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Structural and functional analysis of cloned DNA containing genes responsible for branched-chain amino acid transport in *Escherichia coli*

(cloning/leucine transport genes/*in vitro* expression/processing/minicells)

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ABSTRACT **The four genes encoding the components of the high-affinity branched-chain amino acid transport systems in *Escherichia coli* (*livH, livG, livJ,* and *livK*) have been cloned into λ phage and subsequently into the plasmid vector pACYC184. The presence of the four structural genes and their accompanying regulatory regions on the resultant plasmid, pOX1, was confirmed by genetic complementation analysis and by transport studies carried out on the appropriate transformed mutant strains. When pOX1 DNA was used to direct an *in vitro* transcription/translation system, four major polypeptide products were produced. Immunoprecipitation with antibody directed against the LIV-binding protein identified the two leucine-binding proteins as products of *in vitro* synthesis. The binding proteins were produced in precursor forms and had molecular weights approximately 2500 higher than the processed, mature forms. A minicell-producing strain transformed with plasmid pOX1 produced the binding proteins in the processed form.**

The branched-chain amino acids are transported into *Escherichia coli* by two osmotic-shock-sensitive high-affinity systems. One of these, designated LIV-I, is a general transport system whereas the second, LS, is leucine specific. In addition, there is an osmotic-shock-insensitive low-affinity general system, LIV-II, that transports the branched-chain amino acids (1–4). Genetic studies indicate that there are at least four polypeptides, specified by the *livJ, livK, livH,* and *livG* genes, in the two high-affinity transport systems. A fifth gene, designated *livP,* when mutationally altered, specifically affects the LIV-II transport system (5). The first four genes are clustered at 74 min on the recalibrated *E. coli* linkage map (4). Two of these genes, *livJ* and *livK,* encode the periplasmic LIV-binding protein and LS-binding protein, respectively. These proteins, which serve as amino acid receptors for the two high-affinity transport systems, have been isolated and extensively characterized (6). Genes *livH* and *livG* are both required for high-affinity transport and have only been characterized genetically (4). In order to identify all of the components required for high-affinity branched-chain amino acid transport, we have cloned the *E. coli* chromosomal region containing the branched-chain amino acid transport genes. We introduced this region first into the genome of phage λ and subsequently into a multicopy plasmid vector. Strains with the plasmid showed 3- to 5-fold increased expression of high-affinity leucine transport. The cloned DNA was used to direct *in vitro* and *in vivo* synthesis of transport polypeptides, including the periplasmic binding proteins.

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MATERIALS AND METHODS

Bacterial Strains, Phage, and Plasmids. *E. coli* strain AE168 (F^- *arg his leu trp thy livP livH lstR*) was constructed by standard genetic techniques and has been described (5). Strain X1411, a minicell-producing strain, was obtained from R. Helling. Phage λNM781 (7) and plasmid pACYC184 (8) have been described.

Restriction Endonuclease Digestion and Ligation of DNA Fragments. Endonuclease digestions were performed as described (9). *Sal* I, *Bam*HI, and *Eco*RI restriction endonucleases were purified by the procedure of Greene *et al.* (9). *Hin*dIII was prepared by the heparin-agarose affinity chromatography procedure of Bickle *et al.* (10). All other enzymes were obtained from BRL (Rockville, MD). Plasmid and phage restriction fragments were analyzed on horizontal 0.8% agarose gels as described (11, 12). DNA fragments generated by *Hin*dIII and *Eco*RI restriction of λ DNA were used as molecular weight standards (13). The ligation and transformation steps were carried out as described (12, 14). Plasmid DNA was prepared by a $NaDodSO_4$ lysis procedure and further purified by CsCl ultracentrifugation (15).

Preparation of λNM781–*E. coli* Pool. λNM781, which is a replacement vector for cloning DNA fragments generated by *Eco*RI (7), was used in the production of a bank of *E. coli* genes. Phage DNA was prepared as described by Thomas and Davis (16), and *E. coli* DNA was prepared from strain W3110 by the procedure of Saito and Miura (17). One microgram of phage and *E. coli* DNAs was digested with *Eco*RI, ligated (80 μg/ml), and used to transfect *E. coli* strain SF8 (18). Phage from the resulting plaques (approximately 6300) were harvested and passaged through *E. coli* strain C600 mK^+ rK^- to yield a high-titer stock. This pool was the source of λNM781–*Eco*RI recombinants used in subsequent experiments. Approximately 60% of the phage in the pool contained *E. coli* DNA inserts (7). P1 containment was used according to NIH Recombinant DNA Research Guidelines.

Selection of λ Lysogens. The transport mutant strain, AE168, which is unable to grow on 5 μg of L-leucine or 150 μg of D-leucine per ml, was grown on 0.2% maltose and infected with λNM781–*Eco*RI at 34°C. Cells were plated on medium containing low levels of L-leucine or high levels of D-leucine and incubated at 34°C. Resulting lysogens were screened for leucine transport activity by the rapid transport assay of An-

Abbreviations: Mops, 4-morpholinepropanesulfonic acid; LIV, branched-chain amino acid transport system; LS, leucine-specific branched-chain amino acid transport system; kb, kilobase.

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derson and Oxender (4). Ten percent of the lysogens that could grow on the selective plates showed increased transport activity. One lysogenic strain exhibiting normal transport activity, designated strain AE168 λ5.1, was purified and used for further study.

Construction of *liv* Plasmids. *E. coli* strain AE168 was again used for selection of plasmids containing the *liv* genes as described for the λ5.1 selection. A lysate of λ phages carrying an insert that complemented the *livH* mutant was prepared by heat induction of the AE168 λ5.1 lysogen. The phage DNA (16) was restricted with *Eco*RI endonuclease and mixed with *Eco*RI-restricted pACYC184 plasmid DNA. The mixture was ligated with T4 ligase and the DNA obtained was used to transform strain AE168. Transformants were selected on medium containing 150 μg of D-leucine per ml. The D-leucine, if transported, would satisfy the L-leucine requirement. The plates also contained 10 μg of tetracycline per ml to select for the vector that carries the gene for tetracycline resistance.

Transport and Binding Protein Assays. Transport of L-leucine was carried out on logarithmically growing cells in 4-morpholinepropanesulfonic acid (Mops) minimal medium (19) as described (4). To determine binding protein activity, we osmotically shocked harvested cells and determined the leucine-binding activity by equilibrium dialysis with L-[^{3}H]leucine.

***In Vitro* Synthesis.** Protein was synthesized *in vitro* by the coupled transcription/translation system of Zubay (20) as described (21, 22). Plasmid DNA was used as template. Samples of the S-30 reaction mixture were run on 12.5% acrylamide/$NaDodSO_4$ gels according to Laemmli (23). Immunoprecipitated samples were prepared with specific antisera against the LIV-binding protein as described (4).

Minicell Labeling. Minicells (24) were isolated from mid-logarithmic phase transformed *E. coli* strain X1411. The cells were grown in 300 ml of Mops complete medium (19) (without leucine) containing appropriate antibiotics for maintaining the plasmid. Cells were harvested by centrifugation and suspended in 35 ml of 5 mM phosphate buffer (pH 7.8) containing 150 mM NaCl, 1 mM EDTA, and 100 μg of gelatin per ml. Whole cells were removed by centrifuging twice for 1 min at 3000 × *g*. Centrifugation in a 5–30% sucrose gradient in an SW-27 rotor at 5000 rpm for 5 min resulted in enrichments of greater than 10^4 minicells per whole cell. Minicells (10^9) were incubated for 45 min in 0.1 ml of Mops complete medium lacking leucine and methionine and then labeled for 1 hr by addition of 10 μCi of L-[^{35}S]methionine. Labeled minicells were collected by centrifugation, washed, and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis as described above.

RESULTS

Cloning of Leucine Transport Genes. Selection of transducing phages and plasmids carrying the branched-chain amino acid transport genes was facilitated by the construction of a mutant strain of *E. coli*, AE168, defective for both high-affinity (*livH*) and low-affinity (*livP*) leucine transport. Strain AE168 requires high levels of L-leucine for growth because transport is defective. It cannot grow on either 5 μg of L-leucine or 150 μg of D-leucine per ml. Complementation of the *livP* mutation will, however, allow growth on 5 μg of L-leucine per ml (but not on 150 μg of D-leucine per ml) because the LIV-II system is restored. Complementation of the *livH* mutation restores both phenotypes due to the restoration of the LIV-I system. Strain AE168 also contains the regulatory mutation, *lstR*, which permits constitutive expression of leucine transport genes.

Selection of a λ*liv* Transducing Phage. The initial selection was made for a transducing phage from a λ phage lysate that had been prepared by inserting *Eco*RI DNA restriction fragments from wild-type *E. coli* into λNM781 by the procedures described above. Lysogens of strain AE168 (*livP livH*) able to grow on low L-leucine (5 μg/ml) were divided into two classes by their leucine transport phenotypes (uptake of 0.1 μM L-leucine and ability to grow on 150 μg of D-leucine per ml): those regaining the low-affinity LIV-II transport system (*livP*$^+$) and those regaining the high-affinity LIV-I transport system (*livH*$^+$). Phage λ5.1 was found to carry *livH*$^+$.

Subcloning the *liv* Region. The 13-kilobase (kb) *Eco*RI insert from λ5.1 DNA was recloned into the single *Eco*RI restriction site of plasmid pACYC184 as described in *Materials and Methods*. Transformants complementing *livH* were selected in strain AE168 by growth on minimal medium containing 10 μg of tetracycline per ml and 150 μg of D-leucine per ml as the source of L-leucine. Plasmid DNA was extracted from a representative recombinant plasmid, designated pOX1, and subjected to restriction analysis.

Restriction Map. Restriction of pOX1 DNA with *Eco*RI gave two fragments, one approximately 4 kb, corresponding to the vector (8), and one of 13 kb, the bacterial DNA insert (Fig. 1). The DNA was then subjected to single and mixed digestions by using other restriction endonucleases in the now familiar algebraic method of obtaining a unique orientation of the restriction sites. Our present map of these sites is depicted in Fig. 1. The relatively small number and distribution of the infrequent sites for endonucleases such as *Bam*HI, *Bgl* II, and *Hin*dIII have facilitated further subcloning strategies, to be reported.

Leucine Transport by Transformed Strains. The values of the kinetic variables K_m and V_{max} of leucine uptake in the various strains we have prepared are presented in Table 1. As reported previously (5), no saturable uptake of L-leucine was detectable in the transport mutant strain AE168. The results presented in Table 1 show that the λ5.1 lysogen of strain AE168 regained high-affinity L-leucine uptake, as determined by its low apparent K_m as well as its ability to grow on D-leucine. The latter requires an operational leucine-specific system (4). The K_m and V_{max} values of the lysogen AE168 λ5.1 were similar to those of strain AE137, which had been transduced to *livH*$^+$ by P1 tranduction. The data are consistent with the interpretation that a single *livH*$^+$ allele is present in strain AE168 λ5.1.

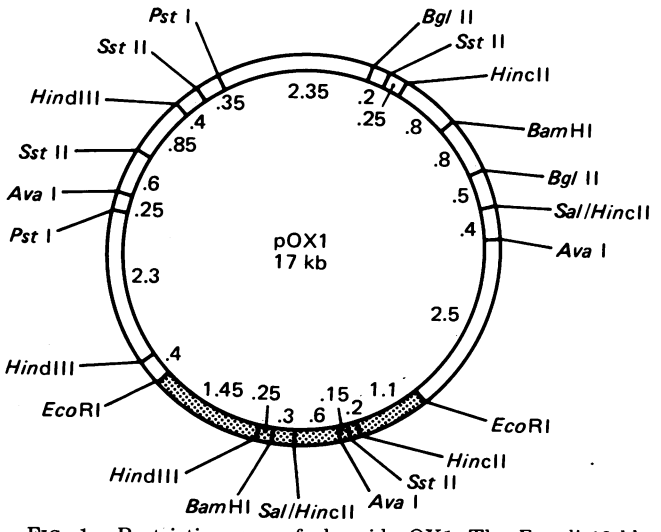

FIG. 1. Restriction map of plasmid pOX1. The *E. coli* 13-kb *Eco*RI insert from phage λ5.1 (text) was ligated into the *Eco*RI site of the plasmid vector pACYC184 (shaded area). The relative positions of restriction sites and the size (in kb) of intervening segments were determined as in *Materials and Methods*.

Table 1. K_m and V_{max} of LIV-I transport system in control and transformed strains

Strain	Added DNA	Chromosomal background	LIV-I transport system* K_m, μM	LIV-I transport system* V_{max}†
AE168	—	*lstR livP livH*	NM	NM
AE137	—	*lstR livP livH*$^+$	0.6 (0.4–0.9)	3.1 (2.4–4)
AE168	λ5.1	*lstR livP livH*	0.6 (0.2–2.0)	2.1 (1.2–2.5)
AE168	pOX1	*lstR livP livH*	0.6 (0.2–0.8)	10.0 (7–14)

* Determined as described in ref. 25. Values in parentheses are 67% confidence limits. NM, not measurable.
† V_{max} is given in nmol/min per mg dry weight.

In contrast, the pOX1 transformed strain, which showed a similar K_m for leucine uptake, had a 3- to 5-fold elevated V_{max}. These data are consistent with the expectation that there are multiple copies of the plasmid and, hence, of the genes coding for the rate-limiting components in leucine transport by the LIV-I system. Although the selection explicitly involved only complementation of *livH*, the fact that the genes for the LIV-I system are closely linked encouraged us to test for increased synthesis of the leucine-binding proteins. Using the equilibrium dialysis binding assay, we found a 5-fold elevation in the levels of the leucine-binding proteins specified by the *livJ* and *livK* genes (data not shown). In these experiments we compared osmotic shock fluids from pOX1-containing strains relative to a nontransformed control.

Regulation of Plasmid-Encoded Transport. The expression of the LIV-I transport system in *E. coli* is regulated by the level of leucine in the medium (26). To determine if plasmid pOX1 contains the regulatory region for the LIV-I genes, we transformed strain AE89 (*livH*) with pOX1 DNA and grew the transformed strain, AE89/pOX1, in the presence and absence of leucine. Table 2 shows that wild-type strain AE62 exhibited a 4-fold repression of leucine transport when grown in the presence of L-leucine. Similarly, strain AE89/pOX1 showed a 5-fold repression of leucine transport by L-leucine. In addition, this strain showed a 4-fold increase in leucine transport over strain AE62 when both strains were grown in the absence of leucine. A determination of the levels of leucine-binding proteins in strains AE62 and AE89/pOX1 grown in the presence and absence of L-leucine established that their synthesis was also regulated (data not shown). These results suggest that the regulatory regions controlling expression of the leucine transport genes are present on pOX1.

Genetic Complementation Analyses. We used genetic complementation analyses to determine how many of the known transport genes were contained on plasmid pOX1. The leucine transport genes *livH*, *livG*, *livJ*, and *livK* are clustered near minute 74 of the *E. coli* genetic map (4). We transformed strains carrying each of the mutant alleles with pOX1 and determined the complementation pattern. *recA* recipient strains were used. We found that pOX1 complemented all four known genes in the *liv* region; these include the structural genes for the LIV-binding protein (*livJ*) and the leucine-specific binding protein (*livK*), and *livH* and *livG*, other genes whose products are required for high-affinity leucine transport (4).

Table 2. Regulation of leucine transport in control and transformed cells

Strain	Genotype	Uptake velocity* No L-Leucine	Uptake velocity* L-Leucine	Factor of repression
AE62	*livH*$^+$	0.52	0.14	4
AE89/pOX1	*livH*$^-$/*livH*$^+$	1.9	0.38	5

* Velocity of uptake of 0.1 μM L-leucine in nmol/min per mg dry weight of cells. Cells were grown with and without 50 μg of L-leucine per ml.

***In Vitro* Synthesis of Leucine-Binding Proteins.** Purified pOX1 DNA was used to direct the synthesis of polypeptides in a coupled transcription/translation system. Fig. 2 shows the pattern of L-[^{35}S]methionine-labeled proteins separated by NaDodSO$_4$/12.5% acrylamide gel electrophoresis. Lane A shows the pattern obtained with vector pACYC184 DNA alone. Lane B shows that several new bands appeared in the pOX1 DNA-directed reaction. Of these, two bands, of approximate molecular weights of 40,000 and 42,000, were specifically precipitated by antibody to the LIV-binding protein (lane C). These polypeptides had molecular weights approximately 2500 higher than the mature forms of the two leucine-binding proteins. The positions of the mature forms of the leucine-binding proteins are shown by the arrows. We interpret these results as indicating that the binding proteins are present in their precursor forms. These forms can be processed *in vitro*, as we will report in a separate publication. It is obvious from inspection of lane B that pOX1 also codes for other polypeptides.

Synthesis and Processing of Binding Proteins in Minicells. The minicell system for protein synthesis has been useful for the *in vivo* identification of plasmid-coded gene products. This is due to the exclusion of chromosomal, but not plasmid, DNA from minicells (24). A minicell-producing strain, X1411, was transformed with either pOX1 or pACYC184 DNA and transformants were used to generate minicells. The minicells were then labeled with L-[^{35}S]methionine. The NaDodSO$_4$ gel patterns of the labeled polypeptides produced are shown in Fig. 3. Lane A shows the pattern of labeled proteins produced by the pACYC184-transformed minicells; lane B shows the complex pattern obtained with the pOX1-transformed strain. One band of 37,000 molecular weight is at the known position of the mature form of the LIV-binding protein. The absence of a band

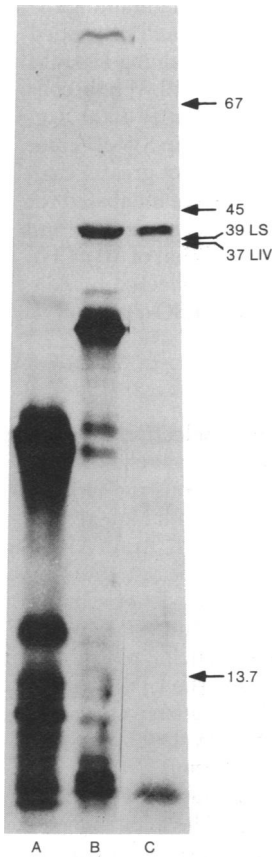

FIG. 2. *In vitro* synthesis of leucine-binding proteins. Purified pACYC184 vector DNA (lane A) and hybrid plasmid pOX1 DNA (lanes B and C) were used as templates in an *in vitro* transcription/translation system. Proteins labeled with L-[^{35}S]methionine *in vitro* were dissolved in NaDodSO$_4$ sample buffer and electrophoresed in 12.5% acrylamide/NaDodSO$_4$ gels. A portion of the reaction mixture with pOX1 DNA was treated with antibody against the LIV-binding protein and the immunoprecipitate was dissolved and run in lane C. Arrows and numerals designate the molecular weights ($\times 10^{-3}$) and positions (top to bottom) of the stained reference proteins bovine serum albumin, ovalbumin, LS-binding protein, LIV-binding protein, and lysozyme.

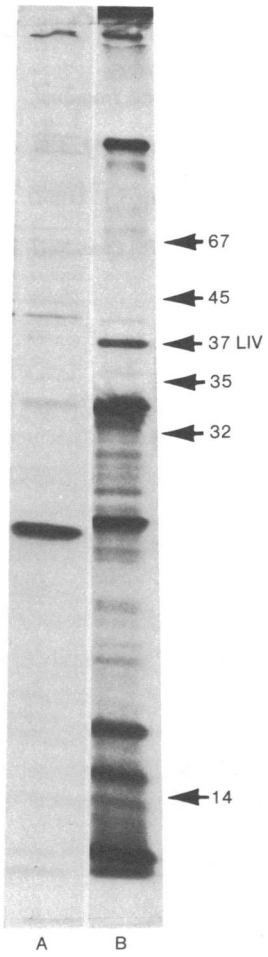

FIG. 3. Protein synthesis in minicells. Minicells were purified and labeled with L-[^{35}S]methionine. The labeled minicells were washed and dissolved in NaDodSO$_4$ sample buffer, and the samples were electrophoresed in 11% acrylamide/NaDodSO$_4$ gels. Arrows and numerals designate marker positions and molecular weights ($\times 10^{-3}$, top to bottom) of bovine serum albumin, ovalbumin, the LIV-binding protein, galactose-binding protein, sulfate-binding protein, and lysozyme. Lane A shows the pattern obtained with minicells containing the vector pACYC184; lane B shows the pattern with minicells containing plasmid pOX1.

of 39,000 molecular weight indicates that minicells process the precursor form of the LIV-binding protein. The mature LS-binding protein, which also has a molecular weight of 39,000, is present in smaller amounts *in vivo* than the LIV-binding protein and, therefore, is not observed under these conditions.

DISCUSSION

The techniques of gene cloning have provided new approaches to the identification and isolation of gene products and for studying gene expression. The data presented here demonstrate that four genes, *livG*, *livH*, *livJ*, and *livK*, necessary for high-affinity branched-chain amino acid transport in *E. coli* K-12, have been cloned into both phage λ and the plasmid pACYC184. Measurement of transport activity in plasmid-bearing strains showed that leucine transport had been increased approximately 4-fold. This increase is similar to the increase in the amounts of periplasmic leucine-binding proteins estimated by equilibrium dialysis of osmotic shock fluid.

Leucine transport activity in wild-type cells is repressed about 4-fold in response to changes in the concentration of leucine (26). The results in Table 2 show that the leucine transport activity of the pOX1-transformed strain is repressed approximately to the normal extent by the presence of L-leucine in the growth medium. Previous studies have shown that mutations altering Rho, leucyl tRNA synthetase, and the products of the *hisT* and *relA* genes influence regulation of branched-chain amino acid transport (26). The *in vitro* expression of the transport genes (Fig. 3) presents the possibility of studying regulation of these genes in a cell-free system. This should permit the identification of regulatory components.

The *in vitro* synthesis studies demonstrate that the binding proteins are initially synthesized in precursor form, as is true of other periplasmic components (27). The molecular weight difference between the mature and the precursor forms is around 2500. This is consistent with the presence of a 20-amino-acid residue "signal" peptide as an NH_2-terminal extension of the mature form. In another report we will show that the LS-binding protein does, in fact, have an NH_2-terminal signal peptide extension of 23 amino acid residues. The accumulation of the precursor forms of the binding proteins in the *in vitro* system should allow us to assay for the precursor processing enzyme(s).

Although two periplasmic components of leucine transport have been characterized, genetic studies indicate that the products of additional genes are required for transport function. These proteins, which we presume are integral membrane components, are specified by *livH* and *livG*. These genes are on plasmid pOX1, as determined by complementation analyses. This offers the opportunity to clone the individual leucine transport genes and to identify their polypeptide products *in vitro* and *in vivo*. Subcloning should also allow us to determine the transport functions of the respective gene products and to study the regulatory mechanisms controlling gene expression.

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