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Cloning and Characterization of *livH*, the Structural Gene Encoding a Component of the Leucine Transport System in *Escherichia coli*

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The physical location of the genetically defined *livH* gene was mapped in the 17-kilobase plasmid pOX1 by using transposon Tn*5* inactivation mapping and further confirmed by subcloning and complementation analysis. These results indicated that the *livH* gene maps 3′ to *livK*, the gene encoding the leucine-specific binding protein. Moreover, the nucleotide sequence of the *livH* gene and its flanking regions was determined. The *livH* gene is encoded starting 47 base pairs downstream from the *livK* gene, and it is transcribed in the same direction as the *livK* gene. The *livK-livH* intergenic region lacks promoter sequences and contains a GC-rich sequence that could lead to the formation of a stable stem loop structure. The coding sequence of the *livH* gene, which is 924 base pairs, specifies a very hydrophobic protein of 308 amino acid residues. Expression of *livH*-containing plasmids in minicells suggested that a poorly expressed protein with an M_r of 30,000 could be the *livH* gene product.

High-affinity transport of the branched-chain amino acids in *Escherichia coli* is mediated by two periplasmic binding protein-dependent transport systems (4, 25). One of these systems, designated leucine specific (LS), contains a periplasmic binding protein, the *livK* gene product, which binds both isomers of leucine. The second system is a general system, designated LIV-I, and utilizes the leucine-isoleucine-valine (LIV) binding protein, the *livJ* gene product. Genetic analysis has indicated that both high-affinity transport systems require at least two additional components, the products of the *livH* and *livG* genes (4, 24, 29). All four genes, *livJ, livK, livH,* and *livG*, are clustered at minute 76 on the *E. coli* linkage map. By using recombinant DNA techniques, this cluster of genes has been cloned, and the exact location of the *livJ* and *livK* genes on plasmid pOX1 has been reported (20, 28). The products of these genes, the LIV and LS binding proteins, have been extensively characterized (20). Recently, the physical location of the *livG* gene on plasmid pOX1 and the identification of a new gene, *livM*, most likely involved in transport, have been reported (24).

Although several binding protein-dependent transport systems have been studied in bacteria, the molecular basis of the transport process is not yet well understood (1, 3, 18, 19). It is known, however, that these systems utilize both periplasmic binding proteins and membrane-associated components. Most binding protein-dependent transport systems derive their energy from either high-energy chemical bonds or a membrane potential (2, 15, 16).

In this report, the location of the *livH* gene carried by pOX1 has been defined by transposon Tn*5*-mediated mutagenesis and genetic complementation studies with pOX1 derivative plasmids. We have determined the complete nucleotide sequence of the *livH* gene and its predicted amino acid sequence. Finally, the *livH* gene product has been tentatively identified as a protein with an M_r of 30,000 in an *E. coli* minicell expression system.

MATERIALS AND METHODS

Bacterial strains, phage, and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. All bacterial strains were *E. coli* K-12 derivatives. Minicell strain X1411 (*minA1 glnU42 minB2*) was obtained from R. Helling, The University of Michigan. Phage λ::Tn*5* was obtained from C. Yanofsky, Stanford University.

Media and chemicals. Restriction endonucleases and bacterial alkaline phosphatase were from New England Bio-Labs and Bethesda Research Laboratories; T4 DNA ligase and T4 kinase were purchased from New England Bio-Labs. L-[^{35}S]methionine and [γ-^{32}P]ATP were from Amersham Corp. [α-^{35}S]dATP, the M13 universal primer, and Klenow polymerase were from New England Nuclear Corp. Acrylamide and *N,N′*-bismethyleneacrylamide were from Bio-Rad Laboratories. Agarose was from Bethesda Research Laboratories. Nonradioactive dideoxy- and deoxynucleotides were purchased from Pharmacia and P-L Biochemicals, Inc. Antibiotics and other chemicals were from Sigma Chemical Co. In most of the experiments, cells were grown in Luria broth medium supplemented with 50 μg of thymine per ml. For the transport assays and for osmotic shock treatment, cells were grown in Vogel-Bonner medium (35) or MOPS (morpholinopropanesulfonic acid) minimal medium (26) supplemented with the appropriate amino acids (4). Where appropriate, antibiotics were used at the following concentrations: ampicillin, 25 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 25 μg per ml; and tetracycline, 15 μg/ml.

DNA manipulations. Restriction endonuclease digestions of plasmid DNA were performed as suggested by the suppliers. DNA ligations and transformations were performed as described previously (22). Plasmid DNA was prepared from a mid-log-phase bacterial culture grown in rich medium by the sodium dodecyl sulfate (SDS) lysis procedure (22). Plasmid DNA was further purified by dye-buoyant density centrifugation in an ethidium bromide-cesium chloride gradient as described previously (22). Rapid preparations of

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

Strain or plasmid	Genotype	Source or reference
Strain		
AE84	*argG6 hisG1 trp-31 thyA746 malA1 rpsL104 mtl-2 araC601 fhuA2 lacY1 supE44 gal-6 gyrA260 xyl-7 pdxC3 livR*	4
AE205	*malA1*$^+$ *glpD livH livJ*; otherwise as AE84	J. Anderson
AE404	*recA glpD malA1*$^+$ *thyA*$^+$ *livH*; otherwise as AE84	25
AE405	*recA glpD malA1*$^+$ *thyA*$^+$ *livG*; otherwise as AE84	25
W3110	F$^-$ λ$^-$	CGSC[a]
Plasmid		
pBR322	*bla*$^+$ *tet*$^+$	
pACYC184	*cat*$^+$ *tet*$^+$	
pOX1	*tet*$^+$ *livJ*$^+$ *livK*$^+$ *livH*$^+$ *livG*$^+$	27
pOX7	*bla*$^+$ *livK*$^+$	28
pOX14	*bla*$^+$ *livH*$^+$	This study
pOX16	*bla*$^+$ *livH*$^+$ *livK*$^+$	This study
pOX17	*cat*$^+$ *livH*$^+$ *livK*$^+$	This study
pOX17A	*cat*$^+$	This study

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

plasmid DNA from small volumes of cultures or from large size colonies were obtained by alkaline lysis and by the boiling method described previously (22). The plasmid DNA and DNA fragments were analyzed by gel electrophoresis in 1 or 2% horizontal agarose slab gels in Tris acetate buffer or in 5% vertical acrylamide gels in Tris borate buffer (22).

Transposon insertion mutagenesis. Transposon insertions with the transposon Tn*5* were introduced into the Tet^r plasmid pOX1 by infection of strain W3110(pOX1) with phage λc1857 b221 carrying the transposable element Tn*5* (λ::Tn*5*) as described previously (7). A 5-ml culture of strain W3110 containing pOX1, growing exponentially in Luria broth containing 0.2% maltose, was collected by centrifugation, suspended in 10 mM $MgSO_4$, and mixed with λ::Tn*5* at a multiplicity of infection of 5 to 10. The mixture was incubated at 30°C for 1 h. The cells were placed on fresh plates containing kanamycin and tetracycline to select for pOX1::Tn*5* transformants, and were incubated 48 h at 30°C. Sodium pyrophosphate was added to prevent phage readsorption. The kanamycin- and tetracycline-resistant colonies were collected by washing the cells off the plates with a small volume of Luria broth, and the plasmid DNA was isolated by a quick-screening method (30). This plasmid DNA was used to transform the double transport mutant strain AE205 (*livJ livH*) to Kan^r and Tet^r. The kanamycin-resistant, tetracycline-resistant colonies were then isolated on minimal media plates containing valine (0.05 mg/ml) to select for valine-resistant colonies, some of which might be LIV-I mutants. Thirty Val^r transformants were isolated and screened for the presence of high-affinity L-valine and L-leucine transport and for the presence of the LIV binding protein.

Transport assays and detection of binding protein. Transport assays were performed on logarithmically growing cells in Vogel-Bonner or MOPS minimal medium as described previously (4). Binding protein was detected in shock fluid as described previously (4).

DNA sequencing analysis. Most of the nucleotide sequences was obtained by the method of Maxam and Gilbert (23). DNA fragments were 5′ labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, as described previously (22), and precipitated from 2.5 M ammonium acetate with ethanol. The labeled fragments were digested with an appropriate restriction endonuclease, separated through a 0.2-mm-thick 5% polyacrylamide gel and purified by the method of Maxam and Gilbert (23). Before the sequencing reactions, the labeled fragments were ethanol precipitated from 50 mM ammonium acetate. The sequencing reactions were performed by the method of Maxam and Gilbert, except for the A > G reaction. This reaction was carried out by a modification (20) of the original procedure. Additional DNA sequencing was carried out by the method of Sanger (32) on both strands of the *Bgl*II-*Sal*I fragment of *livH*, which had been cloned into phages M13mp18 and M13mp19 and linearized with *Bam*HI. The DNA sequence data were analyzed by using the computer program of Allen Delaney of the University of British Columbia, Vancouver, Canada, which operates on The University of Michigan Computer Center system. The probable orientation of the protein within the membrane was determined based on hydrophobicity predictions from the protein sequence by using a program based upon the method developed by Pat Argos, Purdue University, West Lafayette, Ind. (5).

Plasmid DNA expression in minicells. Minicells were purified from the *E. coli* X1411 minicell-producing strain as described previously (9) with minor modifications. Transformed minicell-producing strains were grown to an A_{600} of 0.8 in 250 ml of MOPS-rich medium without leucine and containing the appropriate antibiotics to maintain the plasmids. Cells were harvested by centrifugation at 10,000 × *g* for 15 min at 4°C and suspended in 35 ml of BSGE buffer (150 mM NaCl, 5 mM KH_2PO_4, 5 mM Na_2HPO_4, 1 mM EDTA, 100 mg of gelatin per ml; pH 7.8). Parental cells were partially removed by centrifugation at 3,000 × *g* for 1 min. The supernatant fluid containing the partially purified minicells was centrifuged at 10,000 × *g* for 15 min to pellet the minicells. The contaminating viable cells were removed by using two cycles of centrifugation (Sorvall SW28 rotor at 5,000 rpm for 5 to 10 min) on a 5 to 30% sucrose gradient in BSGE buffer. Before the labeling step, minicells were incubated for 20 min at 37°C in MOPS-rich medium without leucine and methionine, to allow the decay of mRNA (28), and then labeled for 30 to 50 min by addition of 10 μCi of L-[^{35}S]methionine per 0.1 ml. Labeled minicells were subjected to SDS-acrylamide gel electrophoresis as described previously (17).

RESULTS

Location of *livH* gene in the *liv* regulon. We have previously reported the cloning of the *E. coli* high-affinity branched-chain amino acid transport regulon contained on a 13 kilobase (kb) *Eco*RI DNA fragment in plasmid pOX1 (27). The precise locations of the *livJ* and *livK* transport genes on this plasmid have been determined by genetic complementation by using various cloned restriction fragments and DNA sequencing analysis (20) (Fig. 1).

To identify the location of the *livH* gene in the *liv* region, we performed insertion inactivation mapping on the plasmid pOX1. *E. coli* W3110(pOX1) was infected with a temperature-sensitive λ::Tn*5* phage, and the kanamycin- and tetracycline-resistant colonies were isolated as described in Materials and Methods. These conditions provided a selection for random Tn*5* insertions either in the chromosome or in plasmid pOX1 since Tet^r selects for pOX1 and Kan^r selects

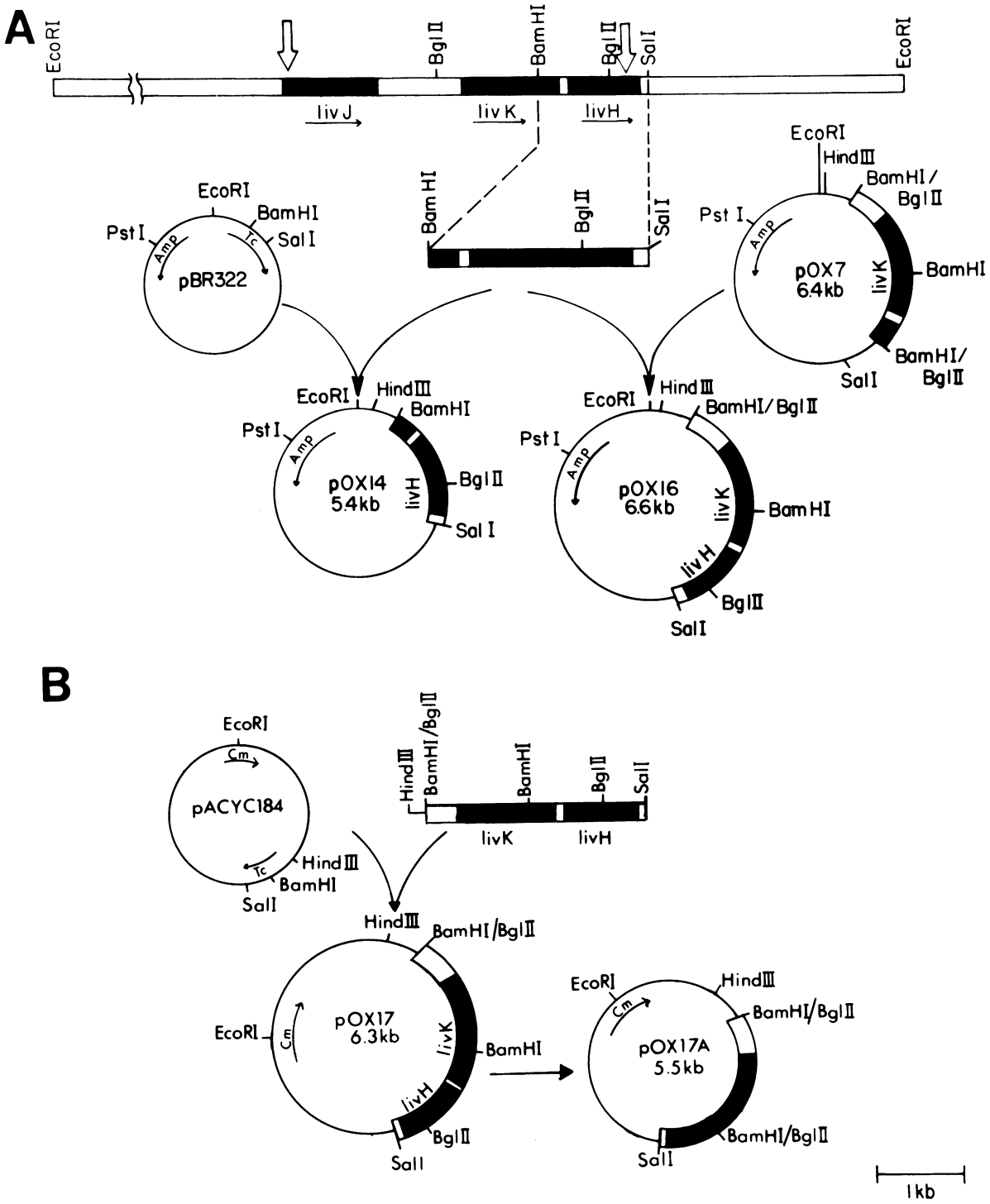

FIG. 1. Construction scheme for plasmids pOX14, pOX16, pOX17, and pOX17A. The top line of panel A represents the 13-kb *Eco*RI insert from the plasmid pOX1 (27). Solid boxes indicate the positions of three of the genes involved in branched-chain amino acid transport as determined from DNA sequencing. Arrows beneath the genes show the direction of transcription. The positions of the Tn*5* insertions are indicated by the vertical arrows. Thin lines represent vector segments, and double lines represent inserted segments derived from pOX1. (A) The 1.3-kb *Bam*HI-*Sal*I fragment from pOX1 was subcloned into pBR322 digested with *Bam*HI and *Sal*I to yield pOX14. This same fragment was also subcloned into pOX7 digested with *Bam*HI and *Sal*I, resulting in pOX16. (B) The 2.85-kb *Hin*dIII-*Sal*I, fragment from pOX16 was subcloned into pACYC184 digested with *Hin*dIII and *Sal*I to produce pOX17. The plasmid pOX17 was digested with *Bam*HI and *Bgl*II and religated to construct pOX17A. In all of these constructions, the designation *Bam*HI/*Bgl*II indicates a hybrid site which cannot be recleaved by either *Bam*HI or *Bgl*II.

TABLE 2. Complementation analysis of *livH* and *livJ* mutations by using pOX1::Tn*5* plasmid derivatives

Strain	Plasmid	*LIV-I Uptake* (nmol/min per mg)[a]	
		L-Valine	L-Leucine
AE84	None	1.0	0.9
AE205	None	0	0
AE205	pOX1	ND[b]	1.3
AE205-8	pOX1 *livH*::Tn*5*-8	0.1	0.1
AE205-24	pOX1 *livJ*::Tn*5*-24	0	0.8
AE404	None	0	0
AE404	pOX1	ND	1.4
AE404	pOX1 *livH*::Tn*5*-8	ND	0.2
AE404	pOX1 *livJ*::Tn*5*-24	ND	1.7
AE405	pOX1 *livH*::Tn*5*-8	ND	0.34
AE405	pOX1 *livJ*::Tn*5*-24	ND	2.0

[a] Cells were grown in 0.2% glucose-VB minimal medium. Uptake is expressed in nanomoles per minute per milligram of dry weight of cells from 0.5 μM L-valine or 0.1 μM L-leucine added.
[b] ND, Not determined.

for Tn*5*. We isolated the crude plasmid DNA mixture from the W3110 (pOX1) strain and used it to transform the double mutant strain AE205 (*livH*, *livJ*) to kanamycin resistance, tetracycline resistance, and valine resistance. Since *E. coli* K-12 strains are very sensitive to valine, Val^r is a phenotype for LIV transport-defective strains (4). Strain AE205 (*livJ livH*) containing a pOX1::Tn*5* derivative plasmid with a Tn*5* insertion in the *liv* locus is likely to be defective in the *livJ* gene or the *livH* gene or both and would be valine resistant. Cultures grown from the Val^r colonies were screened for L-valine transport. Among 30 Val^r isolates, 14 were unable to transport L-valine, suggesting that they contained *livJ*::Tn*5* or *livH*::Tn*5* mutations in plasmid pOX1. A preliminary report of these findings has been published elsewhere (R. Landick, P. Mavromara, and D. L. Oxender, Fed. Proc. **40**:1894, 1981).

To confirm these results, we measured the high-affinity L-leucine transport activities of these transformants. Strain AE205(pOX1::Tn*5*) transformants with a defective *livJ* gene would be expected to show a normal L-leucine transport activity due to the presence of the high-affinity leucine-specific transport system (4), while AE205(pOX1::Tn*5*) transformants with a defective *livH* gene would be expected to have very low L-leucine transport activity since the *livH* gene is a common component for both of the high-affinity transport systems (4, 29). By using these screening methods, we identified both *livJ*::Tn*5* and *livH*::Tn*5* insertions on pOX1. Complementation analysis of representative insertions is described in Table 2. Transformed strain AE205-8 showed no high-affinity uptake for L-valine or L-leucine, suggesting that Tn*5* had inactivated *livH*, whereas transformed strain AE205-24 showed high-affinity uptake for L-leucine only, indicating that Tn*5* had inactivated the *livJ* gene (Fig. 1). The complementing activity of pOX1 *livH*::Tn*5* and pOX1 *livJ*::Tn*5* derivative plasmids was verified by transforming strains AE404 and AE405, *livH* and *livG* mutants, respectively, and by measuring L-leucine transport (Table 2). In addition, the synthesis of the LIV binding protein of the AE205 pOX1::Tn*5*-transformed strains was examined. Strain AE205-8 expressed both the LIV and LS binding proteins, whereas strain AE205-24 synthesized only the LS binding protein (data not shown).

The location of the Tn*5* insertion in the *livH* gene was determined by restriction mapping analysis, taking advantage of the known restriction cleavage sites in plasmid pOX1 and in transposon Tn*5* (27, 31). This analysis indicated that in the pOX1 *livH*::Tn*5* plasmid, Tn*5* was inserted within the 0.5-kb *Bgl*II-*Sal*I segment about 100 base pairs (bp) downstream from the *Bgl*II site (Fig. 1). These data indicate that the *livH* gene is located downstream from *livK*. We assumed that Tn*5* inactivates *livH* gene expression by interrupting the structural gene, although it is also possible that the *livH* gene may be located further downstream from the position of the Tn*5* insertion. In the latter case, *livH* gene expression would be abolished by the polar effects of the transposon (7). To determine whether the Tn*5* location identifies the structural gene for *livH*, we subcloned the region of plasmid pOX1 that contained the *livH*::Tn*5* mutation.

Cloning the *livH* gene. The 1.3-kb *Bam*HI-*Sal*I DNA fragment from pOX1 was cloned into pBR322 that had been digested with *Bam*HI and *Sal*I, yielding a 5.4-kb plasmid identified as pOX14 (Fig. 1). Previous data (24) indicate that this DNA fragment should be large enough to carry the *livH* gene, together with the 3'-terminal portion (360 bp) of the *livK* gene. We also assumed that it would not contain the promoter which serves for expression of the *livH* gene because previous results suggested that the *livH* gene product was translated from a polycistronic mRNA with the transcriptional start site located proximal to the *livK* gene (4, 20). We therefore cloned the *livH* gene within the tetracycline resistance region of pBR322, yielding pOX14, so that expression of the *livH* gene could be achieved from the tetracycline promoter. In parallel to the construction of pOX14, the 1.3-kb *Bam*HI-*Sal*I DNA fragment from pOX1 was cloned into pOX7 which had been digested with *Bam*HI and *Sal*I to produce the 6.6-kb pOX16 plasmid (Fig. 1). Plasmid pOX16 carries the *livH* gene together with the entire *livK* structural gene and its regulatory sequences.

The presence of the *livH* gene within the 1.3-kb *Bam*HI-*Sal*I DNA fragment and the ability of plasmids pOX14 and pOX16 to express this gene were tested for by determining the ability of these plasmids to complement the *livH* mutation of strain AE404. When either pOX14 or pOX16 was introduced into AE404 (*livH*), L-leucine high-affinity transport was restored, whereas, when either plasmid was introduced into strain AE405 (*livG*), complementation of transport activity was not achieved (Table 3). These results suggest that the entire *livH* gene is contained in the 1.3-kb *Bam*HI-*Sal*I DNA fragment and is expressed by both pOX14 and pOX16.

Nucleotide sequence of the *livH* gene. We sequenced the 1.3-kb *Bam*HI-*Sal*I DNA fragment from pOX14 mainly by using the Maxam and Gilbert sequencing procedure with minor modifications, as described previously (20, 23). Each run was repeated at least twice, and the entire sequence has

TABLE 3. Complementation analysis of AE404 (*livH*) and AE405 (*livG*) by pOX plasmids

Plasmid	L-Leucine uptake (nmol/min per mg) in[a]:	
	AE404 (*livH*)	AE405 (*livG*)
None	0	0
pOX1	1.6	1.7
pOX14	1.7	0.1
pOX16	1.7	0.1
pOX17	2.2	0.1
pOX17A	0.1	0.1

[a] Uptake is expressed in nanomoles per minute per milligram (dry weight) of cells from 0.1 μM L-leucine added.

FIG. 2. DNA sequence of the *livH* gene and its flanking regions. The first line contains the stop codon from *livK* (TGA) and the intercistronic DNA sequence between *livK* and *livH*. The numbering is relative to the ATG of *livH*. The box at −8 (AAGG) represents the putative Shine-Dalgarno site for *livH*, while the inverted arrows between −40 and −20 represent a potential stem loop formed by the mRNA. The DNA sequence of the *livH* gene has been translated into the predicted amino acid sequence, and the DNA sequence following the gene is included until the *Sal*I site.

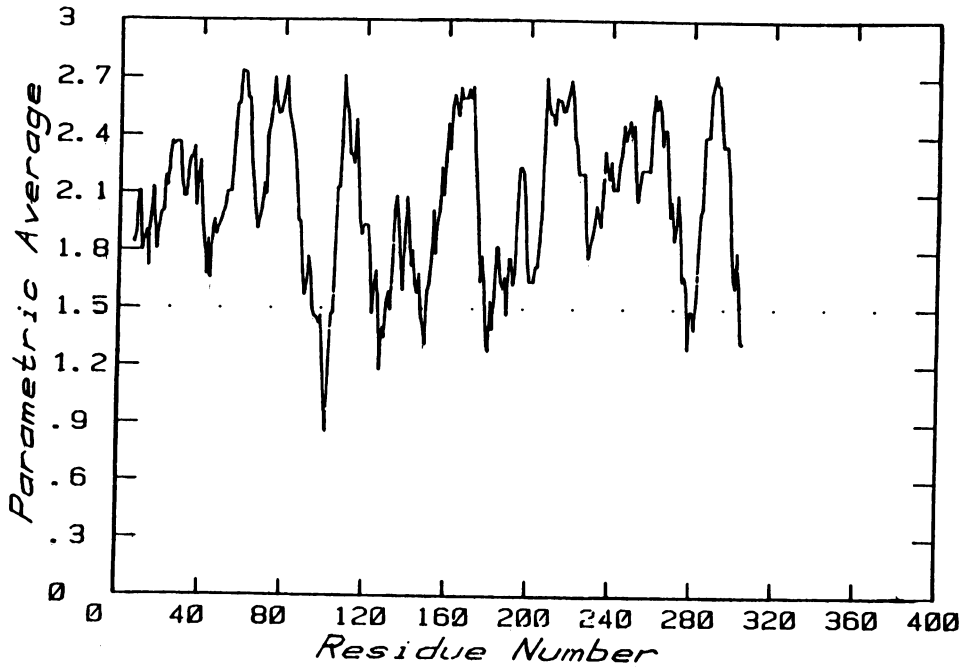

FIG. 3. Hydrophobicity profile of the LivH protein. The average hydrophobicity of an 8-residue segment was determined as the computer program advanced through the protein (N terminus to C terminus). Hydrophobicity values assigned per amino acid were taken from reference 5.

been determined on both strands. A continuous open reading frame of 924 nucleotides was found, starting 47 bases downstream from the *livK* gene. The sequence data indicate that the *livH* gene was transcribed in the same direction as the upstream *livK* gene. Fig. 2 shows the nucleotide sequence of the *livH* gene and the predicted amino acid sequence of the *livH* gene product.

The possible initiation site is preceded at a distance of 7 bp by a nucleotide sequence (AAGG) that could serve as a potential Shine-Dalgarno ribosome-binding site (13). It is predicted, therefore, that this AUG codon (position +1) serves to initiate transcription of the *livH* structural gene (Fig. 2). This prediction has been supported by the construction of a *livH-lacZ* gene fusion that contains only the first ATG codon (position +1) and the next nine codons of the *livH* gene. This fusion produced a hybrid protein with an apparent molecular weight similar to that of wild-type β-galactosidase (data not shown).

The codon usage for the *livH* gene was highly nonrandom and reflected the abundance of the cognate tRNA species present in *E. coli* (data not shown). The ATA (Ile), AAT (Asn), ACA (Thr), CAA (Gln), and AGG (Arg) codons, which are used infrequently in efficiently expressed genes in *E. coli*, were not used more than once in the *livH* gene. Similar results have been reported for the codon usage for genes coding for the membrane proteins of other bacterial transport systems (8, 12, 14).

The DNA sequence of the *livH* gene (924 nucleotides) encodes a protein with a mass of approximately 38,000 daltons. The LivH protein is similar to other membrane-associated transport proteins as it is a hydrophobic protein (more than 70% of its amino acid residues are nonpolar) and appears to be positively charged at neutral pH. Leucine is one of the most abundant amino acid residues in the LivH protein, as was also found for both the LS and LIV binding proteins (20).

The NH_2-terminal sequence of the LivH protein does not contain a typical signal peptide. A cleavable signal peptide, however, has not been found for most of the known inner membrane proteins such as HisP, HisQ, HisM, LacY, MalF, and MalK (8, 10, 12, 14). It is assumed that the internal hydrophobic domains of these proteins are sufficient to insert and assemble the proteins in the cell membrane. To analyze the hydrophobic nature of the LivH protein, we used the method described by Argos et al. (5). The hydrophobicity profile of the LivH protein, calculated with a window of eight residues, is shown in Fig. 3. The LivH protein was very hydrophobic with a mean hydrophobicity of about 2. This value is similar to that calculated for the HisQ protein and bacteriorhodopsin by this same method. There were nine hydrophobic segments within the protein of an average length of 20 amino acid residues or more which were likely to span the cell membrane (Fig. 3). Alternatively, there were only a few hydrophilic regions where most of the charge was located. Interestingly, the longest hydrophilic regions, which contained most of the charged amino acids, occurred in the middle of the protein. It is possible that these segments or domains remained outside the cell membrane, and they might represent regions that could interact with the binding protein-substrate complex or with the energy-coupling component to carry out the transport events.

The *livK-livH* intercistronic region. The intercistronic region between the *livK* and *livH* genes was 47 bp in length and did not appear to contain promoter-like sequences (Fig. 2). This observation, combined with previous data from genetic studies, suggests that the *livH* gene is part of a polycistronic mRNA that uses the *livK* promoter and may also include the downstream *livM* and *livG* genes. An interesting feature of the *livK-livH* intergenic region is the presence of a palindromic sequence of seven bases that could lead to the formation of a stable stem-loop structure in the corresponding transcript with a calculated free energy value (34) of −22

kcal/mol. An almost identical stem-loop structure has been found in the 45-bp intergenic region between the *lacZ* and *lacY* genes (8). This GC-rich secondary structure was very similar to a typical transcription termination site and might represent an intercistronic regulatory element (30) (see Discussion).

Identification of the LivH protein. To identify the *livH* gene product, the 2.85-kb *Hin*dIII-*Sal*I DNA fragment from pOX16 was cloned into the *Hin*dIII and *Sal*I sites of the pACYC184 vector, to give plasmid pOX17 (Fig. 1). We used the plasmid pACYC184 because the vector-coded proteins migrate to a different position on SDS-acrylamide gels than do the *livH* gene product and the β-lactamase that is coded for by the plasmid pBR322. The ability of pOX17 to express the *livH* gene was first examined by transforming pOX17 into the transport mutant strain AE404 (*livH*) and by measuring the high-affinity leucine transport activity of the transformed strain. Plasmid pOX17 was able to complement the transport defect in strain AE404 (*livH*) but not in strain AE405 (*livG*) (Table 3).

The polypeptides encoded by the pOX17 plasmid were expressed in minicells, labeled with L-[^{35}S]methionine, separated by SDS-polyacrylamide gel electrophoresis and visualized by fluorography (Fig. 4). Minicells containing only the plasmid vector pACYC184 produced one predominant polypeptide with an apparent mass of 22 kilodaltons, which was identified as the chloramphenicol resistance gene product (Fig. 4). The additional minor bands on the gel most likely resulted from stable mRNA, which survived in the minicells (21). Minicells containing pOX17, in addition to the chloramphenicol resistance gene product, produced another predominant polypeptide of 39 kilodaltons which is the LS binding protein. A minor protein with an electrophoretic mobility similar to that of beta-lactamase was also synthesized reproducibly by pOX17 minicells but was not made by pACYC184-containing minicells (Fig. 4). These results suggest that this minor protein (30 kilodaltons) may be the *livH* gene product.

Additional evidence that the 30-kilodalton protein is encoded by the *livH* gene was obtained by in vitro deletion mutagenesis including the *livH* gene contained on the pOX17 plasmid. The 0.8-kb *Bam*HI-*Bgl*II DNA fragment was removed by digestion of pOX17 with *Bam*HI and *Bgl*II, followed by religation at a low DNA concentration to produce the plasmid pOX17A. Since the 0.8-kb deleted *Bam*HI-*Bgl*II DNA fragment carries portions of both the *livK* and *livH* genes, it was expected that plasmid pOX17A would be unable to complement the *livH* mutation and would fail to produce the LS binding protein and the LivH protein. pOX17A was not able to restore transport when introduced into strain AE404 (Table 3). Plasmid pOX17A-encoded polypeptides were labeled with [^{35}S]methionine in minicells and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4). Neither the 39-kilodalton binding protein nor the 30-kilodalton protein were made by the pOX17A plasmid, suggesting that the 30-kilodalton protein may be the *livH* gene product.

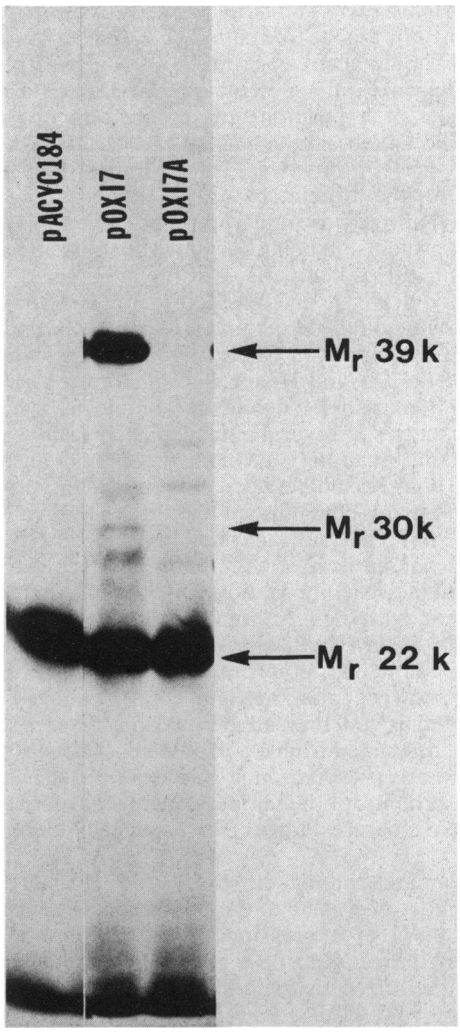

FIG. 4. Analysis of proteins encoded by pACYC184, pOX17, and pOX17A in minicells. [^{35}S]methionine-labeled proteins were separated in a 12.5% SDS–polyacrylamide gel and fluorographed. The band labeled M_r 39k corresponds to the LS binding protein, and the band labeled M_r 30k corresponds to the putative *livH* gene product.

DISCUSSION

By using genetic approaches, we determined that the high-affinity branched-chain amino acid transport in *E. coli* requires at least two additional components, LivH and LivG, besides the periplasmic LIV and LS binding proteins (4, 24). The location and the nucleotide sequence of the *livH* gene has been determined, and a candidate for the LivH protein was tentatively identified by using minicells.

The *livH* gene is contained within a 1.3-kb *Bam*HI-*Sal*I fragment of plasmid pOX1, as judged by the ability of plasmids carrying this subcloned fragment to complement strains carrying a *livH* mutation. The location of *livH* was first mapped on plasmid pOX1 by Tn*5* mutagenesis. We have isolated both *livH*::Tn*5* and *livJ*::Tn*5* insertions by using plasmid pOX1. All of the pOX1 *livJ*::Tn*5* plasmid derivatives had a functional *livH* gene despite the known polar effects of the transposon insertions (7). On the other hand, we found that the *livH*::Tn*5* insertion exerted a polar effect on *livG*, since the plasmid pOX1*livH*::Tn*5* was not able to complement the transport defect of strain AE405 (*livG*) (Table 2). These results are in agreement with other findings from this laboratory, and they suggest the existence of at least two transcriptional units for the *liv* locus, one for the *livJ* gene and another for the *livK*, *livH*, *livM*, and *livG* genes (20, 24).

The nucleotide sequence of the 1.3-kb *Bam*HI-*Sal*I DNA fragment contains a 924-bp open reading frame oriented in the same direction as the *livK* gene which codes for a protein

of approximately 38 kilodaltons. We propose that the start site of the *livH* gene is the ATG codon at position +1, located 47 bp downstream from the *livK* gene. This is based on the presence of a potential Shine-Dalgarno ribosome-binding site at a position appropriate only for this ATG codon. The amino acid sequence of the amino terminus of the LivH protein, however, will unambiguously define the start site for the protein.

The LivH protein is similar to other membrane-associated transport proteins, in being very hydrophobic and somewhat basic (2, 6, 10). A comparison of the amino acid sequences among LivH, HisQ, and HisM proteins did not show any significant homology (H. Nikaido, personal communication). Similarly, a comparison of the amino acid sequences determined for the Liv and His periplasmic binding proteins has shown only marginally significant homologies, although the X-ray structures of several binding proteins show a common bilobate conformation (19). On the other hand, an amino acid sequence comparison between HisP and MalK proteins, the probable energy-coupling components of their transport systems, shows a significant homology (11, 15, 16). Also, an extensive comparison of the amino acid sequences of the MalF protein, a membrane component of the maltose transport system, with the membrane components of the histidine transport system (HisP, HisQ, and HisM) of *S. typhimurium*, as well as to other inner membrane nontransport proteins, showed, again, no significant homology (10). It has been hypothesized that all periplasmic transport systems may have originated from a common ancestor system (18). If this hypothesis is correct, it appears that the transmembrane transport proteins of the various systems have retained their hydrophobic natures rather than specific amino acid sequences.

The identification of the *livH* gene product has been difficult, most likely due to the poor expression of the gene. The low level of expression of the membrane transport components appears to be a common property of all presently characterized osmotic shock-sensitive transport systems (2, 6, 33). The LivH protein has been tentatively identified in minicells as a weak protein band corresponding to a mass of 30 kilodaltons on the basis of the expression of this protein from plasmid pOX17 (*livH*$^+$), but not from the vector pACYC184 or from the plasmid pOX17A (*livH*$^-$). The mobility of the protein is similar to that of the beta-lactamase protein, and preliminary cellular fractionation experiments with minicells have indicated that it is a membrane-associated protein (data not shown). The apparent mass of the putative LivH protein as observed from the electrophoretic mobility of the protein on SDS gels (30 kilodaltons) is smaller than that predicted from the nucleotide sequence (38 kilodaltons). Such discrepancies appear to be common for hydrophobic proteins, presumably due to an abnormally high capacity for binding SDS (8, 10).

The molecular mechanism responsible for the decreased expression of the membrane proteins still remains obscure. In the case of LivH, it is possible to hypothesize that the potential stem-loop structure in the *livK-livH* intergenic region may decrease the level of the mRNA coding for the membrane components either as a transcriptional attenuator or as a recognition site for mRNA processing. Alternatively, since the distance between the potential stem-loop and the potential ribosome binding site is only 7 bp, it is also possible that this structure may reduce ribosome binding and, thereby, alter or inhibit translational initiation. We have recently constructed *livH-lacZ* fusions which contain only the first 10 amino acids of the LivH protein fused in frame to β-galactosidase, and we observed that the *livH-lacZ* hybrid gene is efficiently expressed. These preliminary results could be taken to suggest that a more complex posttranscriptional regulatory mechanism may be involved in the expression of the *livH* gene.

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