JOURNAL OF BACTERIOLOGY, Oct. 1991, p. 6411–6420
0021-9193/91/206411-10$02.00/0
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Vol. 173, No. 20

Lipoic Acid Metabolism in *Escherichia coli*: Isolation of Null Mutants Defective in Lipoic Acid Biosynthesis, Molecular Cloning and Characterization of the *E. coli lip* Locus, and Identification of the Lipoylated Protein of the Glycine Cleavage System

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We report the isolation and genetic characterization of novel Tn*10*dTc and Tn*1000*dKn insertion mutations in and near the *lip* locus of the *Escherichia coli* chromosome. The Tn*10*dTc and Tn*1000*dKn mutations define two genes, *lipA* and *lipB*, involved in lipoic acid biosynthesis. Two representative alleles (*lip-2* and *lip-9*) from the previously reported genetic class of lipoic acid auxotrophic mutants (A. A. Herbert and J. R. Guest, J. Gen. Microbiol. 53:363–381, 1968) were assigned to the *lipA* complementation group. We have cloned the *E. coli lip* locus and developed a recombinant plasmid-based genetic system for fine-structure physical-genetic mapping of mutations in this region of the *E. coli* chromosome. We also report that a recombinant plasmid containing a 5.2-kbp *Pvu*II restriction fragment from the *E. coli lip* locus produced three proteins of approximately 8, 12, and 36 kDa by using either a maxicell or in vitro transcription translation expression system. The 36-kDa protein was identified as the gene product encoded by the *lipA* locus. Finally, we have identified a previously unreported lipoylated protein that functions in the glycine cleavage system of *E. coli*.

R-(+)-Lipoic acid (6,8-thioctic acid) is a widely occurring coenzyme reported in a diverse group of prokaryotic and eukaryotic microorganisms (26) and a variety of plant and animal tissues (for reviews, see references 43 and 44). In the functional protein-bound form of the coenzyme (Fig. 1), the carboxyl group of lipoic acid is bound in amide linkage to the ε-amino group of a lysyl residue (43). The best-understood enzymatic role of lipoic acid as a coenzyme is in the oxidative decarboxylation of α-keto acids (44). Two species of α-keto acid dehydrogenases have been purified from *Escherichia coli* and studied in some detail: the pyruvate dehydrogenase complex (PDH) and the α-ketoglutarate dehydrogenase complex (KGDH). Each complex contains multiple copies of three enzyme subunits, including complex-specific dehydrogenase (E1p or E1o) and dihydrolipoamide acyltransferase (E2p or E2o) subunits and a common lipoamide dehydrogenase (E3) subunit (37). The E2p and E2o subunits, encoded by the *aceF* and *sucB* genes, respectively, are posttranslationally modified by covalent attachment of lipoic acid moieties to specific lysyl residues (44). The lipoyl domains in these subunits are thought to provide a "swinging arm" that permits movement of substrates between the various active sites in the enzyme complex (for review, see reference 44).

Isotope experiments have suggested that octanoic acid is a direct biosynthetic precursor of lipoic acid in *E. coli*. In addition, tritium and deuterium retention experiments have indicated that introduction of sulfur into the octanoic acid carbon skeleton does not involve desaturation of the fatty acyl chain (for review, see reference 39). Mutant strains of *E. coli* defective in lipoic acid biosynthesis (lipoic acid auxotrophs) have been reported (24). These mutants were placed in a single genetic class (designated *lip*); the *lip* mutation maps to ca. min 14.5 on the *E. coli* chromosome (24). Extracts of *lip* mutants grown in the absence of exogenous lipoic acid lack any detectable PDH or KGDH activity (24). However, the specific nature of the genetic defect in these mutants has not been elucidated. A recent finding (1, 2) is that a recombinant E2p subgene overexpressed in a *lip-2* mutant strain produces an octanoylated E2p derivative. Moreover, several lines of evidence indicated that the octanoyl modification occurred on the same lysyl residue that is normally lipoylated (2). It remains to be determined whether this unique modification of the E2 subunit is physiologically relevant (e.g., a precursor in the E2-lipoyl biosynthetic pathway) or merely a secondary consequence of the genetic defect (2).

We have initiated studies aimed at better understanding the biosynthesis and metabolism of lipoic acid in *E. coli*. In this article, we report the isolation of novel Tn*10*dTc and Tn*1000*dKn insertion mutants defining two genes, *lipA* and *lipB*, involved in lipoic acid biosynthesis. We have also determined the nature of the genetic defect in two representative alleles (*lip-2* and *lip-9*) from the previously identified genetic class of lipoic acid auxotrophic mutants (24). In addition, we have cloned the *E. coli lip* locus and examined the organization of structural genes in this region of the *E. coli* chromosome. Finally, we report the identification of a third lipoylated protein in extracts of *E. coli* that is involved in the glycine cleavage enzyme system of this organism.

(This work is in partial fulfillment of the requirements for the Ph.D. degree for K. E. Reed from the University of Illinois.)

MATERIALS AND METHODS

Bacterial and bacteriophage strains. The bacterial strains used in this study are listed in Table 1. Kohara λ phage 1G6

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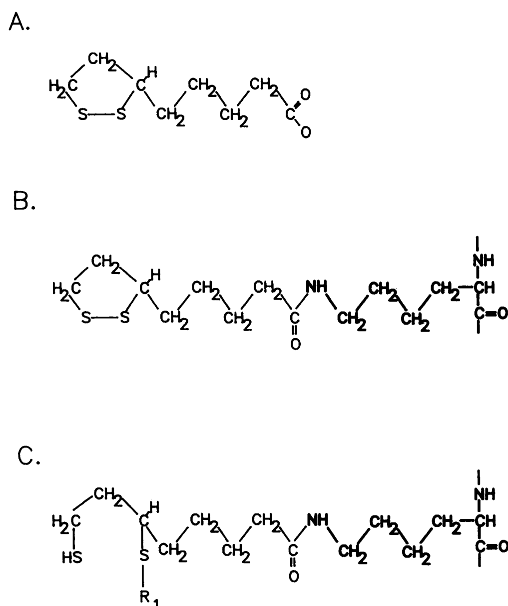

FIG. 1. (A) Chemical structure of lipoic acid. (B) Functional protein-bound form of lipoic acid (lipoyl-lysyl moiety). (C) Modified form of protein-bound lipoic acid. In the α-keto acid dehydrogenases, R_1 is an acetyl or succinyl group. In the glycine cleavage enzyme systems, R_1 is a methyl amine group. In panels B and C, the lysyl moiety is indicated in boldface type.

(miniset number 168) has been described elsewhere (10, 29). The λ::Tn*10*dTc vehicle lambda λNK1098 was obtained from N. Kleckner (53). Strains JRG26 (*lipA2*) and JRG33 (*lipA9*) are thought to be representative mutants from the single genetic class of lipoic acid auxotrophs isolated by Herbert and Guest (24).

Plasmid constructions. Recombinant plasmid vectors pSU19, pMTL22 (8), and pMTL23 (8) were used in this work. Plasmid pSU19 is a derivative of plasmid pSU2719 constructed by Martinez et al. (32). Plasmid pUC4K was used as a source of the kanamycin resistance gene cartridge (41). Plasmid subclones derived from the 18-kbp chromosomal fragment in Kohara phage λ 1G6 are shown in Fig. 2A. Plasmids pCTV601 and pCTV602 contain the 3.8- and 4.2-kbp *Eco*RI restriction fragments, respectively, from Kohara phage λ 1G6 inserted into the *Eco*RI site of plasmid pMTL23. Plasmid pCTV603 contains the 4.0-kbp *Hin*dIII restriction fragment from λ 1G6 inserted into the *Hin*dIII site of plasmid pMTL23. Plasmids pCTV604 and pCTV605 contain the 4.2-kbp *Eco*RI restriction fragment from plasmid pCTV602 inserted in opposite orientations in the *Eco*RI site of plasmid pSU19, as indicated in the legend to Fig. 2A. Plasmid pCTV616 contains the 5.2-kbp *Pvu*II restriction fragment from λ 1G6 inserted into the *Sma*I site of plasmid pSU19. Plasmids pCTV632 and pCTV633 contain the *Bam*HI-*Kpn*I-liberated chromosomal fragment from pCTV 616 ligated to the corresponding *Bam*HI and *Kpn*I sites in plasmids pMTL22 and pMTL23, respectively. Plasmid pIT103 (Fig. 2A) was obtained from I. Takase (50) and contains a 2.8-kbp chromosomal *Sal*I-*Hin*dIII restriction fragment which overlaps the *E. coli lip* locus.

Plasmids pKR58 through pKR67 were constructed as follows. The 1.2-kbp *Bam*HI restriction fragment containing the kanamycin resistance gene from plasmid pUC4K was purified, and the recessed ends were filled in by using the Klenow fragment of DNA polymerase I. The resulting blunt-ended fragment was inserted into the unique *Sma*I site located within the Tn*1000* element in the pCTV plasmids shown in Table 2. We have designated the resulting Tn*1000* derivatives Tn*1000*dKn.

Culture media and growth conditions. The following growth media were routinely used for growth of bacterial strains: minimal E medium (15), modified Davis-Mingioli medium (14), rich broth [RB (16)], and 2XYT medium (35). Solid medium contained 1.5% agar (United States Biochemical). Supplements were added as necessary at the following concentrations unless otherwise indicated: glucose (0.4%), sodium acetate (5 mM), sodium succinate (5 mM), thiamine (1 μg/ml), threonine (20 μg/ml), histidine (22 μg/ml), arginine (22 μg/ml), leucine (20 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%), sodium pp_i (1.2 mM), and DL-lipoic acid (5 ng/ml). All amino acids were of the L-form. Antibiotics were added at the indicated concentrations: chloramphenicol (50 μg/ml), ampicillin (100 μg/ml), kanamycin sulfate (25 or 50 μg/ml), and tetracycline-HCl (2 or 10 μg/ml). In cloning experiments with the α-complementation system, 20 μl of a 100 mM stock solution of isopropylthiogalactoside (IPTG) and 75 μl of a 2% stock solution of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were spread directly on solid agar media 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). Lipoic acid was assayed by a disk microbiological method similar to that described for biotin (17). A protocol for this assay is available upon request from the authors.

Genetic techniques. P1 *vir* transductions and bacterial conjugations were carried out as described by Miller (35). Hfr mapping of Tn*10*dTc insertion mutants was performed with the *E. coli* Hfr mapping kit assembled by Singer et al. (47). Transpositions of Tn*10*dTc were carried out as described by Way et al. with λNK1098 (53). Tn*1000* mutagenesis of plasmid pCTV616 was carried out as follows. Supercoiled monomer form of plasmid pCTV616 was purified from a 1% low-melting-point agarose gel (Seaplaque agarose; FMC Corp.) and used to transform strain JA200. Strain JA200(pCTV616) was subsequently mated with strain DH5α. Samples of the conjugation mixture were plated on minimal E glucose medium supplemented with thiamine and chloramphenicol (50 μg/ml). Plasmid DNA was prepared from purified chloramphenicol-resistant exconjugants and screened for pCTV616::Tn*1000* derivatives. A second method was used to specifically isolate Tn*1000* insertions within the structural gene encoding the 36-kDa protein (see below). Several thousand exconjugants from an experiment performed as described above were pooled, and plasmid DNA was prepared from the pooled culture by a miniprep procedure. This plasmid DNA was used to transform strain KER72 (*lipA2 recA*). The lipoic acid nutritional phenotype of chloramphenicol-resistant transformants was scored on minimal E glucose medium with and without lipoic acid. Transformants which required a lipoic acid supplement for growth contained the desired pCTV616::Tn*1000* derivatives, which no longer complemented the *lipA2* allele (see below).

Recombinant DNA techniques. Recombinant DNA techniques were performed as described by Maniatis et al. (30) and Ausubel et al. (3). Restriction enzymes, T4 DNA ligase, and DNA polymerase I (Klenow fragment) were obtained from Bethesda Research Laboratories, Boehringer Mann-

TABLE 1. Bacterial strains used in this work

Strain	Sex	Genotype[a]	Source or reference
JK1	F^-	*rpsL*	J. Konisky
JRG26	F^-	*supE iclR lipA2*	J. R. Guest (24)
JRG33	F^-	*proA purB his thi rpsL gal lipA9*	J. R. Guest (24)
GP150	F^-	*araD139 ΔlacU169 rpsL thi recA::kan*	T. Silhavy
DH5α	F^-	ϕ80 d*lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA1*	A. Salyers
JA200	F^+	*recA thr leu ΔtrpE5 lacY*	CGSC
KER72	F^-	JR626 Δ(*recA-srl*) *srl*::Tn*10*	This study
TVB98	F^-	JK1 *lipB175*::Tn*10*dTc	This study
TVB99	F^-	JK1 *lipB176*::Tn*10*dTc	This study
TVB100	F^-	JK1 *lipB177*::Tn*10*dTc	This study
TVB101	F^-	JK1 *lipB178*::Tn*10*dTc	This study
TVB102	F^-	JK1 *lipB179*::Tn*10*dTc	This study
TVB103	F^-	JK1 *lipB180*::Tn*10*dTc	This study
TVB104	F^-	JK1 *lipB181*:Tn*10*dTc	This study
TVB106	F^-	TVB98 *recA*::*kan*	This study
TVB107	F^-	TVB99 *recA*::*kan*	This study
TVB108	F^-	TVB100 *recA*::*kan*	This study
TVB109	F^-	TVB101 *recA*::*kan*	This study
TVB110	F^-	TVB102 *recA*::*kan*	This study
TVB111	F^-	TVB103 *recA*::*kan*	This study
TVB112	F^-	TVB104 *recA*::*kan*	This study
TVB115	F^-	JRG33 *recA*::*kan*	This study
CY265	Hfr	HfrC *ΔaceEF*	Laboratory collection
TK3D01	F^-	Δ(*gltA-sdhCDAB-sucAB*) Δ(*gal-bio*)	J. Guest (7)
CAG12149	F^-	MG1655 *zbd-601*::Tn*10*	Singer et al. (47)
PA360	F^-	*thi-1 thr-1 leu-6 argH1 his-1 serA1 mtl-2 malA1 ara-13 xyl-7 gal-6 lacY1 tonA*	CGSC
KER56	F–	JRG26 *zbd-601*::Tn*10*	This study
KER61	F^-	PA360 *lipA2 zbd-601*::Tn*10*	This study
KER62	F^-	PA360 *zbd-601*::Tn*10*	This study
JC7623	F^-	*thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE37 recB21 recC22 sbcB15 sbcC201*	CGSC
KER176	F^-	*rpsL lipA150*:: Tn*1000*dKn	This study
KER177	F^-	*rpsL zbe-2242*:: Tn*1000*dKn	This study
KER178	F^-	*rpsL zbe-2243*:: Tn*1000*dKn	This study
KER198	F^-	*rpsL zbe-2244*:: Tn*1000*dKn	This study
KER184	F^-	*rpsL lipB182*:: Tn*1000*dKn	This study
KER185	F^-	*rpsL zbe-2245*:: Tn*1000*dKn	This study
KER193	F^-	*rpsL zbe-2246*:: Tn*1000*dKn	This study
KER195	F^-	*rpsL lipB183*:: Tn*1000*dKn	This study
KER196	F^-	*rpsL zbe-2247*:: Tn*1000*dKn	This study
KER197	F^-	*rpsL zbe-2248*:: Tn*1000*dKn	This study
KER199	F^-	chgstyle;col;tk;3*rpsL lipA2 zbd-601*::Tn*10*	This study
CAG12206	Hfr	PO1 *thi-1 relA1 spoT1 supQ80 nadA3052*:: Tn*10*kan	Singer et al. (47)
CAG12203	Hfr	PO43 *relA1 zbc-3105*:: Tn*10*kan	Singer et al. (47)

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

heim Biochemical, and New England BioLabs. All enzymatic reactions were carried out according to the manufacturers' specifications. Southern hybridizations were carried out with the Genius (Boehringer Mannheim) nonradioactive nucleic acid detection system (4). Restriction fragments used in cloning experiments and as Southern hybridization probes were purified either by electroelution (52) or by the phenol-freeze extraction method.

SDS-PAGE analysis of in vitro transcription-translation and maxicell 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 approximately 30 μCi of ^{35}S-Trans label (1,074 Ci of [^{35}S]methionine and [^{35}S]cysteine per mmol; ICN Biomedicals). Maxicell experiments were performed as described by DeVeaux et al. (18). Radiolabeled proteins were analyzed on 16% polyacrylamide–sodium dodecyl sulfate (SDS) gels for polyacrylamide gel electrophoresis (PAGE).

In vivo cloning of *lipB*::Tn*10*dTc alleles. Chromosomal insertion mutations were moved to the corresponding cloned segment essentially as described by Chang and Cronan (9). Briefly, purified plasmid DNA (pCTV604 or pCTV616) was irradiated with approximately 100 to 500 J of UV light per m^2. The irradiated plasmid DNA was used to transform various Tn*10*dTc mutant strains. Several thousand chloramphenicol-resistant transformants were pooled, and plasmid DNA was prepared from the pooled cultures by a miniprep procedure (3). This plasmid DNA was subsequently used to transform strain DH5α. Transformants were selected on RB plates containing tetracycline (2 μg/ml) and chloramphenicol (50 μg/ml).

Radiolabeling of cultures with [^{35}S]lipoic acid. *R*-(+)-[^{35}S] lipoic acid was synthesized as described for the nonradioactive compound (21). The di-(*t*-butyl dimethylsilyl) derivative of (6*S*)-isopropyl-6,8-dihydroxyoctanoate was obtained as a gift from W. S. Johnson. The *t*-butyl dimethylsilyl moieties were removed by treatment with Dowex 50X-8 ion-exchange resin (11) to generate isopropyl-6,8-dihydroxyoctanoate. The remainder of the synthesis was done as described by Elliot et al. (21), except for the use of ^{35}S-elemental sulfur (Amersham Corp.) to make the labeled compound. The final product had a specific activity of approximately 0.8 Ci/mmol (estimated by disk bioassay of lipoic acid).

Strains JRG26, CY265, and TK3D01 were cultured at 37°C to 1×10^9 to 2×10^9 cells per ml in minimal E medium containing glucose, thiamine, cysteine and [^{35}S]lipoic acid (8 ng/ml) (0.8 Ci/mmol). Cysteine was added to repress cysteine biosynthesis, thereby precluding labeling of protein by utilization of the [^{35}S]sulfide that contaminated the [^{35}S]lipoic acid. Strains CY265 and TK3D01 were additionally supplemented with acetate and succinate, respectively. Unless otherwise indicated, glycine was added at 100 μg/ml. The cells were harvested and lysed in a solution of 0.1 M

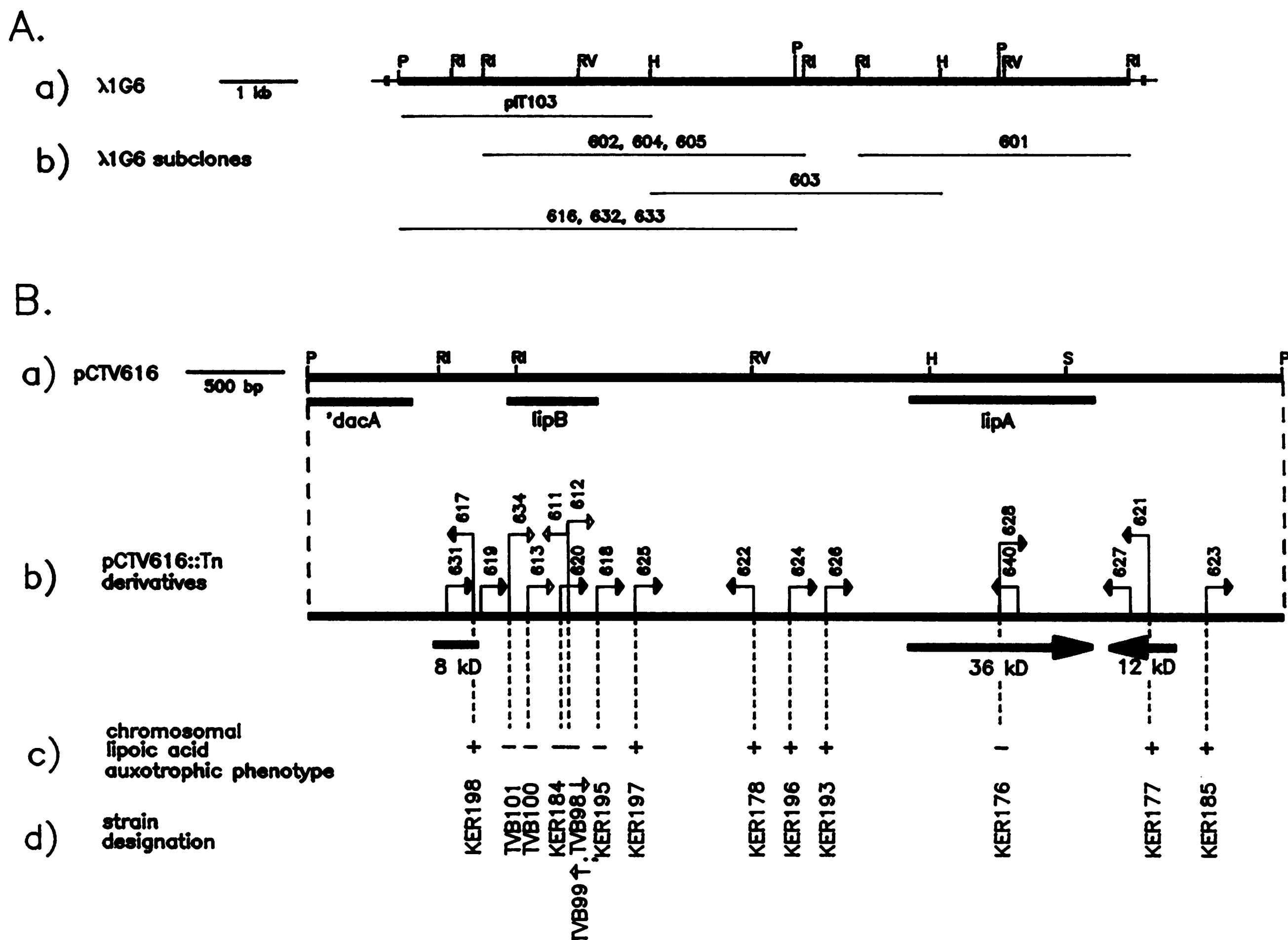

FIG. 2. (A) Recombinant phage and plasmid clones of the *E. coli lip* locus. (a.) Restriction map of the *lip* segment of the chromosomal insert in Kohara phage λ 1G6. (b.) λ 1G6 subclones. For plasmid subclones pCTV605 and pCTV616, the left end of the subcloned restriction fragments (as shown above) is proximal to the *lac* operator of the vector. The insert in plasmids pCTV602 and pCTV604 has the opposite orientation relative to the vector. The orientation of the subcloned restriction fragments was not determined for plasmids pCTV601 and pCTV603. Plasmid pIT103 (50) is also shown. (B) Physical and genetic maps of the *E. coli lip* locus. (a.) Physical-genetic map of the 5.2-kbp *Pvu*II restriction fragment of plasmid pCTV616. Genes identified on this segment of the chromosome are given below the physical restriction map. The location of the *dacA* gene is based on nucleotide sequence data (5). The location of the *lipB* gene is defined by transposon insertion mutations (see panel b, below). The location of the *lipA* gene is based on the location of transposon insertions in this gene and on the apparent molecular weights of full-length and truncated forms of the *lipA* gene product identified in SDS-polyacrylamide gels. (b.) Physical map of transposon insertions in plasmid pCTV616. The numbers refer to the pCTV plasmid designations. The direction of the arrows indicates the orientation of the transposable element. For Tn*10*dTc, right to left corresponds to the orientation of the element depicted by Way et al. (53). For Tn*1000*, right to left corresponds to delta to gamma. Tn*10*dTc elements are designated by open arrowheads, and Tn*1000* elements are designated by solid arrowheads. The lines under the physical map correspond to identified structural genes producing protein gene products from plasmid pCTV616 in the in vitro transcription-translation system. The preliminary assignment of the direction of transcription of the structural genes is indicated by an arrow (see text). The apparent molecular masses of the corresponding proteins are given in kilodaltons below each gene. (c.) The lipoic acid auxotrophic phenotype of isogenic strains harboring chromosomal transposon insertions at the positions shown is indicated as either a plus or minus; a plus indicates that the transposon-containing strain does not require a lipoic acid supplement, and a minus indicates that the transposon-containing strain is a lipoic acid auxotroph. (d.) The strains shown are isogenic and contain the transposon insertions given in panel c. Allele designations are given in Table 1. Abbreviations used: RI, *Eco*RI; RV, *Eco*RV; H, *Hin*dIII; P, *Pvu*II; S, *Sph*I.

Tris-HCl (pH 7.5) containing 8 M urea and 1% SDS. The cell extracts were analyzed on 7.5% polyacrylamide–SDS gels.

Construction of Tn*1000*dKn chromosomal insertions. Allele replacements were accomplished essentially as described by Oden et al. (38). Briefly, plasmids pKR58 through pKR67 were transformed into the *recBC sbcB* strain JC7623. Recombinants were selected on RB medium containing vitamin-free casein hydrolysate, lipoic acid, and kanamycin (25 μg/ml). Colonies were screened for chloramphenicol sensitivity. A P1 *vir* phage lysate grown on one kanamycin-resistant, chloramphenicol-sensitive candidate from each transformation was used to transduce strain TVB98 to kanamycin resistance. Transductants were selected on medium containing 50 μg of kanamycin per ml owing to the low-level kanamycin resistance of the recipient strain TVB98. Representative transductants were screened for sensitivity to tetracycline and for their lipoic acid nutritional phenotype. The lipoic acid growth phenotype was scored on

TABLE 2. Plasmids used in allele replacement experiments[a]

Plasmid	Parent plasmid	Mutant strain
pKR58	pCTV621	KER177
pKR59	pCTV622	KER178
pKR60	pCTV628	KER176
pKR61	pCTV617	KER198
pKR62	pCTV620	KER184
pKR63	pCTV623	KER185
pKR64	pCTV618	KER195
pKR65	pCTV624	KER196
pKR66	pCTV625	KER197
pKR67	pCTV626	KER193

[a] pKR plasmids contain a Tn*1000*dKn insertion element, and pCTV plasmids contain a Tn*1000* insertion element.

minimal E medium plates containing glucose, thiamine, and kanamycin (50 μg/ml) with or without lipoic acid (5 ng/ml).

Glycine cleavage assays. Glycine cleavage enzyme assays were performed by the method of Sagers and Gunsalus (46), as modified by Meedel and Pizer (34). Total protein content of crude cell extracts was determined by the microbiuret assay of Itzhaki and Gill (27).

RESULTS

Molecular cloning of the *E. coli lip* locus. Kohara λ phages (miniset numbers 165 to 170) containing chromosomal fragments in the vicinity of the previously identified *lip* locus (24) of the *E. coli* chromosome were screened for their ability to rescue the *lipA2* mutation, as described elsewhere (10). A series of overlapping *Eco*RI, *Hin*dIII, and *Pvu*II restriction fragments from Kohara phage λ 1G6 were recovered in either the high-copy-number (500 to 700 per cell) plasmid vectors pMTL22 and pMTL23 or the intermediate-copy-number (30 to 50 copies per cell) vector pSU19, as described under Materials and Methods (Fig. 2A). The *Pvu*II restriction fragment in plasmid pCTV616 was targeted for cloning because the leftmost *Pvu*II site (as shown in Fig. 2A) was thought to lie within the nearby *dacA* structural gene (5). *dacA* is the last gene in a 7.2-kbp sequenced cluster of genes involved in cell wall biosynthesis (33). We sought to avoid cloning the intact *dacA* structural gene because cells containing multicopy plasmids carrying this gene are unusually detergent sensitive and have an aberrant cell shape (31). The orientation of the cloned *Pvu*II chromosomal fragment in plasmid pCTV616 was verified by DNA sequencing (data not shown). We found that our sequence overlapped the carboxy-terminal coding portion of the *dacA* gene (5).

Analysis of proteins expressed from plasmids carrying *lip* region chromosomal segments. Radiolabeled gene products encoded by recombinant plasmids were prepared by the in vitro transcription-translation system (Materials and Methods). The resulting proteins were analyzed on either 10 or 16% polyacrylamide–SDS gels. Although 16% polyacrylamide gels resulted in broadening of the chloramphenicol acetyltransferase band, these gels were routinely used in most experiments because the 8-, 12-, and 36-kDa proteins were resolved in a single gel. The results of this analysis (data not shown) support the chromosomal order of genes shown in Fig. 2B. Plasmids pCTV604 and pCTV605 contain the 4.2-kbp chromosomal *Eco*RI fragment in opposite orientations in plasmid pSU19, and both produce the 12- and 36-kDa proteins in the in vitro transcription-translation system. Similarly, the pattern of expression of the 8-, 12-, and

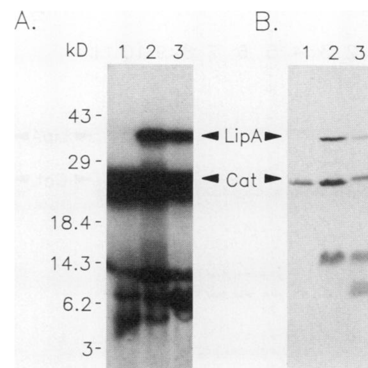

FIG. 3. Pattern of proteins produced from plasmids pSU19, pCTV604, and pCTV616. Protein products were analyzed in 16% polyacrylamide–SDS gels. (A) Proteins produced in the in vitro transcription-translation expression system. (B) Proteins produced in the maxicell expression system. Lane 1, pSU19; lane 2, pCTV604; lane 3, pCTV616. Cat, chloramphenicol acetyltransferase.

36-kDa proteins is independent of the orientation of the 5.2-kbp *Pvu*II fragment in plasmids pCTV632 and pCTV633 (data not shown). The pattern of proteins produced in the in vitro transcription-translation system with plasmids pCTV604 and pCTV616 was identical to that produced in a maxicell expression system (Fig. 3). Plasmid pIT103, constructed by I. Takase and coworkers (50) in their analysis of the cluster of penicillin-binding protein genes located near the *lip* locus, produced a single protein of approximately 8 kDa in the in vitro transcription-translation system (data not shown).

Organization of genes in the *dacA-lip* region of the *E. coli* chromosome. In order to determine more precisely the location of the genes contained within the *Pvu*II restriction fragment in plasmid pCTV616, the plasmid was mutagenized with Tn*1000*. A set of Tn*1000* insertion derivatives of this plasmid spanning the 5.2-kbp *Pvu*II chromosomal fragment were assembled through restriction analysis of independent pCTV616::Tn*1000* candidates (Fig. 2B). Our original screen of 48 pCTV616::Tn*1000* derivatives yielded a single insertion (pCTV628) in the gene encoding the 36-kDa polypeptide, despite the relatively large target size of this gene (≈1.0 kbp). Three additional Tn*1000* insertions disrupting the gene encoding the 36-kDa protein were isolated by using the modified Tn*1000* mutagenesis protocol described under Materials and Methods (including plasmid pCTV640; Fig. 2B; see also Fig. 4). The gene products produced by this set of plasmids in the in vitro transcription translation system were analyzed as described above. The identification of truncated polypeptides produced by plasmids pCTV628, pCTV640, and pCTV627 is consistent with the indicated directions of transcription of the genes encoding the 12- and 36-kDa proteins (Fig. 2B). Plasmids pCTV628 and pCTV640 produce truncated forms of the 36-kDa protein of approximately 27 and 30.5 kDa, respectively (Fig. 4B).

The *lip-2* and *lip-9* alleles define a single genetic complementation group encoding the 36-kDa protein. As indicated above, a *lip-2* mutant strain was used to identify the Kohara λ phage 1G6 which carried the corresponding chromosomal *lip* DNA segment. Recombinant plasmids derived from λ 1G6 (pCTV601, pCTV602, pCTV603, pCTV604, and pCTV616) were transformed into the *lip-2 recA* strain KER72. Plasmids pCTV602, pCTV604, and pCTV616 com-

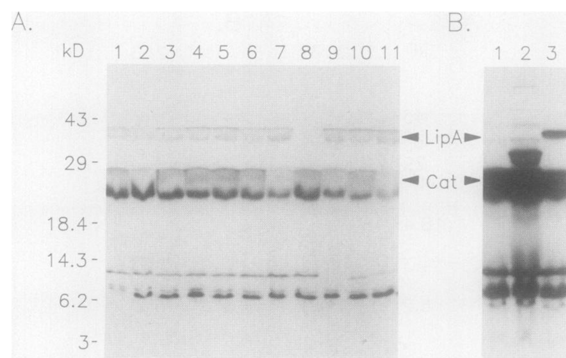

FIG. 4. Pattern of proteins produced from pCTV616::Tn*1000* derivatives in the in vitro transcription-translation system. Proteins were analyzed in a 16% polyacrylamide–SDS gel. (A) Lane 1, pCTV617; lane 2, pCTV620; lane 3, pCTV618; lane 4, pCTV625; lane 5, pCTV622; lane 6, pCTV624; lane 7, pCTV626; lane 8, pCTV628; lane 9, pCTV621; lane 10, pCTV627; lane 11, pCTV623. (B) Lane 1, pCTV628; lane 2, pCTV640; lane 3, pCTV616. The truncated form of the LipA protein produced by plasmid pCTV628 migrates slightly above the *cat* gene product. The presence of this species was verified in in vitro transcription-translation extracts lacking the Cat protein (data not shown). Cat, chloramphenicol acetyltransferase.

plemented the defect in the *lip-2* strain (Table 3). This pattern of complementation among the above recombinant plasmids suggested that the *lip-2* allele encoded a defective 36-kDa protein. Transformation of these plasmids into the *lip-9 recA* strain TVB115 yielded identical results. In order to confirm that the *lip-2* and *lip-9* strains encoded defective 36-kDa proteins, a pCTV616 derivative containing a Tn*1000* insertion which disrupted the cloned structural gene for this protein was isolated (pCTV628). Plasmid pCTV628 no longer complemented the *lip-2* and *lip-9* mutations (Table 3) and produced only the 8- and 12-kDa proteins in the in vitro transcription-translation system. We conclude that the *lip-2* and *lip-9* mutations are allelic and that they define the gene

TABLE 3. Complementation of *lipA* and *lipB* mutant strains with recombinant plasmids

Strain	Relevant genotype[a]	Complementation[b] with plasmid:					
		pCTV 604	pCTV 616	pCTV 628	pCTV 618	pCTV 634	Vector
KER72	*lipA2*	+	+	−	+	+	−
TVB115	*lipA9*	+	+	−	+	+	−
TVB127	*lipA150*::Tn*1000*dK	+	+	−	+	+	−
TVB106	*lipB175*::Tn*10*dTc	+	+	+	−	−	−
TVB109	*lipB178*::Tn*10*dTc	+	+	+	−	−	−

[a] Only the relevant *lip* allele is given. All of the strains are also *recA*.

[b] The indicated recombinant plasmids were transformed into the mutant strains shown. Transformants were selected on RB medium supplemented with chloramphenicol and 5 ng of lipoic acid per ml. Fifty independent transformants were patched onto minimal E glucose plates with and without lipoic acid. Generally, from 90 to 100% of the transformants patched from RB medium grew on nutritionally permissive minimal medium. In order to reliably score complementation of *lipB* alleles, transformants were restreaked or replicated from the original minimal E glucose plate to fresh medium. A positive complementation result indicates that the mutant strain containing the recombinant plasmid was able to grow on minimal E glucose medium lacking lipoic acid. Growth was scored after 48 h at 37°C. Fifty independent transformants for each plasmid-mutant strain combination were tested in order to avoid ambiguities due to lipoic acid carryover, shiftdown phenomena, and reversion events. All of the plasmids are derivatives of plasmid pSU19.

encoding the 36-kDa protein. We have designated this genetic locus *lipA* in accordance with the nomenclature of Herbert and Guest (24).

Tn*10*dTc insertion mutants (lipoic acid auxotrophs) define a second genetic complementation group defective in lipoic acid biosynthesis. Strain JK1 was mutagenized with Tn*10*dTc as described under Materials and Methods. Four thousand tetracycline-resistant colonies from RB plates supplemented with sodium acetate, sodium succinate, sodium pp_i, and tetracycline (10 μg/ml) were patched onto minimal E glucose medium supplemented with sodium acetate, sodium succinate, and tetracycline (10 μg/ml). The use of patched (50 isolates per plate) replica master plates was necessary in order to avoid cross-feeding phenomena. Following 24 h of growth at 42°C, this master plate was replicated onto minimal E glucose plates with or without lipoic acid. This screen yielded seven independent transposon insertion-generated lipoic acid auxotrophs. The tetracycline resistance and lipoic acid auxotrophic markers were 100% linked in P1 *vir* transduction experiments. The Tn*10*dTc mutants were indistinguishable under a variety of nutritional conditions. All of the mutants grew on minimal E glucose medium supplemented with either lipoic acid or acetate plus succinate.

The mutations were localized to between 12 and 17 min on the *E. coli* genetic map by Hfr mapping with strains CAG12206 and CAG12203 (35, 47). Since this region included the cloned *lip* locus described above, we attempted to recover the chromosomal Tn*10*dTc mutations onto a plasmid by the in vivo cloning strategy described previously (9). All of the mutations were successfully recombined into either plasmid pCTV604 or pCTV616. The in vivo cloning strategy was unsuccessful with the corresponding higher-copy-number pMTL23-derived clones, presumably due to the negative gene dosage effects associated with the *tetA* gene (19). However, this problem was avoided by use of pSU19-derived clones and a tetracycline concentration of 2 μg/ml (versus 10 μg/ml) to select Tn*10*dTc-containing recombinants. The location and orientation of the transposon insertion mutations within the cloned *lip* DNA segment were then determined by restriction mapping of the pCTV604::Tn*10*dTc and pCTV616::Tn*10*dTc derivatives (Fig. 2B).

Several independent isolates for each construction gave identical restriction patterns. Interestingly, all of the Tn*10*dTc insertion mutations mapped within an approximately 500-bp region of DNA at least 1.4 kbp removed from the *lipA* locus (Fig. 2B). Analysis of the products produced by Tn*10*dTc-containing plasmids pCTV634, pCTV613, pCTV611, and pCTV612 in the in vitro transcription-translation system indicated that these mutations did not affect the expression of any of the three identified polypeptides encoded by the *Pvu*II chromosomal fragment of plasmid pCTV616 (data not shown). In order to verify that the mutants represented simple insertions into the expected region of the *E. coli* chromosome, Southern hybridizations of each of the mutant strains and corresponding plasmids were performed. This analysis demonstrated that all insertions were the products of simple insertion events within the expected *E. coli* DNA segment (data not shown). For the pCTV616::Tn*10*dTc derivative pCTV612, this was also confirmed by reconstructing the original insertion mutant from the Tn*10*dTc-containing clone by the allele replacement procedure described for Tn*1000*dKn mutants (Materials and Methods). All of the resulting recombinants from the allele replacement experiment had the predicted lipoic acid auxotrophic phenotype. Recombinant plasmids containing representative Tn*10*dTc insertion mutations were transformed

into strain KER72 to test for complementation of the *lipA2* allele (Table 3). All Tn*10*dTc-containing derivatives of plasmid pCTV616 complemented the *lipA2* defect but failed to complement any of the other Tn*10*dTc mutations (Table 3). We have designated the genetic locus defined by the Tn*10*dTc mutants *lipB*. Our analyses of the proteins produced by plasmid pCTV616 or by any of the pCTV616 derivatives in the in vitro transcription-translation system have thus far failed to identify a protein product associated with the *lipB* locus.

Isolation and characterization of chromosomal Tn*1000*dKn insertion mutants. As detailed above, mutational analysis of plasmid pCTV616 revealed genes encoding 8- and 12-kDa proteins adjacent to the *lipB* and *lipA* genes, respectively. In order to determine whether these gene products are directly involved in lipoic acid biosynthesis, chromosomal Tn*1000*d Kn insertion mutations were constructed in each gene, as described under Materials and Methods. The chromosomal Tn*1000*dKn insertions showed 100% linkage in P1 *vir* transductions with the Tn*10*dTc *lipB175* allele in strain TVB98, as predicted. The constructions were also verified by Southern hybridization analysis of the Tn*1000*dKn mutant and isogenic wild-type strains (data not shown). The Tn*1000*dKn mutant strains KER183 and KER177, defective for the 8- and 12-kDa protein, respectively, were tested for lipoic acid auxotrophy. Both strains were able to grow on minimal E glucose medium lacking lipoic acid. Thus, these proteins do not appear to be directly involved in lipoic acid biosynthesis.

In order to gain additional insight into the genetic organization of the *E. coli lip* locus, a collection of Tn*1000*dKn insertion mutations located within the DNA segment between the *lipA* and *lipB* genes were constructed and characterized (Fig. 2B). Mutant strains KER184 and KER195 were *lipB* mutants. These strains were nutritionally indistinguishable from the isogenic Tn*10*dTc *lipB* mutants TVB98, TVB99, TVB100, and TVB101. Plasmids pCTV616 and pCTV628 complemented all of the *lipB* mutations (Table 3). Plasmids harboring any of the *lipB* alleles (e.g., pCTV618 and pCTV634, Fig. 2B) failed to complement any other *lipB* mutation (Table 3). Interestingly, several Tn*1000*dKn insertion mutations mapping between the *lipA* and *lipB* genes (Fig. 2B) failed to confer lipoic acid auxotrophy. We have not yet identified any proteins encoded by this 1.4-kbp DNA segment.

We have also compared the growth characteristics of isogenic strains harboring the *lipA2*, *lipA150*::Tn*1000*dKn, and *lipB182*::Tn*1000*dKn alleles. The *lipA2* and *lipB182*::Tn*1000*dKn mutants appear to be leakier than the *lipA150*::Tn*1000*dKn mutant strain (data not shown).

Lipoylated proteins of *E. coli*. It has long been known that *E. coli* contains two lipoylated proteins, the *aceF* subunit of pyruvate dehydrogenase and the *sucB* subunit of α-ketoglutarate dehydrogenase (37). To our knowledge, no other lipoylated proteins have been reported for this organism, but no direct data were available. We report the presence of a third lipoylated protein detected by labeling *E. coli* with [^{35}S]lipoic acid.

Two proteins of approximately 82 and 51 kDa were detected in [^{35}S]lipoic acid-labeled extracts of strain JRG26 (*lipA2*) grown in minimal E glucose medium. The 82- and 51-kDa proteins were readily identified as the lipoylated subunits of pyruvate dehydrogenase (*aceF*, encoding E2p) and α-ketoglutarate dehydrogenase (*sucB*, encoding E2o), respectively, by use of the appropriate deletion strains (Fig. 5A) (13, 40). Extracts of JRG26 labeled with [^{35}S]lipoic acid in an amino acid-containing medium (25) contained an additional protein of approximately 30 kDa apparent molecular mass (data not shown). The presence of the third protein only in cells grown in the presence of amino acids suggested that it might be a component of the glycine cleavage system.

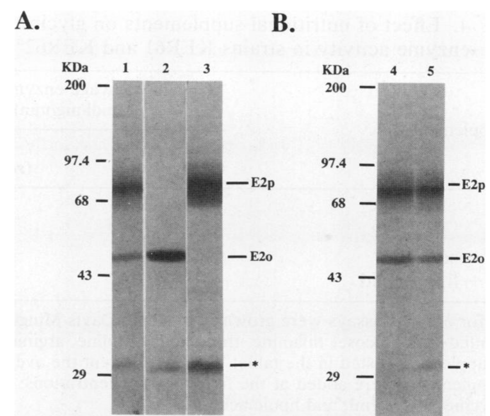

FIG. 5. Analysis of [^{35}S]lipoic acid-labeled proteins in *E. coli* extracts. Strains were grown in minimal E glucose medium. Extracts were analyzed in 12% polyacrylamide–SDS gels. (A) Strains grown in the presence of glycine. Lane 1, JRG26; lane 2, CY265 (*aceF*); lane 3, TK3D01 (*sucB*). *, unidentified lipoylated protein. (B) Strain JRG26 grown in the absence (lane 4) or presence (lane 5) of glycine.

The glycine cleavage system of vertebrates (22, 36) and the bacteria *Peptococcus glycinophilus* (45) and *Arthrobacter globiformis* (28) has been reported to contain an essential small lipoylated protein. This lipoylated protein serves as an acceptor of a methylamine group from glycine (Fig. 1). In the overall reaction, glycine is cleaved to CO_2, NH_3, and 5,10-methylene tetrahydrofolate while generating one reducing equivalent. The presence of the glycine cleavage system in *E. coli* has been reported, but only for cells grown in the presence of glycine (34, 42). Thus, we tested whether the presence of the 30-kDa lipoylated protein depended on growth in the presence of glycine. As shown in Fig. 5B, the 30-kDa protein was found only in cells grown with glycine supplementation. Only basal levels of the 30-kDa protein were found in serine-supplemented cells (Fig. 5B, lane 4), consistent with the induction specificity of the glycine cleavage system (34).

The specific induction of the 30-kDa lipoylated protein argued that this protein was a component of the glycine cleavage system and hence that lipoic acid should be required for glycine cleavage in *E. coli*. We have tested this requirement by both in vivo and in vitro assays. The in vivo assay depends on the growth phenotypes of serine auxotrophs of *E. coli*. Serine auxotrophs (*serA*, *-B*, or *-C*) grow well when supplemented with either serine or glycine (34). Serine auxotrophs additionally blocked in the glycine cleavage pathway are unable to use glycine as a serine source (34) because they are unable to form sufficient 5,10-methylene tetrahydrofolic acid. Strains harboring the *lipA2* allele require either exogenous lipoic acid or acetate plus succinate for growth (24). To determine the effect of the *lipA2* mutation on the glycine cleavage system, a *serA1 lipA2* double mutant (KER62) was constructed. The *serA1* allele was required because a defective glycine cleavage phenotype can only be observed in a *ser* background (42). The medium used in the plate growth tests was modified Davis-Mingioli medium supplemented with glucose, sodium acetate, sodium succi-

TABLE 4. Effect of nutritional supplements on glycine cleavage enzyme activity in strains KER61 and KER62[a]

Supplement	Glycine cleavage enzyme sp act[b] (pmol/mg/min)	
	KER62 (*serA1*)	KER61 (*serA1 lipA2*)
Serine	48	<23[c]
Glycine	457	ND[d]
Glycine + serine	362	<23[c]
Glycine + lipoic acid	ND	131

[a] Cells for enzyme assays were grown in modified Davis-Mingioli medium supplemented with glucose, thiamine, threonine, histidine, arginine, leucine, and the supplements listed in the table. Values represent the average of two trials. Supplements were added at the following concentrations: serine, 200 μg/ml; glycine, 300 μg/ml; and lipoic acid, 100 ng/ml.

[b] Specific activities are in picomoles of formaldehyde generated per milligram of protein per minute.

[c] Sodium acetate (5 mM) and sodium succinate (5 mM) were added as additional growth supplements.

[d] ND, not done.

nate, thiamine, threonine, histidine, arginine, leucine, and one of the following: serine (100 μg/ml), glycine (100 μg/ml), or glycine (100 μg/ml) plus lipoic acid (50 ng/ml). Growth was scored after incubation at 37°C for 48 h. As expected, strain KER62 (*serA1*) grew when supplemented with serine, glycine, or glycine plus lipoic acid. In contrast, strain KER61 (*serA lipA2*) grew with a serine supplement, but only grew with glycine supplementation if lipoic acid was also added to the medium. Thus, lipoic acid was required for utilization of glycine as a serine source.

Glycine cleavage enzyme assays were performed on extracts of strains KER61 and KER62 (Materials and Methods) to determine whether the inability of strain KER61 to use glycine as a serine source in the absence of lipoic acid was due to altered activity of the glycine cleavage system (Table 4). Consistent with previous work (34), we found that the glycine cleavage activity of the *serA1* strain KER62 was induced by glycine and unaffected by the presence of serine as long as glycine was present. Strain KER61 (*serA1 lipA2*) showed no detectable glycine cleavage activity when grown in the presence of glycine and serine. However, when strain KER61 was grown with a lipoic acid supplement (in addition to serine and glycine), the glycine cleavage activity increased greatly, to reach a level roughly one-third of that observed in the induced *serA1* strain KER62 (Table 4).

DISCUSSION

Our understanding of lipoic acid metabolism in *E. coli* is severely limited (20). Initial efforts at a genetic approach to studying lipoic acid biosynthesis in this organism (24) were hampered by the inability to isolate (or to distinguish between) different classes of lipoic acid auxotrophic mutants. In this article, we report the construction of a recombinant plasmid-based system which permits fine-structure physical-genetic mapping of mutations within the *lip* locus of the *E. coli* chromosome. We have used this system to identify two genes, *lipA* and *lipB*, involved in lipoic acid biosynthesis. Representative mutations (*lip-2* and *lip-9*) from the previously isolated group of 36 independent *lip* mutants (24) were assigned to the *lipA* complementation group. The isolation of this group of mutants by Herbert and Guest involved a penicillin enrichment step (24). Since *lipB* alleles appear to be leakier than *lipA* alleles, this class of mutants may have been lost during this procedure. Alternatively, *lipB* mutants may be represented among the remaining 34 mutants in the Guest collection. Our plasmid-based genetic system should permit the ready assignment of these alleles.

It is interesting that no *lipA* alleles were recovered among our collection of seven independent Tn*10*dTc insertion mutants, given the predicted target size of this gene (≈1,000 bp). We have examined the nutritional characteristics of the *lipA150*::Tn*1000*dKn null mutant (KER176) by using the plating conditions employed during the Tn*10*dTc mutagenesis experiment. Our results suggest that a *lipA*::Tn*10*dTc mutant should have survived our screening procedure (data not shown). Thus, the cause of our failure to isolate *lipA*::Tn*10*dTc mutants remains unclear. The distribution of Tn*10*dTc mutations might simply reflect the insertion specificity of Tn*10* insertions (23). Our mutagenesis results with Tn*10*dTc underscore the utility of the more directed mutagenesis approach in this study by use of Tn*1000*dKn chromosomal insertions.

Our analysis of the genetic organization of the *dacA-lip* region of the *E. coli* chromosome permitted the localization of the corresponding structural genes encoding the 8-, 12-, and 36-kDa proteins produced by plasmid pCTV616 (Fig. 2B). These genes, in addition to the *dacA* coding sequence, account for roughly 40% of the potential coding capacity of the 5.2-kbp chromosomal *Pvu*II restriction fragment present in plasmid pCTV616. We have identified the 36-kDa protein as the gene product encoded by the *lipA* gene. This result is in agreement with the preliminary assignment made by Spratt et al. (48), who used defective λ transducing phages. We have thus far not identified a protein product associated with the *lipB* gene by either the maxicell or in vitro transcription-translation plasmid expression systems. It is of interest that Spratt et al. (48) reported two proteins of approximately 11 and 20 kDa encoded between the *dacA* and *lipA* genes. It seems possible that our 8-kDa protein corresponds to the 11-kDa species reported previously. The coding capacity of the DNA segment between the structural gene for the 8-kDa protein and the *lipA* gene is more than necessary to encode the 20-kDa protein described earlier. However, we have not detected this protein species in the expression systems and SDS-PAGE formats described above. This discrepancy might be due to differences in the cloned DNA segments or in the expression systems used. (The results of Spratt and coworkers are based on the pattern of [^{35}S]methionine-labeled proteins produced in cells infected with defective λ transducing phages.) It is also possible that the 20-kDa protein was produced from a transcript originating from λ DNA sequences, since the putative DNA segment encoding this gene was located at the end of the chromosomal insert of the defective transducing phage analyzed (48). Nonetheless, the rescue of chromosomal *lipB* mutants in a *recA* genetic background with a recombinant plasmid containing the *lipB* gene indicates that a *trans*-acting product is encoded by this DNA segment.

The assignment of the direction of transcription of the genes encoding the 12-kDa protein and the LipA protein is tentative because it is based on the identification of truncated polypeptides in the in vitro transcription-translation system. This system occasionally produces truncated polypeptides due to premature translational termination which could be erroneously attributed to the location of the Tn*1000* insertion. In addition, the determination of the direction of transcription of the structural gene encoding the 12-kDa protein is based on the assumption that a Tn*1000* insertion resulting in no visible 12-kDa protein (or truncated deriva-

tive) is located in the amino terminus-proximal portion of the coding sequence relative to a Tn*1000* insertion which produces a truncated polypeptide species. Plasmid pCTV627 produces a slightly truncated derivative of the 12-kDa protein, whereas plasmid pCTV621 produces no visible 12-kDa species at all in the in vitro transcription-translation system (Fig. 2B and 4).

Our findings that a lipoylated protein in *E. coli* is inducible with glycine and that glycine cleavage activity is dependent on lipoic acid supplementation in a *serA1 lipA2* mutant strain strongly suggest that the glycine cleavage enzyme system in this organism includes a lipoylated protein similar to those reported in other microorganisms and eukaryotic cells (22, 28, 36, 45). This conclusion is further supported by the recent report by Steiert et al. (49) that glycine cleavage activity in *E. coli* is dependent on the *lpd* gene, which encodes the lipoamide dehydrogenase activity associated with the PDH and KGDH enzyme complexes. It will be of interest to examine the primary sequence of the *gcv* protein for a consensus lipoylation site (6).

The novel Tn*10*dTc and Tn*1000*dKn *lip* mutants, as well as the plasmid clones containing *lip* DNA sequences, should be useful in a variety of genetic and biochemical studies of lipoic acid biosynthesis in *E. coli*. In addition, the use of *E. coli* as a model system should facilitate studies directed at understanding the link between lipoic acid and fatty acid metabolism owing to the large number of well-characterized lipid mutants of this organism available (12, 51). Since transposon insertion mutations affecting fatty acid biosynthesis should be lethal, the Tn*10*dTc and Tn*1000*dKn *lip* mutants described above probably represent mutations in the terminal steps in the lipoic acid-biosynthetic pathway. These steps involve sulfur addition to the octanoic acid carbon skeleton and formation of the dithiolane ring (20, 39). Virtually nothing is known about the immediate sulfur donors involved in lipoic acid biosynthesis (20). In vivo radioisotope tracer studies are necessarily ambiguous because of the numerous and complex fates of ^{35}S donor molecules supplied exogenously. It seems likely that an in vitro system will be required to elucidate the terminal steps in lipoic acid biosynthesis. The availability of the cloned structural genes for putative enzymes or regulatory factors involved in lipoic acid biosynthesis should facilitate the overexpression, purification, and subsequent characterization of these activities.

ACKNOWLEDGMENTS

We gratefully acknowledge the following individuals for providing bacterial and bacteriophage strains or plasmids used in this work: B. Bachmann, F. de la Cruz, J. R. Guest, N. Kleckner, J. Konisky, F. Neidhardt, T. Silhavy, G. Stauffer, and I. Takase. We also thank W. S. Johnson for kindly providing the precursor used in the synthesis of [^{35}S]lipoic acid. Finally, we thank S. Khambatta for her expert technical assistance and D. Keating for critical reading of the manuscript.

This work was supported by Public Health Service grant AI15650 from the National Institute of Allergy and Infectious Diseases.

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