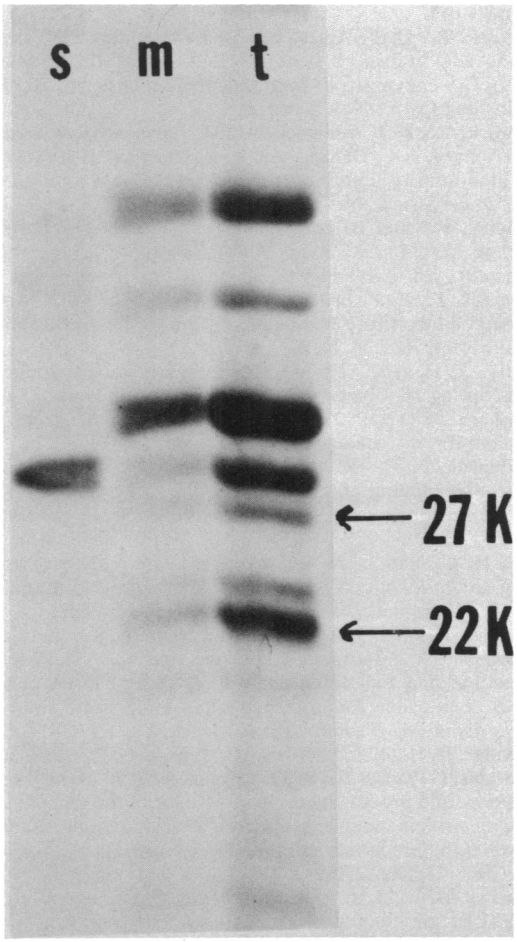

FIG. 4. Fluorogram of 12.5% SDS–polyacrylamide gel electrophoresis, illustrating the cellular fractionation of L-[^{35}S]methionine-labeled proteins synthesized by plasmid pOX19 in minicells. Lane s contains the soluble fraction (cytosol plus periplasm); lane m contains the membrane fraction, approximately 50% of the total sample; lane t contains unfractionated minicells.

not shown) but was not made in detectable amounts by pOX21 plasmid containing minicells (Fig. 3). The small (22K) polypeptide, however, was present in pOX21-containing minicells. It appears that neither the 27K protein nor the 22K protein is synthesized by pOX1-containing minicells in detectable levels (20). We assumed that the reason that both proteins are very poorly expressed by pOX1-containing minicells is that they are under the normal chromosomal promoter control. The fact that the 27K protein was not encoded by the pOX21 plasmid, even though this plasmid has *livG* complementation activity, makes this polypeptide an unlikely candidate for the *livG* product, leaving the 22K protein the most likely candidate. We tentatively conclude that the 27K protein may be the product of an additional gene (*livM*) lying between the *livH* and *livG* genes (see Discussion).

To determine the cellular location of the *livG* protein, pOX19 plasmid-containing minicells were labeled and fractionated into periplasmic, cytoplasmic, and membrane fractions as described in Materials and Methods. The labeled polypeptides of each fraction were analyzed on a 12.5% SDS–polyacrylamide gel. We found that under the conditions used for fractionation, both the 22K (*livG*) protein and the 27K (*livM*) protein were associated predominantly with the membrane fraction (Fig. 4).

DISCUSSION

We have previously shown that high-affinity branched-chain amino acid transport in *E. coli* requires two periplasmic binding proteins and at least one membrane-associated component (4). In those studies, the mutator phage Mu was used to induce mutants in the high-affinity branched-chain amino acid transport in *E. coli*, but because of the nature of the mutations, we were not able to perform complementation studies. In the present study, we have used additional genetic approaches combined with recombinant DNA techniques, to identify one of the membrane-associated components of this system, *livG*. A combination of less stringent selective conditions and the use of derepressed starting strains made it possible to isolate EMS-induced mutants in the three previously identified genes, *livH*, *livJ*, and *livK*, and in a new gene, *livG*. The tentative identification of the *livG* mutation was shown by genetic complementation studies. Since *livH* and *livG* mutations have similar phenotypes, they are difficult to distinguish. Similar results have been reported for the histidine transport system in *Salmonella typhimurium* (2, 10). We were able to use the cloned *liv* regulon to confirm the results of the genetic studies. The *livG* gene was mapped both functionally and physically by using a number of recombinant plasmids carrying different portions from the pOX1 plasmid. We found that plasmid pOX20, which carries a defective *livH* gene; plasmid pOX21, which lacks the *livH* gene; and plasmid pOX20A, which carries a defective *livH* gene and a 300-bp *Ava*I deletion approximately 300 bp downstream from the *livH* gene, all contain a functional *livG* component. These results suggested that *livG* defines a separate component for the high-affinity branched-chain amino acid transport systems, a component which maps downstream from the *livH* gene. The fact that plasmid pOX20A carried *livG* complementing activity and plasmid pOX20B (which lacks a 1.1-kb *Hin*cII fragment) did not suggests that the *livG* gene begins approximately 600 to 1,000 bp downstream from the *livH* gene, within the *Ava*I-*Hin*cII fragment.

A DNA sequence with an open reading frame lies between the *livH* and *livG* gene with a coding capacity for a protein with a molecular weight of approximately 30,000. This observation, taken together with the results from the minicell expression experiments, suggests that an additional gene may be located between the *livH* and *livG* and that the 27K protein present in the pOX19- and pOX20-containing minicells may be the product of this gene. The additional putative gene was named *livM* and is currently under further investigation. The 22K protein produced by the pOX19-, pOX20-, and pOX21-containing minicells is tentatively identified as the *livG* gene product since its presence corresponds to the *livG*-complementing activity of these plasmids. Both *livG* and the putative *livM* gene products appear to be membrane associated. The existence of multiple components for the shock-sensitive, high-affinity, branched-chain amino acid transport systems appears to be a common feature for other binding protein-dependent transport systems (1, 13, 30).

A common property of the membrane components of shock-sensitive transport systems is that they are often expressed at very low levels (2, 9, 27, 28). We attempted to amplify the expression of the *livG* gene by cloning the gene after a strong, controllable promoter, such as the *lac* promoter. We found that the 22K and 27K proteins are both

expressed at higher levels from the pOX19 plasmid, which is under *lac* promoter control, than from the pOX1 plasmid, which is under chromosomal control (21).

As shown by the complementation studies, the *livK* mutation that we have examined in strain AE300 failed to complement either the *livH* or the *livG* mutations. Although the nature of the mutation in the *livK* gene is not known, it appears to be a polar mutation since the selection scheme we used demanded that it also be defective in the LIV-I transport system. These and other results suggest that *livK*, *livH*, and *livG* genes are located on the same transcriptional unit (unpublished data).

We are currently studying the nature of the membrane-associated transport components by taking advantage of the amplified expression of the cloned genes. For these studies, we have constructed a number of β-galactosidase gene fusions to the *livH*, *livM*, and *livG* genes.

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