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lep Operon Proximal Gene Is Not Required for Growth or Secretion by *Escherichia coli*

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Leader peptidase is an essential enzyme of *Escherichia coli* and is required for protein export. The structural gene for leader peptidase (*lep*) is separated from its promoter by an upstream gene of unknown function (*lepA*). The gene *lepA* was shown by the use of minicell analysis and overproduction to encode a protein of 74,000 daltons. To determine whether this 74,000-dalton protein functions in protein export, a mutant of *E. coli* H560 was constructed which has a 1.5-kilobase-pair deletion in the *lepA* gene. The *lepA* deletion mutant had no apparent defect for growth or protein export, indicating that *lepA* is nonessential and that the two cotranscribed genes *lepA* and *lep* probably have unrelated functions.

Leader peptidase is a membrane protein of *Escherichia coli* which is responsible for removing leader (signal) peptides from precursors of secreted and outer membrane proteins (25). The enzyme has been purified to homogeneity (25, 28), and its structural gene (*lep*) has been isolated (5), sequenced (26), and mapped to minute 55 of the *E. coli* chromosome (20). Leader peptidase is essential for cell growth in *E. coli* (4). The absence of leader peptidase activity in vivo leads to the accumulation of precursor forms of several exported proteins and finally to cell death (3a). Date and Wickner (5) found that expression of leader peptidase required a putative promoter region of 350 base pairs that was approximately 1,500 base pairs distant from the structural gene. Subsequent sequencing studies of *lep* (26) and the upstream region (14) have established that *lep* is the second gene in a transcriptional unit that includes a promoter-proximal upstream gene of unknown function (*lepA*).

We have established by minicell analysis that *lepA* encodes a protein of 74 kilodaltons (kDa). To determine whether this 74-kDa protein had a function related to leader peptidase, a mutant strain of *E. coli* H560 was constructed by gene eviction. The mutant has a 1,500-base-pair deletion within the *lepA* gene. Transcription and translation of active leader peptidase is not affected by this deletion. A comparison of the *lepA* mutant with an isogenic *lepA*$^+$ parental strain did not reveal any differences in the rates of growth or protein export, indicating that *lepA* is nonessential and does not have an essential role in protein export.

MATERIALS AND METHODS

Bacteria and growth conditions. *E. coli* H560 *polA1 rpsL endI tsx* and JM103 Δ(*lac pro*) *thi rpsL supE endA sbcB15 hsdR4* F′ *traD36 proAB lacI*^{q}*Z*Δ*M15* were obtained from D. Ray. To provide easily assayed markers during the gene eviction, a derivative of strain H560 was constructed that was resistant to rifampin and auxotrophic for cysteine. The minicell-forming strain p678-5 was obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn. Strains were grown on TYE, minimal M9, or M63 (17). The *E. coli* H560 *rif cys* derivative was supplemented with 50 μg of cysteine per ml for growth on minimal medium.

Plasmids. Plasmid pTD101 is a derivative of pBR322 that has a 4.8-kilobase-pair (kb) *Bam*HI-*Sal*I insert from minute 55 of the *E. coli* chromosome which includes *lepA* and *lep* (5). Plasmids pTD126 and pTD142 are deletion derivatives of pTD101 and are described in Fig. 1. Plasmid pAC142 is a derivative of pACYC184 (3) that has a 1.5-kilobase-pair *Hin*dIII fragment containing Δ*lepA* from pTD142 cloned into the *Hin*dIII insertion site of pACYC184. Plasmid pPW1701 is an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression vector constructed from pACYC184. The *Hin*dIII-*Bam*HI restriction fragment from pRD540 (19), which contains a *trp*::*lac* fusion promoter, was inserted into the *Hin*dIII-*Bam*HI site in pACYC184. Subsequently, a *Hpa*I-*Mst*II restriction fragment from pTD101, which contains the entire *lepA* structural gene without the promoter region or *lep*, was inserted in both orientations under the control of the *trp*::*lac* fusion promoter of pPW1701. These derivatives are named pPW1706 (correct orientation) and pPW1707 (incorrect orientation).

Construction of the Δ*lepA* deletion mutant. The Δ*lepA* gene of pAC142 was used to replace the wild-type chromosomal copy of *lepA* in *E. coli* H560 by gene eviction (8). The integration of pACYC184 into the chromosome of *E. coli* H560 to give chloramphenicol-resistant integrate strains was as previously described (7). Segregants of the integrate strain that spontaneously lost pAC142 and either *lepA* or Δ*lepA* were isolated as previously described (7) and screened for the loss of either *lepA* or Δ*lepA* by Southern hybridization.

Southern hybridization. Chromosomal DNA was prepared from *E. coli* (7), digested with *Pvu*II, electrophoresed through a 1% agarose gel, and transferred to nitrocellulose (21). The filters were probed with a ^{32}P-labeled oligonucleotide (12) of the sequence CGCCATTCAGGCAGCGATTG, which is complementary to both *lepA* and Δ*lepA*.

Mini-cell analysis. The minicell-forming strain *E. coli* p678-5 was used to identify plasmid-encoded proteins. Strain p678-5 was transformed to ampicillin resistance with the indicated plasmids and then grown to saturation in M9 medium supplemented with 0.5% glucose and 100 μg of ampicillin per ml. Minicells were harvested by centrifugation through two sucrose gradients (5 to 20% in phosphate-

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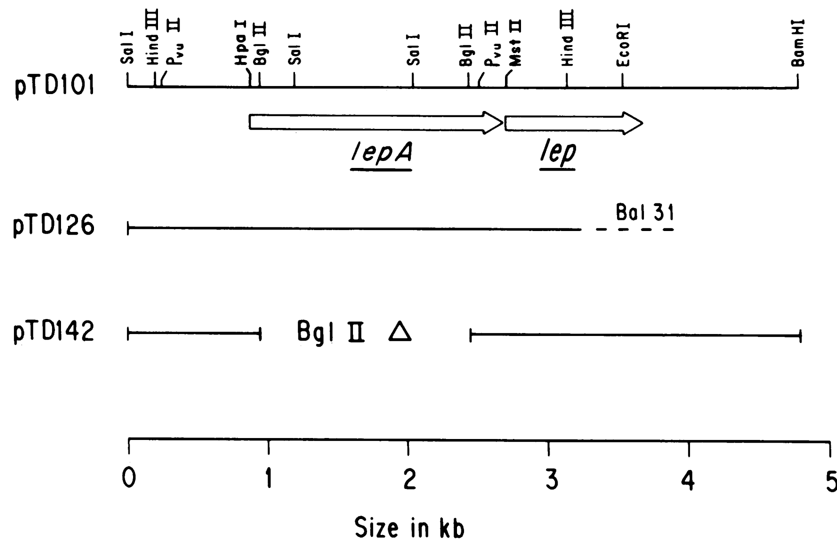

FIG. 1. Restriction map of the 4.8-kb *Sal*I-*Bam*HI insert of pTD101 that contains the *lep* operon of *E. coli*. The arrows indicate the positions and sizes of the *lepA* and *lep* structural genes. Derivatives of pTD101 that have deletions of the *lep* (pTD126) or *lepA* (pTD142) genes are also shown. Plasmid pTD126 was constructed by digestion with BAL 31. The exact position of the deletion junction is not known (-----). Plasmid pTD142 was constructed by deleting a 1.5-kb *Bgl*II fragment within the *lepA* gene.

buffered saline, pH 7.4). Minicells were labeled with 10 μCi of [^{35}S]methionine for 30 min at 37°C. Cell lysates were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and fluorography.

Labeling conditions. Cells were grown in minimal M9 medium containing 0.5% glycerol and 50 μg of each amino acid except methionine per ml at 37°C to mid-log phase. For pulse-labeling, culture samples were labeled with 50 μCi of [^{35}S]methionine (>1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) for 15 s followed by an excess of nonradioactive methionine. Ice-cold trichloroacetic acid was added to a final concentration of 10% (wt/vol).

Immunoprecipitation and gel electrophoresis. Immunoprecipitation of outer membrane protein A (ompA) and maltose-binding protein (MBP) and analysis by SDS-polyacrylamide gel electrophoresis were carried out as previously described (26).

RESULTS

The proximal gene of the *lep* operon encodes a protein of 74 kDa. The *lep* operon consists of two genes, *lep*, which encodes leader peptidase, and *lepA*, a promoter-proximal gene of unknown function (14). To identify the protein encoded by *lepA* we undertook a minicell analysis of the products of pTD101, which is a derivative of pBR322 that contains the entire *lep* operon (5) and two deletion derivatives pTD126 and pTD142 (4) as shown in Fig. 1.

The results of minicell analysis of pBR322, pTD101, pTD126, and pTD142 are shown in Fig. 2. Three polypeptides with molecular sizes of 25, 37, and 74 kDa are encoded in pTD101 (lane 2), but not in pBR322 (lane 1), indicating that at least three polypeptides are encoded by the 4.8-kb insert of pTD101. Plasmid pTD126 (lane 3) is known not to encode active leader peptidase because of a BAL 31-induced deletion at the 3′ end of the *lep* gene (4). As expected, the 37 kDa protein which is leader peptidase is absent from lane 3. Plasmid pTD142 (lane 4) encodes active leader peptidase, indicating that both the *lep* gene and its promoter are intact, but has a deletion of approximately 1,500 base pairs from the *lepA* gene (4). This deletion is not polar for expression of the *lep* gene, because minicells containing pTD142 synthesize leader peptidase (37 kDa) and the 25-kDa polypeptide (lane 4). The absence of the 74-kDa protein in pTD142-transformed cells (lane 4) and its presence in pTD126-transformed cells (lane 3) indicates that the *lepA* gene encodes a 74-kDa protein.

The 25-kDa polypeptide band from pTD101-containing cells (Fig. 2, lane 2) may contain at least two comigrating species. This region is less intense in cells transformed with pTD126 (lane 3) or pBR322 (lane 1). These results suggest that a gene coding for a 25-kDa protein is located downstream from the *lep* gene and is deleted in pTD126. This gene is probably *rnc* which has recently been shown to be present on pTD101 downstream of the *lep* operon (13, 23) and which encodes RNase III, which has a molecular weight of 25,218 (23). The remaining 25-kDa polypeptide encoded by pTD126 is probably also encoded by pBR322 because a faint band of this size can be seen in lane 1.

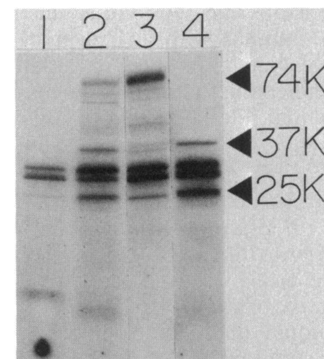

FIG. 2. Minicell analysis of the products of the following plasmids. Lanes: 1, pBR322; 2, pTD101; 3, pTD126; 4, pTD142. The minicell strain p678-5 was used as described in Materials and Methods. Labeled proteins were detected by fluorography after SDS-polyacrylamide gel electrophoresis. Arrows indicate the positions of the three polypeptides encoded by pTD101.

To confirm that the *lepA* gene codes for the 74-kDa polypeptide, an *Mst*II-*Hpa*I restriction fragment from pTD101 (which contains the entire *lepA* structural gene without the promoter region or the *lep* gene) was cloned into pPW1701, an IPTG-inducible expression vector derived from pACYC184. The recombinant plasmids place the *lepA* gene under control of the *trp*::*lac* fusion promoter (6) in either the correct (pPW1706) or incorrect (pPW1707) orientation. Induction of transformants of JM103 containing pPW1706 results in the production of a 74-kDa protein (Fig. 3, lane 2). Transformants containing the *lepA* gene in the opposite orientation do not produce the *lepA* gene product (Fig. 3, lane 4). Overproduction of the *lepA* product does not affect cell viability; nor does it affect the secretion of MBP or assembly of the ompA (data not shown). With the exception that in our expression vector overproduction of the *lepA* product is not lethal, these results confirm the observations of March and Inouye (14).

The *lepA* gene is nonessential. The recent development of gene eviction techniques allows the replacement of chromosomal genes of *E. coli* with mutant alleles cloned on a plasmid (8). Plasmid pTD142 encodes active leader peptidase (4), indicating that both the *lep* gene and its promoter are intact, but makes no detectable *lepA* product because it has a 1.5-kb deletion of *lepA* (see Fig. 2). These results indicated that it would be possible to replace the chromosomal *lepA* gene with the deleted *lepA* gene (*lepA*) of pTD142 without disrupting expression of the leader peptidase gene, which is essential for the growth of *E. coli* (4). The inability to isolate such chromosomal recombinants would suggest that the *lepA* gene is essential for cell viability.

To construct a Δ*lepA* strain, the Δ *lepA* gene on a *Hin*dIII restriction fragment from pTD142 was first subcloned into the plasmid pACYC184. This step was necessary because pACYC184 encodes resistance to chloramphenicol, a bacteriostatic antibiotic which facilitates susequent steps of gene eviction. The derivative plasmid pAC142 was used to transform a *polA* strain of *E. coli* H560 by selecting for chloramphenicol resistance. Because pAC142 cannot replicate in a *polA* host, transformants occurred at a frequency of only 10^{-9} (10). This low frequency of transformation is consistent with the frequency of integration of pAC142 into the chromosome by homologous recombination at the *lep* locus. No transformants were obtained with pACYC184, which lacks the *lep* and *lepA* genes.

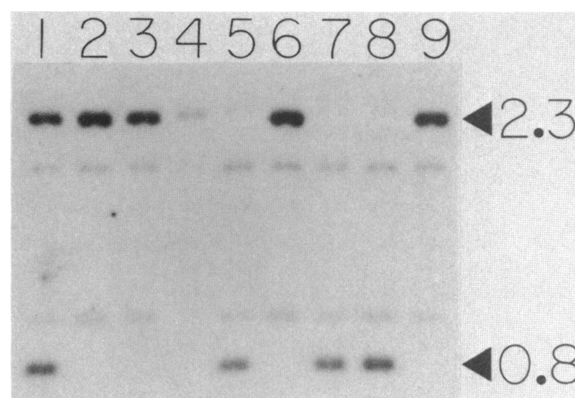

FIG. 4. Southern hybridization analysis of chromosomal DNA prepared from a Δ*lepA* integrate strain (lane 1), a *lepA*$^+$ parental strain (lane 2), and from seven independent segregant strains (lanes 3 through 8). Chromosomal DNA was digested with *Pvu*II, fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and then probed with a ^{32}P-labeled oligonucleotide complementary to both *lepA* and Δ*lepA*. Arrows indicate the *Pvu*II fragments of 2.3 and 0.8 kb which contain sequences complementary to the oligonucleotide (see Materials and Methods).

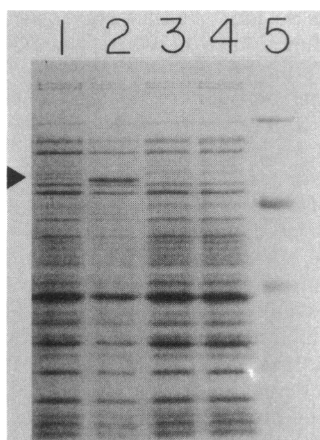

FIG. 3. SDS-polyacrylamide gel analysis of extracts of *E. coli* JM103 transformed with either pPW1706 or pPW1707. Lanes: 1, pPW1706-JM103 noninduced; 2, pPW1706 induced with IPTG; 3, pPW1707 noninduced; 4, pPW1707 induced with IPTG; 5, molecular weight markers β-galactosidase, ovalbumin, and bovine serum albumin. Plasmids pPW1706 and pPW1707 have the *lepA* genes in the correct and incorrect orientations, respectively. The arrow indicates the position of the overproduced product of the *lepA* gene. Strains were grown in TYE at 37°C to mid-log phase in the presence or absence of IPTG and then prepared for gel electrophoresis in SDS. Gels were stained with Coomassie blue.

Cells containing an integrated copy of pAC142 (subsequently referred to as integrate strains) should have both a wild-type and deleted copy of the *lepA* gene (8). To test this, chromosomal DNA from a wild-type and an integrate strain were compared with Southern hybridization. DNA from *E. coli* H560 and an integrate strain was digested with *Pvu*II and hybridized, after agarose gel electrophoresis and transfer to nitrocellulose, with a ^{32}P-labeled oligonucleotide which is complementary to both *lepA* and Δ*lepA* (23). A *Pvu*II restriction fragment of 2.3 kb was detected in DNA from strain H560 (Fig. 4, lane 2). This fragment comigrated with the *Pvu*II fragment of pTD101, which contains the *lepA* gene (data not shown). For the integrate strain (Fig. 4, lane 1), the oligonucleotide hybridized to two fragments of 2.3 and 0.8 kb. The 0.8-kb fragment comigrated with the *Pvu*II fragment of pAC142 (data not shown). This fragment is only 0.8 kb in size because of the deletion of 1.5 kbp from the *lepA* gene (see Fig. 1). The size and intensity of the hybridizing bands from the integrate strain (lane 1) are consistent with the interpretation that the integrate strain has a single copy of both *lepA* and Δ*lepA* as a result of the recombination of pAC142 at the *lep* locus.

Segregants of the integrate strain which have lost the integrated plasmid and either *lepA* or Δ*lepA* would be predicted to occur at a low frequency as the result of intrachromosomal recombination between the wild-type and mutant *lepA* genes (8). Either *lepA* or Δ*lepA* could be lost, depending on whether recombination occurred 5′ or 3′ to the deletion junction of the mutant *lepA* gene. However, segregants that have lost the wild-type *lepA* gene would not be observed if the *lepA* gene has an essential function. There-

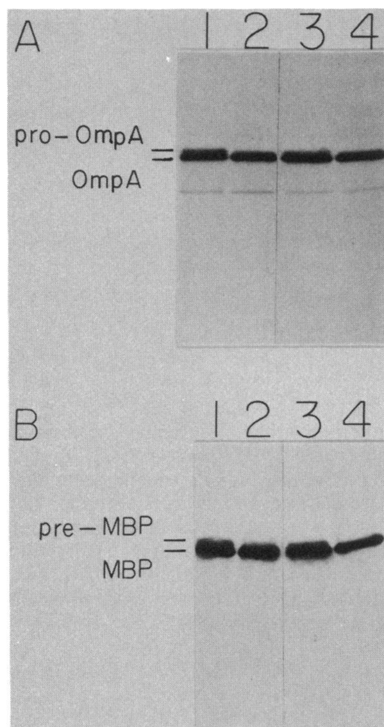

FIG. 5. Comparison of the rates of processing of the precursors of MBP and the ompA in a *lepA*$^+$ and Δ*lepA* strain. (A) Processing of ompA. Lanes: 1, *lepA*$^+$ strain labeled for 15 s with [^{35}S]methionine; 2, *lepA*$^+$ strain labeled for 15 s with [^{35}S]methionine and then chased for 40 s with an excess of unlabeled methionine; 3, Δ*lepA* strain treated as described for lane 1; 4, Δ*lepA* strain treated as described for lane 2. (B) Processing of MBP. Lanes 1 through 4 were treated as described for panel A. MBP and ompA were recovered by immunoprecipitation with specific antisera and anlyzed by SDS gel electrophoresis and fluorography.

fore, analysis of *lepA* segregants from an integrate strain will determine whether *lepA* is an essential gene.

Chloramphenicol-sensitive segregants arose spontaneously from an integrate strain at a frequency of 10^{-5}. The segregants were first enriched by treatment with chloramphenicol and cycloserine (8) and then identified by screening for chloramphenicol sensitivity. Chromosomal DNA was prepared from seven independent chloramphenicol-sensitive segregants and analyzed by Southern hybridization for loss of either *lepA* or Δ*lepA*. A total of three of the seven segregants had lost the wild-type *lepA* gene (Fig. 4, lanes 5, 7, and 8). The remainder had lost the deleted *lepA* gene (lanes 3, 4, 6, and 9). Clearly, either *lepA* or Δ*lepA* are lost at approximately equal frequencies without affecting cell viability. These results indicate that the wild-type *lepA* gene is not essential for cell growth under the conditions used. The growth rate of *lepA* segregants was found to be similar under all conditions tested, including growth at 30 or 42°C on rich (TYE) or minimal media (M9 or M63) as well as on a variety of carbon sources (data not shown). In addition, both *lepA* and *lepA*$^+$ segregants made equal amounts of leader peptidase, as assessed by immunoblotting with anti-leader peptidase antibody (data not shown).

The *lepA* deletion mutant is not defective for protein export. To determine the effects of the loss of the *lepA* gene on protein translocation, the rates of export of MBP, a periplasmic protein, and an ompA, an outer membrane protein, were compared in *lepA*$^+$ and Δ*lepA* strains. Cells were labeled for 15 s with radioactive methionine and then precipitated by the addition of trichloroacetic acid. A sample of the same culture was also labeled for 15 s with radioactive methionine, but it was then chased with an excess of unlabeled methionine for 40 s, followed by acid precipitation. Proteins were recovered by immunoprecipitation with specific antisera and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. A comparison of lanes 1 and 2 in Fig. 5A shows that the small amount of pro-ompA made during the 15-s pulse-labeling of a *lepA*$^+$ strain was processed to mature ompA within 40 s. Identical treatment of the Δ*lepA* strain (lanes 3 and 4) shows no significant accumulation of the precursor to ompA. The rates of processing of the precursor of the MBP to its mature form in the *lepA*$^+$ and Δ*lepA* strains were compared by the same procedure in Fig. 5B. Again, there was no discernible difference in the rates of processing of pre-MBP to its mature form in either the *lepA*$^+$ (lanes 1 and 2) or Δ*lepA* (lanes 3 and 4) strains. Furthermore, all of the mature MBP could be released by osmotic shock from both the wild-type and mutant *lepA* strains (data not shown).

DISCUSSION

Polycistronic transcripts in *E. coli* usually encode products involved in a common biosynthetic pathway, for example, the *trp* and *lac* operons or components of multisubunit complexes, for example, the *unc* and *frd* operons (2). Exceptions to this general rule include an operon, which codes for sigma factor (*rpoD*), DNA primase (*dnaG*), and a component of the 30S ribosomal subunit (*rpsU*) (11, 24), and the *lsp* locus, which codes for the lipoprotein-specific signal peptidase and *ileS*, an Ile-tRNA synthetase (9, 27).

The genes *lep* and *lepA* are transcribed from the same promoter (14). Gene *lep* is the last gene of the operon and encodes leader peptidase which is an essential membrane enzyme that cleaves leader (or signal) peptides from exported proteins both in vitro (25) and in vivo (Dalbey and Wickner, in press). The function of the *lepA* gene is unknown. Because cotranscribed genes often have related functions, it was decided to test whether the *lepA* gene has a protein export function by constructing and then characterizing a *lepA* deletion mutant.

To identify the product of the *lepA* gene, the plasmids pTD101, which contains both *lep* and *lepA* (4), and pTD142, which codes for leader peptidase but has a 1.5-kilobase-pair deletion within the *lepA* gene, were analyzed in minicells. The absence of a 74-kDa protein among the products of pTD142 and its presence in pTD101-transformed cells indicates that the *lepA* gene encodes a protein of 74 kDa. This result was confirmed by cloning the *lepA* gene into an inducible expression vector. Induction of this plasmid leads to the overproduction of a protein of 74 kDa. These observations confirm those of March and Inouye (14), who determined the sequence of the *lepA* gene and identified a protein of 76 kDa encoded by the *lepA* gene. They further showed that the *lepA* gene product fractionated with the inner membrane of *E. coli*, and they have recently demonstrated homologies between the amino-terminal domain of LepA and several GTP-binding proteins (15).

It was possible to replace the chromosomal copy of the *lepA* gene with the deleted *lepA* gene (Δ*lepA*) of plasmid pTD142 by gene eviction (7, 8). The ability to isolate Δ*lepA* strains indicated that the wild-type *lepA* gene is not required for the growth of *E. coli* under the conditions used. The Δ*lepA* mutant, which still makes active leader peptidase, was found to grow at the same rate as a *lepA*$^+$ strain on both

minimal and rich media, strongly suggesting that the *lepA* gene does not have an essential function for the growth of *E. coli*.

To determine whether the *lepA* gene has a role in protein export, the Δ*lepA* and *lepA*$^+$ strains were compared for differences in the rates of export of MBP and an ompA. Both proteins are highly sensitive to a number of mutations which affect protein export (16). The Δ*lepA* strain exported MBP and ompA at rates identical to those of an isogenic *lepA*$^+$ strain, suggesting that the *lepA* gene does not code for an essential component of the secretory apparatus. The organization of the *lep* operon therefore resembles the *lsp* locus (22). Both code for enzymes which are involved in the maturation of protein precursors but are cotranscribed with genes which do not code for proteins with any obviously related functions (9, 27).

The *lep* operon has been mapped to minute 55 on the *E. coli* chromosome (20). The tentative gene order in this region is *purL azl rnc lep pdxJ nadB* (2). The gene *lepA* is not *purL*, *pdxJ*, or *nadB* because the Δ*lepA* mutation does not cause auxotrophy (1). Mutations at *azl* cause resistance to azaleucine (18). Similarly, the *lepA* gene is not *azl* because the Δ*lepA* strain is not resistant to azaleucine. The gene *rnc* is located next to the *lep* operon and encodes RNase III, a protein of only 25 kDa (13, 23).

In summary, *lepA* is a new gene of *E. coli* that has yet to be assigned a function. The recent determination of the sequence of the *lepA* gene (14), the location of its product to the inner membrane of *E. coli*, and its possible role in GTP binding (15) may suggest other possible functions for this protein. The Δ*lepA* mutant described here should facilitate further tests of the role of the *lepA* gene in *E. coli*.

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