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Lipoic Acid Metabolism in *Escherichia coli*: Sequencing and Functional Characterization of the *lipA* and *lipB* Genes

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Two genes, *lipA and lipB*, involved in lipoic acid biosynthesis or metabolism were characterized by DNA sequence analysis. The translational initiation site of the *lipA* gene was established, and the *lipB* gene product was identified as a 25-kDa protein. Overproduction of LipA resulted in the formation of inclusion bodies, from which the protein was readily purified. Cells grown under strictly anaerobic conditions required the *lipA* and *lipB* gene products for the synthesis of a functional glycine cleavage system. Mutants carrying a null mutation in the *lipB* gene retained a partial ability to synthesize lipoic acid and produced low levels of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase activities. The *lipA* gene product failed to convert protein-bound octanoic acid moieties to lipoic acid moieties in vivo; however, the growth of both *lipA* and *lipB* mutants was supported by either 6-thiooctanoic acid or 8-thiooctanoic acid in place of lipoic acid. These data suggest that LipA is required for the insertion of the first sulfur into the octanoic acid backbone. LipB functions downstream of LipA, but its role in lipoic acid metabolism remains unclear.

R-(+)-Lipoic acid (6,8-thioctic acid) is a sulfur-containing coenzyme found throughout nature. The physiological role of lipoic acid as a cofactor in the oxidative decarboxylation reactions of α-keto acids (34) and the glycine cleavage reaction (14) has been well established. In the functional form of the coenzyme, lipoic acid is covalently bound to proteins via an amide linkage to the ε-amino group of specific lysine residues. There are three lipoyl-proteins in *Escherichia coli*: the dihydrolipoamide acyltransferase subunits of pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KGDH), E2p (40) and E2o (39), respectively; and the H protein of the glycine cleavage system (42). The lipoyl domains in these subunits are thought to be extremely mobile, facilitating the movement of substrates between active sites within these large, multicomponent enzyme complexes (34).

Although a great deal is known about the enzymes in which lipoic acid plays a functional role, little is known about lipoic acid biosynthesis or the mechanism by which lipoic acid becomes covalently attached to proteins. Recently, we identified two genes, *lipA and lipB*, involved in lipoic acid biosynthesis (42). Both genes are located at min 14.5 on the *E. coli* chromosome. Representative mutations (*lip-2* and *lip-9*) from the previously isolated group of 36 *lip* mutants (19) were assigned to the *lipA* complementation group (42). Both *lipA* and *lipB* mutants required either exogenous lipoic acid or acetate plus succinate supplements to grow on minimal glucose medium. Interestingly, the *lipA* and *lipB* genes are separated by approximately 1.5 kb of DNA. Vanden Boom et al. (42) showed that insertions in this region of DNA failed to confer lipoic acid auxotrophy. Whether this region plays a role in lipoic acid metabolism remains to be determined.

Several isotope experiments have suggested that octanoic acid is a direct precursor of lipoic acid in *E. coli* (31). In addition, White (43) showed that *E. coli* could convert 8-thiooctanoic acid to lipoic acid, suggesting that this octanoic acid derivative is a possible intermediate in lipoic acid biosynthesis. Recently, Ali et al. (1) showed that overexpression of a recombinant lipoyl-accepting domain in a lipoic acid-auxotrophic mutant resulted in the accumulation of a domain modified with an octanoyl group. It is not known, however, whether protein-bound octanoate is a normal precursor in the lipoic acid biosynthetic pathway.

We have conducted experiments aimed at better understanding the role of *lipA* and *lipB* in lipoic acid metabolism. Here, we describe the characterization of representative *lipA* and *lipB* null mutants in terms of their growth characteristics and lipoic acid content. We also report the DNA sequences of the *lipA* and *lipB* genes.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. The bacterial strains used in this study are listed in Table 1. Strain KER310 (*lipA150*::Tn*1000*dKn *lipB175*::Tn*10*dTc) was obtained by transduction of strain KER176 to tetracycline resistance with P1*vir* grown on strain TVB98. Since the *lipA* and *lipB* alleles are >99% linked, a backcross to a wild-type strain was performed to confirm that tetracycline and kanamycin resistance were cotransducible. Strains KER332 and KER318 were obtained by transduction of strain GS395 to kanamycin resistance with P1*vir* grown on strain KER176 and strain KER184, respectively. Strain KER319 was derived by transduction of strain GS395 to tetracycline resistance with P1*vir* grown on strain KER310 and screened for cotransduction of kanamycin resistance. A P1*vir* stock grown on strain CAG18436 was used to transduce strain CY14 to tetracycline resistance. One candidate which could not use oleate (0.1% in 1% Brij-58) as a sole carbon source (i.e., *fadE*) was retained as strain KER287. Strain KER293 was obtained by transduction of strain KER176 to tetracycline resistance with P1*vir* grown on strain KER287 and screened for cotransduction of *fadE*. Strain KER296, a tetracycline-sensitive derivative of strain KER293, was ob-

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TABLE 1. *E. coli* strains used in this work

Strain	Sex	Relevant genotype[a]	Source or reference
JK1	F⁻	*rpsL*	J. Konisky
JRG26	F⁻	*supE iclR lipA2*	19
GS395	F⁻	*lysA serA25 thi-1*	32
DH5α	F⁻	φ80d *lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA1*	A. Salyers
BL21(λDE3)	F⁻	*gal hsdS* λDE3	41
CY14	Hfr	HfrC *metB fadE* T6^r	Laboratory collection
TVB98	F⁻	JK1 *lipB175*::Tn*10*dTc	42
KER176	F⁻	*rpsL lipA150*::Tn*1000*dKn	42
KER184	F⁻	*rpsL lipB182*::Tn*1000*dKn	42
KER199	F⁻	*rpsL lipA2 zbd-601*::Tn*10*	42
KER310	F⁻	*rpsL lipA150*::Tn*1000*dKn *lipB175*::Tn*10*dTc	This study
KER287	Hfr	HfrC *metB fadE* T6^r *zae-502*::Tn*10*	This study
KER293	F⁻	*rpsL lipA150*::Tn*1000*dKn *fadE zae-502*::Tn*10*	This study
KER296	F⁻	*rpsL lipA150*::Tn*1000*dKn *fadE*	This study
KER332	F⁻	*lysA serA25 thi-1 rpsL lipA150*::Tn*1000*dKn	This study
KER318	F⁻	*lysA serA25 thi-1 rpsL lipB182*::Tn*1000*dKn	This study
KER319	F⁻	*lysA serA25 thi-1 rpsL lipA150*::Tn*1000*dKn *lipB175*::Tn*10*dTc	This study

[a] Allele designations are those of the *E. coli* Genetic Stock Center (CGSC), Yale University, New Haven, Conn.

tained as described by Maloy and Nunn (25). Kohara λ phage 1G6 (miniset number 168) has been described elsewhere (7).

Plasmid constructions. Recombinant plasmid vectors pMTL20, pMTL21, pMTL22, and pMTL23 (6) were used in this work. Plasmids pKR109 and pKR116, which carry the *lipA* gene under control of the *tac* and T7 promoters, respectively, were constructed as follows. Plasmid pCTV604 (42) was digested with *Bst*XI, and the ends were filled in with the Klenow fragment of DNA polymerase I. The resulting DNA fragments were digested with *Nsi*I, and a 1.2-kbp *Bst*XI-*Nsi*I fragment was ligated to pMTL22 digested with *Sma*I and *Nsi*I. Ligation of a filled *Bst*XI site to a *Sma*I site generated a new *Nco*I site. The resulting plasmid, pKR106, encoded a LipA protein which was lacking the five amino-terminal amino acids. Plasmid pKR109 was constructed by ligating the 1.8-kbp *Nco*I-*Nsi*I fragment from pKR106 to the 4.6-kbp *Nco*I-*Pst*I fragment from pTM48-1 (T. Morris; a pKK233-2 derivative) to form pKR109. Plasmid pKR109 carries *lipA* downstream of a *tac* promoter. Plasmid pKR116 was constructed by ligating a 1.8-kbp *Nco*I-*Xho*I fragment from pKR106 into pET16b (Novagen) digested with the same enzymes. Plasmid pKR116 carries *lipA* downstream of a T7 promoter. Plasmid pKR111, which carries the entire *lipB* structural gene, was constructed by ligating a 1.2-kbp *Cla*I-*Bam*HI fragment from pCTV625 (42) into pMTL23 digested with the same enzymes. Plasmid pKR111 was digested with *Sst*I and *Stu*I, and the 900-bp fragment was ligated into pCKR101 (24) digested with *Sna*BI and *Sst*I to form pKR112. Plasmid PKR112 carries *lipB* downstream of a *tac* promoter. Plasmid pKR115, which carries *lipB* downstream of a T7 promoter, was constructed by ligating a 900-bp *Cla*I-*Sst*I fragment from pCTV625 into pGEM-7Zf(−) (Promega) digested with the same enzymes.

Plasmid pKR122 was constructed by ligating a 300-bp *Eco*RV-*Xho*I fragment from pKR43 (33) into pKR112 digested with *Sna*BI and *Sal*I. Plasmid pKR122 encodes a fusion protein with 120 amino-terminal residues from LipB and 71 carboxyl-terminal residues from *Klebsiella pneumoniae* oxaloacetate decarboxylase α subunit (LipB-KPBT fusion). This fusion protein was specifically labeled with [^{3}H]biotin as described previously (8).

Culture media and growth conditions. The following growth media were routinely used for growth of bacterial strains: minimal E medium (10), M9 minimal medium (27), rich broth (11), and 2XYT medium (27). Solid medium contained 1.5% agar (United States Biochemical). Supplements were added as necessary at the following concentrations unless otherwise indicated: sodium acetate, 5 mM; sodium succinate, 5 mM; thiamine, 1 μg/ml; serine, 100 μg/ml; glycine, 100 μg/ml; lysine, 88 μg/ml; methionine, 20 μg/ml; cysteine, 1 mM; vitamin-free casein hydrolysate, 0.1%; and DL-lipoic acid, 5 ng/ml. All amino acids were of the L-form. Glucose (0.4%), acetate (50 mM), or succinate (50 mM) was used as a sole carbon and energy source in minimal medium. To determine the crossfeeding ability of acid-hydrolyzed cell extracts, solid medium consisting of 1.5% agar, minimal E medium, succinate (50 mM), vitamin-free casein hydrolysate, and acid-hydrolyzed extract equivalent to approximately 40 μg of protein was used. Antibiotics were added at the following concentrations: chloramphenicol, 50 μg/ml; ampicillin, 100 μg/ml; kanamycin sulfate, 25 or 50 μg/ml; and tetracycline-HCl, 10 μg/ml. In cloning experiments with the α-complementation system, 20 μl of a 100 mM stock solution of isopropylthiogalactopyranoside (IPTG) and 75 μl of a 2% stock solution of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were spread directly on solid agar medium before transformed cells were plated.

Growth of bacterial cultures was routinely monitored with a Klett-Summerson colorimeter with a green filter (1 Klett unit equals approximately 5×10^6 cells per ml). The lipoic acid content of acid-hydrolyzed extracts was determined as described by Herbert and Guest (20) except that KER176 (*lipA150*::Tn*1000*dKn) was used as the assay strain. Acid hydrolysis of extracts was achieved by autoclaving 1 mg of protein for 2 h in the presence of 6 N H_2SO_4. Following hydrolysis, the extracts were neutralized by the addition of 4 N NaOH.

Radiolabeling of cultures with [1-^{14}C]octanoic acid. Cultures were labeled with [1-^{14}C]octanoate (55 mCi/mmol; American Radiolabeled Chemicals) as follows. In general, strains were cultured at 37°C to 1×10^9 to 2×10^9 cells per ml in minimal E medium containing glucose, acetate, succinate, and 3 to 5 μCi of [1-^{14}C]octanoate (50 to 100 nmol/ml). The cultures were precipitated with 10% trichloroacetic acid, and the acid precipitates were washed with acetone and

boiled in denaturing sodium dodecyl sulfate (SDS) buffer. The cell extracts were analyzed on 10% polyacrylamide–SDS gels.

In the experiment designed to study protein-bound lipoate synthesis, strain KER296 was grown to 5×10^8 cells per ml in M9 minimal medium containing glucose, maltose (0.4%), acetate, succinate, vitamin-free casein hydrolysate, kanamycin (25 μg/ml), and [1-^{14}C]octanoate (200 nmol/ml). Maltose was added to induce the λ receptor. The cells were harvested, washed with 10 ml of the above medium without [1-^{14}C]octanoate, and resuspended in the same medium. The culture was then placed at 37°C for 10 min. The culture was divided in half, and Kohara λ phage 1G6 was added at a multiplicity of 7 phage per cell to one culture; the other was left uninfected. At 3 h postinfection, the infected and uninfected cultures were divided in half. Each culture was adjusted to 10% trichloroacetic acid. The acid precipitates were washed several times with acetone, and one sample from the infected and uninfected cultures was boiled in denaturing buffer and electrophoresed on a 10% polyacrylamide–SDS gel. The bands were detected by fluorography with preflashed film. The remaining precipitates were resuspended in 6 N H_2SO_4 and autoclaved for 2 h at 121°C. The acid-hydrolyzed extracts were adjusted to pH 7.0 by the addition of 4 N NaOH.

Synthesis of putative lipoic acid precursors. 6-Thiooctanoic acid was synthesized without isotopic labeling exactly as described by White (43). 6-Ketooctanoic acid and 6-hydroxyoctanoic acid were intermediates in this synthesis. 8-Hydroxyoctanoic acid was synthesized by reduction of suberic acid monomethyl ester with a stoichiometric amount of borane in tetrahydrofuran (5), followed by saponification of the methyl ester. 8-Thiooctanoic acid was obtained by reaction of 8-bromooctanoic acid with thiourea in refluxing ethanol for 2 h (15) or by reaction of 8-hydroxyoctanoic acid with thiourea in refluxing hydroiodic acid overnight (37). The resulting thiouronium salts were then cleaved to the thio with sodium hydroxide (37). All acids were pure, as determined by thin-layer chromotography in the oxidized state. The precursors and reagents were from Aldrich Chemical.

DNA sequencing. Plasmids carrying Tn*1000* insertions in and adjacent to *lipA* and *lipB* were digested with various restriction enzymes so that each Tn*1000* inverted repeat and adjacent *lip* locus DNA could be subcloned into appropriate sites within the pMTL vectors. A synthetic DNA primer (9) that annealed to a sequence in the terminal inverted repeats of Tn*1000* was used to prime DNA sequence into adjacent *lip* locus DNA. Additional plasmids carrying *lip* locus DNA were constructed to complete sequencing of both strands of *lipA* and *lipB*. Double-stranded DNA sequencing was performed by the method of Sanger et al. (36) with Sequenase 2.0 (United States Biochemical Corp.).

SDS-PAGE analysis of in vitro transcription-translation products. In vitro transcription-translation reactions were carried out with S30 extracts obtained from Promega. Reaction mixes contained 2 to 3 μg of purified plasmid DNA and ^{35}S-Trans label (30 μCi of 1,074 Ci/mmol; a mixture of [^{35}S]methionine and [^{35}S]cysteine; ICN Biomedicals). Radiolabeled proteins were analyzed by polyacrylamide gel electrophoresis (PAGE) on 13% polyacrylamide–SDS gels. The proteins are labeled primarily with [^{35}S]methionine because of the presence of nonradioactive cysteine in the translation mixture.

PDH and KGDH assays. PDH and KGDH assays were performed as described by Guest and Creaghan (17). The total protein content of crude cell extracts was determined by the microbiuret assay of Itzhaki and Gill (22).

Amino acid sequence analysis. Amino acid sequencing was performed by automatic Edman degradation in a gas phase system (Applied Biosystems). Protein products from plasmid pCTV616 (42) were labeled with ^{35}S-Trans label in an in vitro transcription-translation reaction as described above. Radiolabeled proteins were separated on a 13% polyacrylamide–SDS gel and electroblotted to a polyvinylidene fluoride membrane. The membrane was exposed to film, and the 36-kDa ^{35}S-labeled protein band was used directly for amino acid sequencing. A sample was collected from each cycle of the amino acid sequence analysis, and radioactivity was counted in Biosafe II (Research Products International) liquid scintillation fluid in a scintillation counter.

Nucleotide sequence accession numbers. The sequence data presented in this article can be obtained from the GenBank data library under accession number L07636.

RESULTS

DNA sequences of *lipA* and *lipB*. The Tn*1000* insertions in plasmid pCTV616 (42) provided a means to prime dideoxy sequence analysis from various locations within the *lip* locus. Our approach was to clone segments of plasmids carrying various Tn*1000* insertions into pMTL20, pMTL23, or pSU19 and then use a synthetic oligonucleotide complementary to the inverted repeats found at the outside ends of the transposon (9) to prime DNA synthesis into the *lip* locus (Fig. 1). Since each subclone carried only one end of the transposon, unique sequences could be obtained from both strands by using only one primer. Sequencing of both strands was completed by constructing appropriate subclones with the restriction sites deduced from transposon-primed sequences.

The sequence of the *lipA* locus contained an open reading frame coding for a putative gene product of 321 amino acids, with a calculated molecular weight of 36,061 (Fig. 2). This predicted molecular weight of LipA is in agreement with the measured molecular weight observed from in vitro transcription-translation analysis of plasmid-encoded LipA protein (42). In addition, the measured sizes of truncated LipA proteins expressed from pCTV628 and pCTV640 (42) are consistent with the predicted sizes of these proteins. After completion of our sequence, Hayden et al. (18) reported the sequence of the *lip* (i.e., *lipA*) gene. Our sequence agrees with theirs except that nucleotides 90 and 91 are inverted in their sequence. Hayden et al. (18) reported a deduced molecular mass of 31.4 kDa for LipA, whereas we previously reported a molecular mass of 36 kDa for the in vitro transcription-translation product of the *lipA* gene (42). Because of this discrepancy and because there are several possible ATG initiation codons near the 5′ end of the *lipA* gene, we experimentally determined the translational start site. The LipA protein was radiolabeled with ^{35}S-Trans label and subjected to amino acid analysis. A sample from each cycle from the amino acid analysis was examined for radioactivity. The peak of radioactivity at cycles 7 and 8 (Fig. 3) is consistent with the presence of a methionine residue at the seventh amino acid in the LipA protein (Fig. 2). Thus, *lipA* translation starts 40 codons upstream of the position reported by Hayden et al. (18). The codon preference (16) of 1.08 (versus 0.53 for randomized sequence) for the *lipA* gene indicates a moderately expressed protein in *E. coli*. The stop codon of the *lipA* gene is followed by an inverted repeat sequence that could form a stem-loop structure, followed by

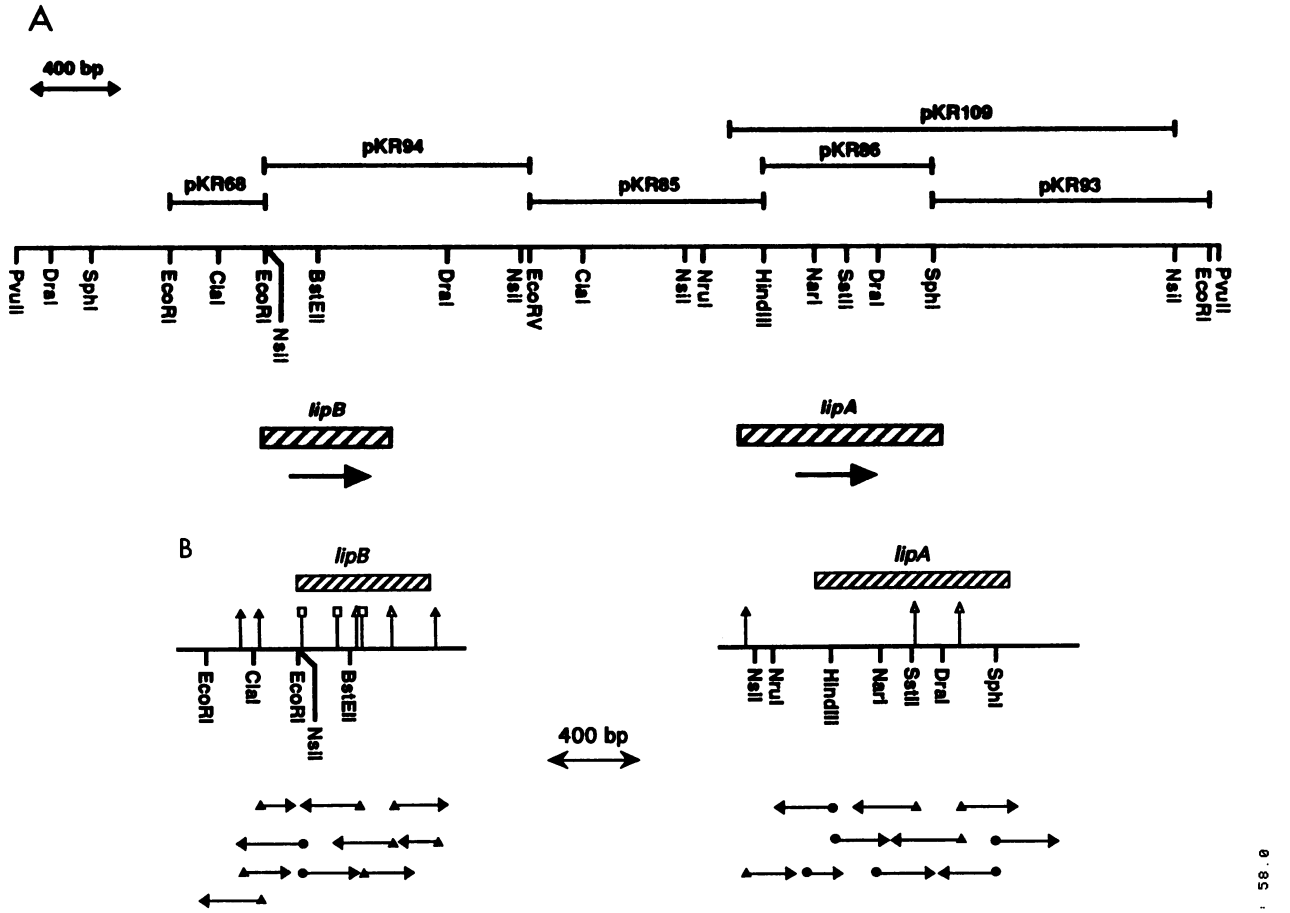

FIG. 1. Restriction map and sequencing strategy for the *E. coli lip* locus. (A) Restriction map of the 5.2-kbp *Pvu*II fragment from plasmid pCTV616. The locations and orientations of the *lipA* and *lipB* genes are indicated below the restriction map. Plasmid subclones carrying various segments of the *lip* locus are indicated above the restriction map. (B) Strategy for sequencing *lipA* and *lipB*. Subclones of plasmids pCTV619, pCTV620, pCTV618, pCTV625, pCTV626, pCTV628, and pCTV640 were used to determine the Tn*1000* insertion points and to sequence the adjacent *lip* locus DNA by the dideoxy method with a synthetic deoxyribonucleotide primer (5'-CGTTTTCGTTCCATTGGC CCCTC-3') that annealed to a sequence in the terminal inverted repeats of Tn*1000*. Subclones of plasmids pCTV634, pCTV613, pCTV611, and pCTV612 were used to determine the location of the Tn*10*dTc insertions by sequencing from the ends of the transposon with the synthetic deoxyribonucleotide primer 5'-CAAAATCATTAGGGGATTCA-3'. The left and right ends of the plasmid subclones shown in panel A were sequenced with commercially available reverse and universal primers (United States Biochemical). The arrows below the restriction maps show the sequencing strategy. Solid triangles indicate sequence determined from Tn*1000* insertions, and solid circles indicate sequence determined from the plasmid subclones shown in panel A. The vertical lines above the restriction maps show the locations of the Tn*1000* and Tn*10*dTc insertions. Triangles represent Tn*1000* insertions, and squares represent Tn*10*dTc insertions. The insertions which confer lipoic acid auxotrophy are indicated by open symbols.

a run of uridines. This structure is indicative of a rho-independent transcriptional terminator.

The sequence of the *lipB* locus revealed an open reading frame coding for a putative gene product of 191 amino acids, with a calculated molecular weight of 21,339 (Fig. 4). The codon preference of 0.72 (versus 0.52 for randomized sequence) for the *lipB* gene and the occurrence of rarely used codons at the 3' end of the gene are consistent with the inability to detect a plasmid-encoded LipB protein from pCTV616 by either in vitro transcription-translation or maxicell analyses (42).

The 1.5-kb region of DNA between the *lipA* and *lipB* genes (Fig. 1) contains an open reading frame coding for a putative protein with a molecular mass of 25.5 kDa (data not shown). We have thus far failed to identify a gene product associated with this locus.

The LipB and LipA proteins. To confirm the authenticity of the *lipB* sequence, expression of this gene was placed under control of the *tac* promoter. In vitro transcription-translation analysis of pKR112 (which carries *lipB* under *tac* control) revealed a 25-kDa protein which was absent in the vector control (Fig. 5). Attempts to overexpress the *lipB* gene product in vivo under control of either the *tac* or T7 promoter were unsuccessful (data not shown). In order to visualize the *lipB* gene product in vivo, we constructed a heterologous fusion protein which could be specifically labeled with [^{3}H]biotin in vivo. Expression of the LipB-KPBT fusion under *tac* control revealed a poorly biotinated protein of the expected molecular weight on an SDS-polyacrylamide gel (data not shown). The fact that the LipB-KPBT fusion competed poorly with BCCP (biotin carboxyl carrier protein) for biotination suggested that *lipB* was poorly expressed even under the control of a heterologous promoter and/or that the LipB protein is very labile.

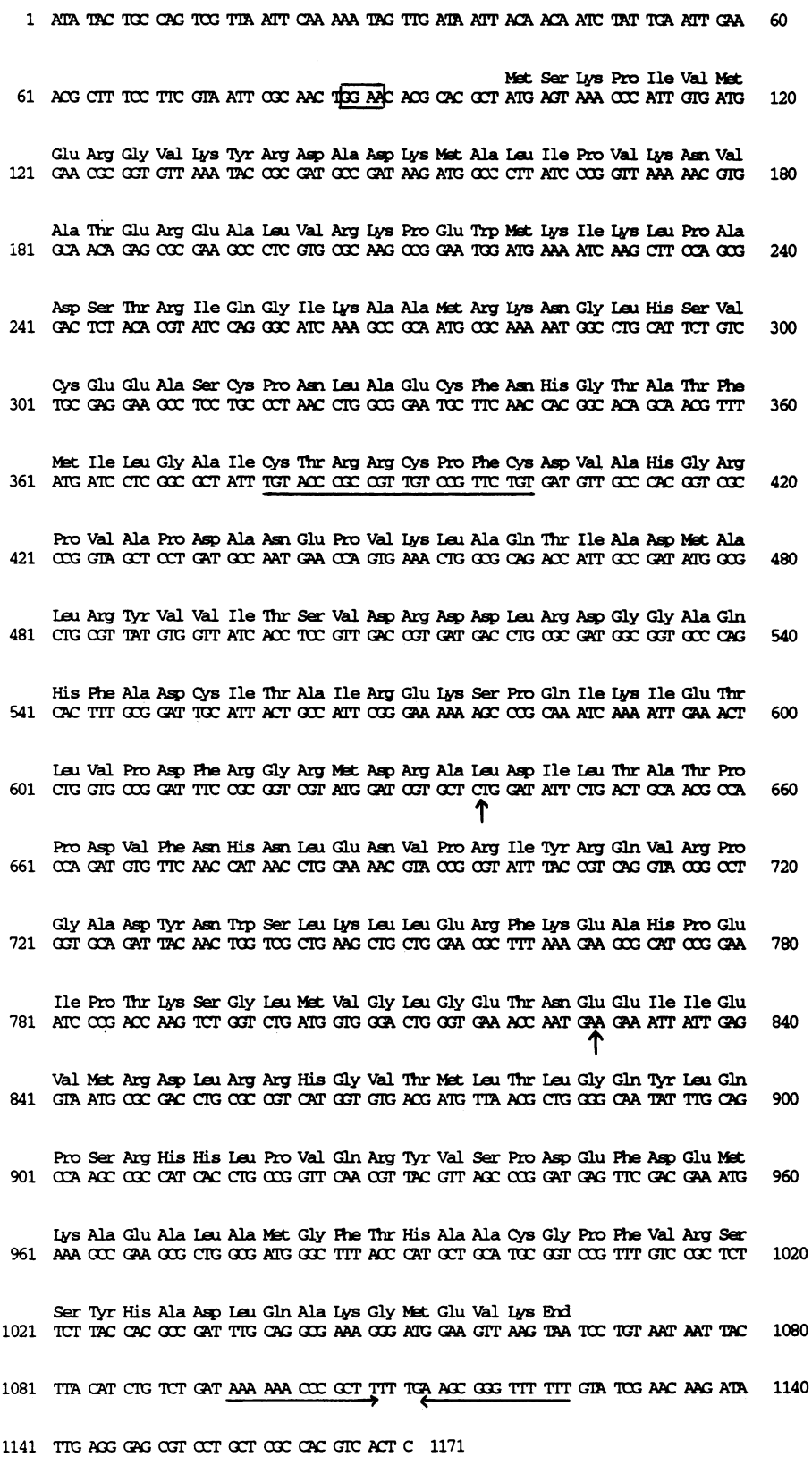

FIG. 2. Nucleotide sequence and deduced amino acid sequence of the *E. coli lipA* gene. The boxed region is the putative ribosome-binding (Shine-Dalgarno) site. The vertical arrows indicate the locations of Tn*1000* insertions. A possible stem-loop transcriptional terminator is indicated by arrows beneath the sequence. The CXXXCXXC motif also found in the biotin synthase proteins of *E. coli* (30) and *Bacillus sphaericus* (29) is underlined.

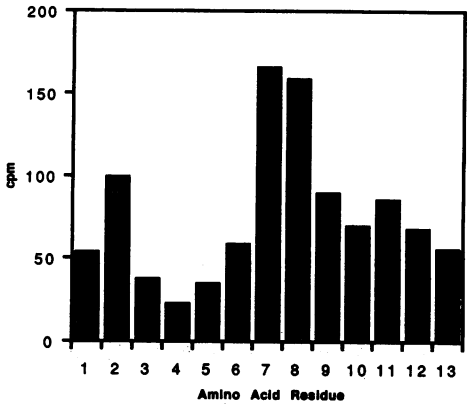

FIG. 3. Release of [^{35}S]methionine from LipA protein. The LipA protein was radiolabeled with ^{35}S-Trans label in an in vitro transcription-translation system, electrophoresed on a 13% polyacrylamide–SDS gel, and transferred to a polyvinylidene fluoride membrane. The membrane was exposed to film, and the band corresponding to the 36-kDa LipA protein was excised. The protein was subjected to amino acid analysis as described under Materials and Methods. A sample from each cycle of the amino acid analysis was collected, and radioactivity was counted in a liquid scintillation counter.

We attempted to overproduce the LipA protein because it would facilitate both the development of an assay for lipoic acid biosynthesis and purification of the LipA protein. The *lipA* gene was placed downstream of the *tac* promoter of pKK233-2 and the T7 promoter of pET16b. In both cases, the *lipA* gene was cloned so that the expressed LipA protein was lacking the first five amino-terminal amino acids. Both plasmids complemented the *lipA150*::Tn*1000*dKn mutation upon induction of *lipA* expression, whereas only pKR109 (in which *lipA* is under *tac* control) complemented the same mutation in the absence of induction. Although a protein of the expected size was expressed in vitro from plasmid pKR109, no overproduction was observed in vivo (data not shown). However, marked overproduction of the LipA protein was observed in vivo when *lipA* was transcribed from a T7 promoter (Fig. 6). Unfortunately, the overproduced LipA protein formed insoluble aggregates even when the cells were induced under conditions designed to decrease inclusion body formation (growth at 30°C and induction with submaximal IPTG concentrations) (Fig. 7). It did appear, however, that under these conditions, a small amount of soluble LipA protein was obtained which could be precipitated in an ammonium sulfate fraction of 30 to 60% (Fig. 7). It was assumed that the soluble LipA protein obtained in this experiment was active, since pKR116 complements the *lipA150*::Tn*1000*dKn mutation under these conditions.

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1   CTC CAC GGA GAT GCC GTT TTG TAT CAG GAT AAA ATT CTT GTC CGC CAG CTC GGT CTT CAG   60

                                          Met His Glu Phe Thr Asp Thr Arg Asp Asp Asp Thr
61  CCT TAC GAG CCA ATC TCC CAG GCT ATG CAT GAA TTC ACC GAT ACC CGC GAT GAT GAT ACC  120

    Leu Asp Glu Ile Trp Leu Val Glu His Tyr Pro Val Phe Thr Gln Gly Gln Ala Gly Lys
121 CTT GAT GAA ATC TGG CTG GTC GAG CAC TAT CCG GTA TTC ACC CAA GGT CAG GCA GGA AAA  180

    Ala Glu His Ile Leu Met Pro Gly Asp Ile Pro Val Ile Gln Ser Asp Arg Gly Gly Gln
181 GCG GAG CAC ATT TTA ATG CCG GGT GAT ATT CCG GTG ATC CAG AGC GAT CGC GGT GGG CAG  240

    Val Thr Tyr His Gly Pro Gly Gln Gln Val Met Tyr Val Leu Leu Asn Leu Lys Arg Arg
241 GTG ACT TAT CAC GGG CCG GGG CAA CAG GTG ATG TAT GTG TTG CTT AAC CTG AAA CGC CGT  300

    Lys Leu Gly Val Arg Glu Leu Val Thr Leu Leu Glu Gln Thr Val Val Asn Thr Leu Ala
301 AAA CTC GGT GTG CGT GAA CTG GTG ACC TTG CTT GAG CAA ACA GTG GTG AAT ACC CTG GCT  360

    Glu Leu Gly Ile Glu Ala His Pro Arg Ala Asp Ala Pro Gly Val Tyr Val Gly Glu Lys
361 GAA CTG GGT ATA GAA GCG CAT CCT CGG GCT GAC GCG CCA GGT GTC TAT GTT GGG GAA AAG  420
                  ↑
    Lys Ile Cys Ser Leu Gly Leu Arg Ile Arg Arg Gly Cys Ser Phe His Gly Leu Ala Leu
421 AAA ATT TGC TCA CTG GGT TTA CGT ATT CGA CGC GGT TGT TCA TTC CAC GGT CTG GCA TTA  480

    Asn Val Asn Met Asp Leu Ser Pro Phe Leu Arg Ile Asn Pro Cys Gly Tyr Ala Gly Met
481 AAC GTC AAT ATG GAT CTT TCA CCA TTT TTA CGT ATT AAT CCT TGT GGG TAT GCC GGA ATG  540
                                          ↑
    Glu Met Ala Lys Ile Ser Gln Trp Lys Pro Glu Ala Thr Thr Asn Asn Ile Ala Pro Arg
541 GAA ATG GCT AAA ATA TCA CAA TGG AAA CCC GAA GCG ACG ACT AAT AAT ATT GCT CCA CGT  600

    Leu Leu Glu Asn Ile Leu Ala Leu Leu Asn Asn Pro Asp Phe Glu Tyr Ile Thr Ala End
601 TTA CTG GAA AAT ATT TTA GCG CTA CTA AAC AAT CCG GAC TTC GAA TAT ATT ACC GCT TAA  660

661 TTC CAT ACA TCA ATG GCC CAA TTT ACA CTG GGT CAT TAC TC 701
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FIG. 4. Nucleotide sequence and deduced amino acid sequence of the *E. coli lipB* gene. The vertical arrows indicate the locations of Tn*1000* insertions. The boxed region is the putative ribosome-binding (Shine-Dalgarno) site.

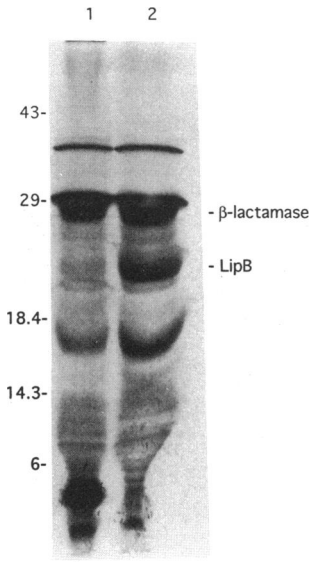

FIG. 5. Production of the LipB protein. Patterns of proteins produced from plasmids pCKR101 (lane 1) and pKR112 (lane 2) in the in vitro transcription-translation system. Protein products were analyzed on a 13% polyacrylamide–SDS gel. The mobilities of protein molecular mass standards (in kilodaltons) are shown to the left.

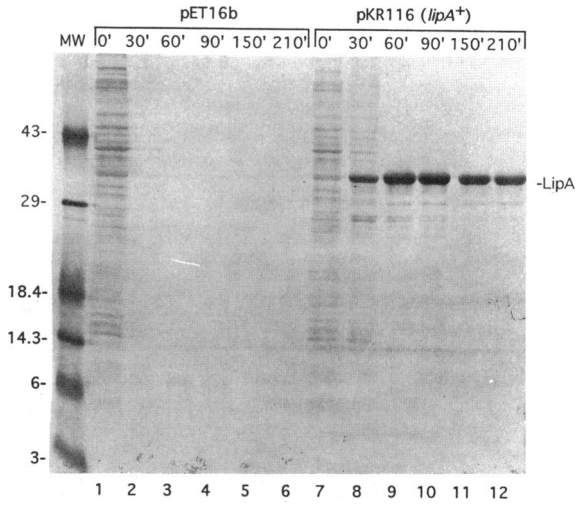

FIG. 6. Expression of the *lipA* gene product from plasmid pKR116 in the T7 expression system. Strain BL21 (λDE3) harboring vector pET16b (lanes 1 to 6) or pKR116 (*lipA*+) (lanes 7 to 12) was grown in rich broth at 37°C. When the cells reached an optical density at 600 nm of 0.5, IPTG was added at a final concentration of 1 mM. The cultures were incubated for 30 min at 37°C, and rifampin (200 μg/ml) was added. At 0 min (lanes 1 and 7), 30 min (lanes 2 and 8), 60 min (lanes 3 and 9), 90 min (lanes 4 and 10), 150 min (lanes 5 and 11), and 210 min (lanes 6 and 12) after the addition of IPTG, a 0.4-ml aliquot was removed from each culture and labeled with 10 μCi of ^{35}S-Trans label (ICN Biomedicals) for 10 min. Cultures were precipitated in 10% trichloroacetic acid. Precipitated protein pellets were washed with acetone and boiled in SDS-containing sample buffer. A volume of each lysate corresponding to approximately 5×10^6 cells was electrophoresed on a 13% polyacrylamide–SDS gel. The gel was exposed to preflashed film and developed by fluorography. Lane MW, protein size standards. Sizes are shown in kilodaltons.

Another method used to obtain soluble LipA protein was to solubilize the inclusion bodies from cells harboring the insoluble LipA aggregates in guanidine-HCl and gradually dialyze the protein solution against renaturation buffer to allow the proteins to refold. This technique was useful for obtaining LipA protein at about 80% purity (Fig. 7), although in the absence of an in vitro assay (the sulfur donor is not known), it is not known whether the refolded protein is active.

Aerobic growth characteristics of *lip* mutants. As described elsewhere (42), a set of Tn*1000* insertion derivatives of plasmid pCTV616, which encodes the *E. coli lip* locus, were assembled. These insertions defined two genes, *lipA* and *lipB*, involved in lipoic acid metabolism. Consistent with the work of Herbert and Guest (19), *lipA* and *lipB* null mutants grew on minimal glucose medium supplemented with either lipoic acid or acetate plus succinate. A comparison of the growth characteristics of isogenic strains harboring the *lipA2*, *lipA150*::Tn*1000*dKn, and *lipB182*::Tn*1000*dKn mutations revealed that the *lipA2* and *lipB182*::Tn*1000*dKn mutants required less lipoic acid to obtain maximal growth than the *lipA150*::Tn*1000*dKn mutant strain (Fig. 8). In addition, spontaneous suppressors of the *lipB182*::Tn*1000*dKn mutation were obtained at a frequency of approximately 10^{-5} in liquid minimal glucose medium.

The partial growth and suppression of *lipB* null mutants in the absence of exogenous lipoic acid suggested that the product of a second gene could replace (at least in part) the function of *lipB* in lipoic acid metabolism, and hence it seemed likely that the *lipB* null mutants may contain low levels of lipoyl-proteins when grown in the absence of lipoic acid.

An indication that *lipB* null mutants are not completely defective in production of lipoyl-proteins is that these strains grow when only the products of the KGDH reaction are added to minimal medium lacking lipoic acid. Strain KER184 grew in succinate minimal medium supplemented with lysine plus methionine, and this growth in the absence of acetate indicates that some PDH activity is present under these conditions. The addition of lysine plus methionine spares the succinyl-coenzyme A requirement for the synthesis of these amino acids. It should be noted that strain KER184 failed to grow on minimal acetate medium supplemented with lysine plus methionine, indicating that even under these sparing conditions, there is insufficient KGDH for the synthesis of other succinyl-coenzyme A-derived compounds. An assay of the PDH and KGDH activities of extracts of strain KER184 grown on minimal medium supplemented with acetate and succinate showed that these cells do indeed retain about 20% of the enzyme activity found in the wild-type strain (Table 2). Moreover, analysis of such *lipB* mutant cells showed that they contain about 10% of the wild-type level of lipoic acid. In contrast, cells of similarly grown *lipA* mutant strains contain no detectable lipoic acid or dehydrogenase activities (Table 2).

Since strain KER184 exhibited a 10-fold decrease in protein-bound lipoic acid levels compared with the wild type, we tested whether there was a concomitant increase in free lipoic acid in this strain. It did not appear that strain KER184 was excreting free lipoic acid, since this strain was unable to crossfeed strain KER176 on minimal succinate medium. To determine whether strain KER184 accumulated intracellular pools of free lipoic acid, a cell extract of KER184 grown on minimal glucose medium containing

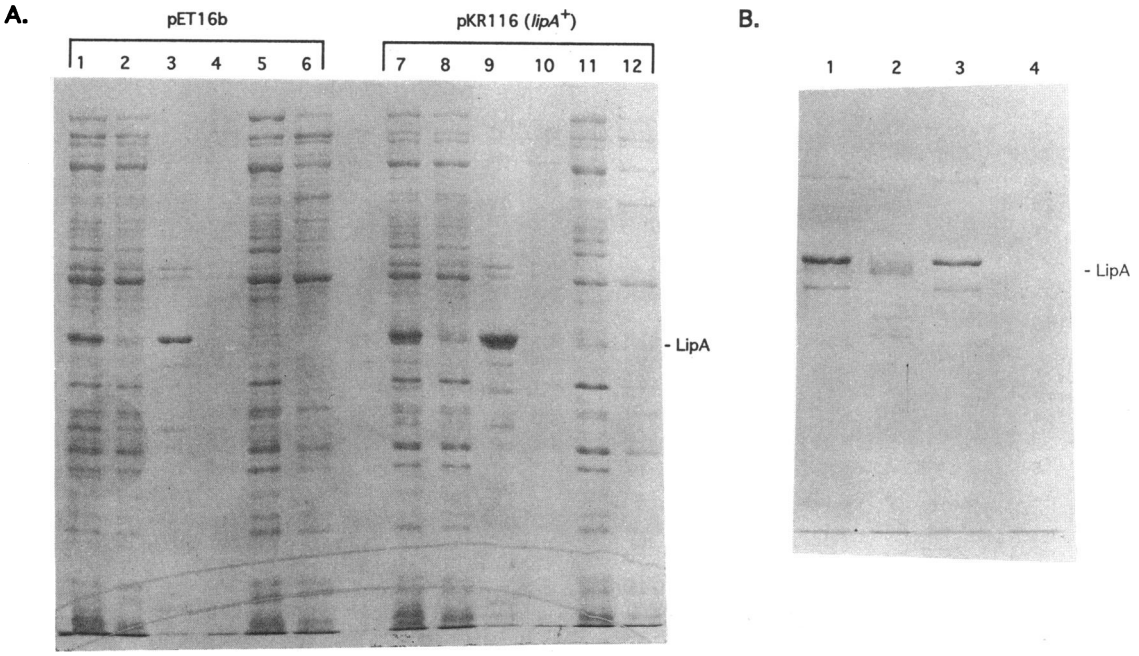

FIG. 7. Partial purification of the *lipA* gene product. (A) Production of partially soluble LipA protein in vivo. Strain BL21(λDE3) harboring vector pET16b (lanes 1 to 6) or pKR116 (*lip*$^+$) (lanes 7 to 12) was grown and cell extracts were fractionated as described under Materials and Methods. Lanes 1 and 7, whole-cell extracts; lanes 2 and 8, low-speed centrifugation supernatants; lanes 3 and 9, low-speed centrifugation pellets; lanes 4 and 10, 0 to 30% $(NH_4)_2SO_4$ saturation pellets; lanes 5 and 11, 30 to 60% $(NH_4)_2SO_4$ saturation pellets; lanes 6 and 12, 60 to 80% $(NH_4)_2SO_4$ saturation pellets. (B) Solubilization of low-speed centrifugation fraction. The low-speed centrifugation pellets from BL21(λDE3) harboring pKR116 (lanes 1 and 2) or pET16b (lanes 3 and 4) were solubilized as described under Materials and Methods. Lanes 1 and 3, guanidine-insoluble protein; lanes 2 and 4, guanidine-soluble protein. Proteins were separated on SDS-polyacrylamide gels and stained with Coomassie blue R.

acetate (4 mM) and succinate (4 mM) was divided in half, and one half was acid hydrolyzed as described under Materials and Methods. The free lipoic acid in the hydrolyzed and unhydrolyzed samples was extracted with ethanol-ether (50:50), and each ethanol-ether extract was analyzed for its ability to feed KER176 on minimal succinate medium. Only the acid-hydrolyzed extract was able to support the growth of KER176.

Growth of *lipA* and *lipB* mutants on possible lipoic acid precursors. In order to better understand the nature of the genetic defects in *lipA* and *lipB* mutants, a variety of potential lipoic acid precursors were synthesized. Each of the putative lipoic acid precursors was examined for its ability to feed KER176. While 3 pmol of lipoic acid per ml allowed KER176 to reach half-maximal growth in minimal glucose medium, concentrations of 35 nmol of 6-thiooctanoate and 2 nmol of 8-thiooctanoate per ml were required for KER176 to reach similar growth yields. No significant replacement or sparing was observed with 6-ketooctanoate, 6-hydroxyoctanoate, or 8-hydroxyoctanoate at a 500 mM final concentration. Qualitatively, the growth of KER184 on agar plates of minimal E glucose medium containing each of the precursors was similar to that observed for KER176. That is, only 6-thiooctanoate or 8-thiooctanoate could substitute for the lipoic acid requirement of KER184. The results of quantitative analysis of KER184 growth in liquid culture were ambiguous because of the ability of *lipB* mutant strains to grow in minimal glucose medium lacking lipoic acid.

Anaerobic synthesis and function of lipoic acid. Many of the possible mechanisms for introduction of sulfur into hydrocarbon chains require molecular oxygen; thus, we tested whether anaerobically grown cells synthesize lipoic acid. Since PDH and KGDH function only in aerobic metabolism (their metabolic functions being replaced by the anaerobic enzymes pyruvate formate lyase and fumarate reductase, respectively) and the synthesis of these proteins is repressed during anaerobic growth, there is no obvious need for anaerobic lipoic acid synthesis. Previous workers had reported that anaerobically grown cells contain lipoic acid and PDH activity (detectable in vitro) (21, 23, 38), but criteria indicating strict anaerobiosis throughout growth were not given. We have therefore reinvestigated this question with cells grown for eight to nine generations under strictly anaerobic conditions (those used for methanogenic organisms) and found that *E. coli* grown under these conditions contains approximately 8 ng of lipoic acid per mg of protein.

A possible reason for anaerobic lipoic acid synthesis is the glycine cleavage enzyme, which requires this cofactor to carry the methylamine group (14). To our knowledge, anaerobic glycine cleavage has not been reported for *E. coli*, and thus we tested whether this reaction proceeds in anaerobically grown cells. This was done by use of *serA* mutant strains, which require either serine or glycine for growth (26), with glycine cleavage activity being required for the utilization of glycine. A *serA* mutant strain grew well under anaerobic conditions when supplemented with either serine or glycine, whereas *serA* mutant strains that also carried *lipA* or *lipB* mutations grew on glycine only when lipoic acid was added. Since the growth of these strains on serine proceeds with or without lipoic acid, anaerobically grown *E. coli* must synthesize an active lipoic acid-containing glycine cleavage

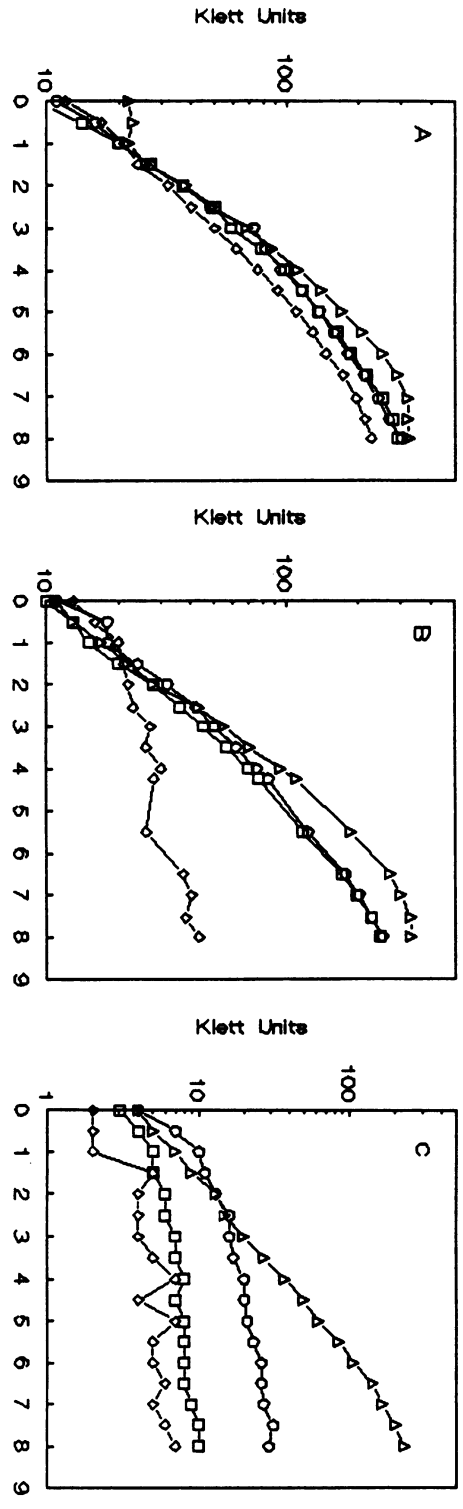

FIG. 8. Growth characteristics of *lip* mutants. Strains were grown in minimal E glucose medium in a shaking waterbath at 37°C. Additional supplements were added as indicated below. Growth was monitored with a Klett-Summerson colorimeter with a green filter (1 Klett unit equals ≈5 × 10^6 cells per ml). Overnight cultures used as the starting inocula were grown in either thiamine plus lipoate (A and B) or thiamine plus acetate and succinate (C). Cells from the overnight cultures were harvested by centrifugation and resuspended in minimal E glucose medium before subculturing to avoid carryover of nutritional supplements. (A) Growth in thiamine plus 0.5 ng of lipoic acid per ml. (B) Growth in thiamine plus 0.06 ng of lipoic acid per ml. (C) Growth in thiamine plus acetate (5 mM) and succinate (5 mM). △, JK1 (wild type); ◇, KER176 (*lipA150*::Tn*1000*dKn); ○, KER184 (*lipB182*::Tn*1000*dKn); □, KER199 (*lipA2*).

enzyme. Thus, the anaerobic synthesis of lipoic acid may be rationalized as being essential to the function of this enzyme of single-carbon compound metabolism.

Is protein-bound octanoic acid a lipoic acid precursor? Isotope labeling studies have indicated that octanoic acid is a direct precursor of lipoic acid in *E. coli* (31). A recent finding that a recombinant E2p subgene overexpressed in a *lipA2* mutant strain produces an octanoyl-E2p derivative (1) raises questions about whether this unique modification is an intermediate in lipoic acid biosynthesis or merely a secondary consequence of the genetic defect. Therefore, we tested whether the *lipA* gene product could convert an octanoyl-protein species to a lipoyl-protein species in vivo. We first developed the means to produce radioactively labeled octanoyl-proteins in vivo with a label specific to the octanoyl moieties. Since the octanoyl moieties observed previously (1) were derived from in vivo fatty acid synthesis, we tested whether these moieties could be labeled by exogenously added [1-^{14}C]octanoic acid. A *fadE* mutation, which blocks β-oxidation, was found to be necessary to prevent the degradation of octanoate to acetyl coenzyme A and subsequent incorporation of this intermediate into amino acids and then protein. After labeling of a *lipA fadE* mutant strain grown in the absence of lipoic acid, two proteins, E2p and E2o, which corresponded to those labeled with [^{35}S]lipoic acid (Fig. 9), were observed on SDS-polyacrylamide gels. The octanoyl-E2o reproducibly migrated faster than lipoyl-E2o. We have used this altered mobility to ascertain whether protein-bound octanoyl moieties can be converted to lipoic acid moieties in vivo.

Strain KER296 (*lipA150*::Tn*1000*dKn *fadE*) was labeled with [1-^{14}C]octanoate as described under Materials and Methods. The cells were washed free of exogenous [1-^{14}C]octanoate and then either infected with Kohara phage λ 1G6 or left uninfected. As described previously, Kohara phage λ 1G6 contains a chromosomal fragment which rescues the *lipA2* mutation (7). We could find no evidence for the conversion of octanoyl-E2o to lipoyl-E2o in the extract of the phage-infected cells (Fig. 9). It must be noted, however, that a limited conversion of octanoyl- to lipoyl-E2o might not be detected by this assay. It seems likely that the phage-infected cells were expressing *lipA*, since *lip*$^+$ recombinants could be isolated from the infected culture, whereas no *lip*$^+$ revertants were isolated from the uninfected culture. The inability to convert octanoyl-E2o to lipoyl-E2o was confirmed by the inability of acid-hydrolyzed extracts of phage-infected cells to feed KER176 on minimal succinate medium. These results, although not conclusive, strongly suggest that the accumulation of octanoyl-proteins in *lipA* strains is not physiologically relevant but merely reflects the ability of a lipoyl-protein ligase to use octanoate as an alternative substrate under lipoate-deficient conditions.

DISCUSSION

Recently, a recombinant-plasmid-based system which permitted fine-structure mapping of the *E. coli lip* locus was used to identify two genes, *lipA* and *lipB*, both of which appeared to be involved in lipoic acid biosynthesis (42). Initial characterization of *lipA* and *lipB* null mutants indicated that *lipB* mutants were leakier than *lipA* mutants (42). In order to better understand the physiological role of these genes in lipoic acid metabolism, we compared the growth characteristics of *lipA* and *lipB* null mutants. Interestingly, functional *lipA* and *lipB* genes were required to produce functional lipoyl-protein complexes aerobically as well as

TABLE 2. Lipoic acid content and α-ketoacid dehydrogenase activity of *lip* mutants[a]

Strain	(+)-α-Lipoic acid content (ng of lipoate/mg of protein)	Dehydrogenase complex activity[b] (μmol/mg/h)	
		PDH	KGDH
JK1 (*lip*$^+$)	33.8	0.047	0.540
KER184 (*lipB182*::Tn*1000*dKn)	3.2	0.009	0.100
KER176 (*lipA150*::Tn*1000*dKn)	<0.1	<0.005	<0.005
KER310 (*lipA150*::Tn*1000*dKn *lipB175*::Tn*10*dTc)	<0.1	<0.005	<0.005

[a] Each strain was grown in minimal E medium supplemented with 0.4% glucose, 4 mM acetate, and 4 mM succinate. Values represent the averages of at least two experiments.

[b] The dehydrogenase activities for pyruvate and α-ketoglutarate are given as micromoles of 3-acetylpyridine adenine dinucleotide reduced per milligram of protein per hour for extracts of the same batches of cells used for assaying lipoic acid content.

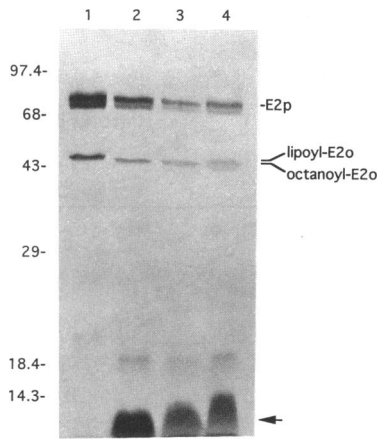

FIG. 9. Analysis of [^{35}S]lipoic acid- and [1-^{14}C]octanoic acid-labeled proteins in *E. coli* extracts. Strain KER296 was labeled with either [^{35}S]lipoic acid (lane 1) or [1-^{14}C]octanoic acid (lanes 2 to 4) as described under Materials and Methods. After labeling, cultures were either harvested directly (lanes 1 and 2) or washed free of exogenous label and then infected with Kohara phage λ 1G6 (lane 3) or left uninfected (lane 4). Cultures were adjusted to 10% trichloroacetic acid, and precipitated proteins were washed with acetone. Proteins were analyzed on a 10% polyacrylamide–SDS gel. The gel was exposed to preflashed film by fluorography. The arrow indicates an unidentified octanoyl-protein species. The mobilities of protein size standards are shown at the left (in kilodaltons).

anaerobically. Initially, the rationale for anaerobic lipoic acid biosynthesis seemed unclear, since both PDH and KGDH are inhibited by anaerobiosis (12, 23, 38). However, the finding that a *serA25 lipA150*::Tn*1000*dKn mutant strain required exogenous lipoic acid to utilize glycine as a serine source anaerobically demonstrated that the glycine cleavage system functions under these conditions.

A distinct difference between *lipA* and *lipB* mutants was that *lipB* mutants exhibited lipoic acid auxotrophy only under certain growth conditions. In fact, *lipB* mutants could grow on any medium which spared the requirement for KGDH activity. While no protein-bound lipoic acid could be detected in KER176 (*lipA150*::Tn*1000*dKn) grown in the absence of exogenous lipoic acid, about 1/10 of the wild-type level of protein-bound lipoic acid could be detected in KER184 (*lipB182*::Tn*1000*dKn) grown under the same conditions. The inability to detect the accumulation or excretion of lipoic acid in KER184 indicated that this strain possessed functional but attenuated lipoic acid biosynthetic activity. Since the *lipA150*::Tn*1000*dKn *lipB*::Tn*10*dTc double mutant exhibited the same growth characteristics as KER176, the *lipA* gene probably defines an earlier step in the lipoic acid biosynthetic pathway than *lipB*. The findings that a *lipA2* mutant accumulated octanoyl-protein species (1) and a *lipA150*::Tn*1000*dKn null mutant could utilize 6- or 8-thiooctanoic acid but not the keto- or hydroxy-derivatives of octanoic acid strongly suggest that LipA is required for insertion of at least the first sulfur into the octanoic acid backbone. This conclusion is supported by the finding that the deduced amino acid sequence of *lipA* shows some similarity to the amino acid sequence of biotin synthase (18). Biotin synthase catalyzes the terminal step in biotin biosynthesis, the incorporation of sulfur into the tetrahydrothiophene ring (13). The sequence similarity between the *lipA* and *bioB* gene products suggests that a common sulfur donor may be involved in both biotin and lipoic acid biosynthesis. Of particular interest is the conserved CXXXCXXC motif (Fig. 2), which may represent a metal-binding site. Recent reports have suggested that the introduction of sulfur into biotin may involve iron-sulfur chemistry (2, 3).

The inability of LipA to convert octanoyl-E2o to lipoyl-E2o in vivo supports the hypothesis that the synthesis of lipoic acid precedes its covalent attachment to protein. The finding that both KER176 and KER184 can utilize 6- and 8-thiooctanoic acid indicates that another unidentified activity may be responsible for the insertion of the second sulfur and formation of the dithiolane ring. These results support the following reaction sequence for the de novo synthesis of protein-bound lipoic acid in *E. coli*:

$$\text{octanoate} \xrightarrow{lipA} \text{6- or 8-thiooctanoate} \rightarrow \text{lipoate} \xrightarrow{lipB} \text{lipoyl-protein}$$

Initial efforts to identify a protein product associated with the *lipB* locus were unsuccessful (42). When the *lipB* gene was placed under the control of a heterologous promoter, an abundant 25-kDa protein was observed in vitro, whereas very little protein could be observed in vivo (Fig. 5). This result may reflect a difference in the expression of *lipB* between the in vitro and in vivo systems or a difference in the stability of LipB in vitro and in vivo. The deduced amino acid sequence of the *lipB* gene product did not show any convincing homology with any proteins in the GenBank data base. Thus, the role of *lipB* in lipoic acid metabolism remains unclear. Recent biochemical and genetic evidence suggests that *E. coli* contains two lipoyl-protein ligases (4, 28). The finding that overexpression of a gene that encodes one of these lipoyl-protein ligases (28) suppresses the *lip B184*::Tn*1000*dKn defect suggests that *lipB* may play a role in a lipoyl-protein ligase reaction. All attempts to associate a lipoyl-protein ligase activity with the *lipB* gene product have been unsuccessful thus far. However, it is possible that *lipB* encodes a single component of a multisubunit ligase enzyme. The lipoyl-protein ligase from *Streptococcus faecalis* contains two separable components (35). An alternative explanation for the attenuated production of lipoyl-proteins in *lipB* mutants is that the *lipB* gene product is a positive regulator of the lipoic acid biosynthetic genes and/or the lipoyl-protein ligase gene(s). Clearly, further genetic and biochemical analyses will be required to elucidate the role of *lipB* in lipoic acid metabolism.

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