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Lipoic Acid Metabolism in *Escherichia coli*: the *lplA* and *lipB* Genes Define Redundant Pathways for Ligation of Lipoyl Groups to Apoprotein

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Lipoic acid is a covalently bound disulfide-containing cofactor required for function of the pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and glycine cleavage enzyme complexes of *Escherichia coli*. Recently we described the isolation of the *lplA* locus, the first gene known to encode a lipoyl-protein ligase for the attachment of lipoyl groups to lipoate-dependent apoenzymes (T. W. Morris, K. E. Reed, and J. E. Cronan, Jr., J. Biol. Chem. 269:16091–16100, 1994). Here, we report an unexpected redundancy between the functions of *lplA* and *lipB*, a gene previously identified as a putative lipoate biosynthetic locus. First, analysis of *lplA* null mutants revealed the existence of a second lipoyl ligase enzyme. We found that *lplA* null mutants displayed no growth defects unless combined with *lipA* (lipoate synthesis) or *lipB* mutations and that overexpression of wild-type LplA suppressed *lipB* null mutations. Assays of growth, transport, lipoyl-protein content, and apoprotein modification demonstrated that *lplA* encoded a ligase for the incorporation of exogenously supplied lipoate, whereas *lipB* was required for function of the second lipoyl ligase, which utilizes lipoyl groups generated via endogenous (*lipA*-mediated) biosynthesis. The *lipB*-dependent ligase was further shown to cause the accumulation of aberrantly modified octanoyl-proteins in lipoate-deficient cells. Lipoate uptake assays of strains that overproduced lipoate-accepting apoproteins also demonstrated coupling between transport and the subsequent ligation of lipoate to apoprotein by the LplA enzyme. Although mutations in two genes (*fadD* and *fadL*) involved in fatty acid failed to affect lipoate utilization, disruption of the *smp* gene severely decreased lipoate utilization. DNA sequencing of the previously identified *slr1* selenolipoate resistance mutation (K. E. Reed, T. W. Morris, and J. E. Cronan, Jr., Proc. Natl. Acad. Sci. USA 91:3720–3724, 1994) showed this mutation (now called *lplA1*) to be a G76S substitution in the LplA ligase. When compared with the wild-type allele, the cloned *lplA1* allele conferred a threefold increase in the ability to discriminate against the selenium-containing analog. These results support a two-pathway/two-ligase model of lipoate metabolism in *E. coli*.

The disulfide-containing cofactor (*R*)-(+)-lipoic acid (6,8-thioctic acid or 1,2-dithiolane-3-pentanoic acid) is an extremely widely distributed protein bound cofactor which is essential for activity of a variety of enzyme complexes that catalyze oxidative decarboxylations. *Escherichia coli* cells express three such lipoate-dependent multienzyme complexes, including the well-studied pyruvate and α-ketoglutarate dehydrogenases (9, 26) as well as the recently identified glycine cleavage enzyme (35, 39). The functional form of lipoate is attached by amide linkage to a single specific lysyl residue within the lipoate-accepting domains of the E2p subunit of pyruvate dehydrogenase, the E2o subunit of α-ketoglutarate dehydrogenase, and the H-protein subunit of the glycine cleavage complex. The redox-active disulfide bond of lipoylated proteins functions as a covalently bound carrier of reaction intermediates between successive active sites within these large multisubunit enzyme complexes. Highly specific lipoyl-protein ligases are thus required to attach lipoate to the appropriate lysyl residues of the lipoate dependent enzymes. We have recently described the *lplA* gene of *E. coli*, which encodes lipoate-protein ligase A (LplA), the first lipoyl ligase gene and enzyme to be isolated and studied in detail (20). As reported here, analysis of *lplA* null mutants has focused renewed attention on poorly understood aspects of lipoate metabolism and revealed that lipoate uptake, ligation of lipoyl groups to apoproteins, and lipoate synthesis are interconnected processes.

Although many organisms are capable of synthesizing lipoate, the biosynthetic pathway has not been fully described in any system. In *E. coli*, labeling experiments (40, 41) have implicated octanoic acid, 8-thiooctanoic acid, and 6-thiooctanoic acid as lipoate precursors, while molecular genetic and feeding studies (30, 39) have demonstrated that the *lipA* gene product is required for the as yet undefined insertion of sulfur into octanoate to form lipoate. While the *lipA* gene is required for de novo lipoate synthesis, the activity of the LipA protein has not been demonstrated in vitro. Sequence similarities between the LipA proteins of *E. coli*, *Saccharomyces cerevisiae*, and *Pelobacter carbinolicus* (24, 30, 36) and the BioB biotin synthetases of *E. coli*, *S. cerevisiae*, and *Bacillus sphaericus* (23, 25, 42) suggest that the mechanisms of sulfur insertion into lipoate and biotin are closely related. A second *E. coli* gene of unknown function (*lipB*) was isolated during selection for mutants defective in lipoate synthesis (39). Strains with null mutations in *lipB* continue to synthesize lipoyl groups, however, and instead display a 10-fold reduction in the accumulation of

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TABLE 1. Bacterial strains used

Strain	Genotype	Plasmid(s) present	Reference or source
JK1	*rpsL*		30
KER176	*rpsL lipA150*::Tn*1000*dKn		30
KER184	*rpsL lipB182*::Tn*1000*dKn		30
KER264	*rpsL lplA1*(*slr1*)		31
KER282	*rpsL lplA1*(*slr1*) *thr*::Tn*10*		This study
KER296	*rpsL lipA150*::Tn*1000*dKn *fadE*		30
KER310	*rpsL lipA150*::Tn*1000*dKn *lipB175*Tn*10*dTc		30
KER316	*rpsL lplA1*(*slr1*) *thr*::Tn*10 recA*::*kan*		This study
KER332	*serA25 lipA150*::Tn*1000*dKn *lysA thi-1*		This study
TVB57	*rpsL* Δ(*recA-srl*) Tc^s		38
TVB129	*rpsL lipA150*::Tn*1000*dKn *fadL*::Tn*10*		This study
JC10240	Hfr(PO 45) *recA56 srl*::Tn*10 the ilv rpsE*		CGSC[a]
LS7072	*fadR fadD zea*::Tn*10*		17
JC7623	*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*		15
TM125	*rpsL lipA150*::Tn*1000*dKn *fadD zea*::Tn*10*		This study
TM131	*rpsL lipA150*::Tn*1000*dKn *lplA148*::Tn*10*dTc *fadE*		20
TM132	*rpsL lipA150*::Tn*1000*dKn *lplA329*::Tn*10*dTc *fadE*		20
TM134	*rpsL lplA148*::Tn*10*dTc		20
TM135	*rpsL lplA329*::Tn*10*dTc		20
TM136	*rpsL lipB182*::Tn*1000*dKn *lplA148*::Tn*10*dTc		This study
TM137	*rpsL lipB182*::Tn*1000*dKn *lplA329*::Tn*10*dTc		This study
TM140	*rpsL lipA150*::Tn*1000*dKn	pKR56, pMS421	20
TM141	*rpsL lipA150*::Tn*1000*dKn	pKK223, pMS421	20
TM174	*rpsL lipB175*Tn*10*dTc	pKR56, pMS421	This study
TM175	*rpsL lipB175*Tn*10*dTc	pMR16, pMS421	This study
TM176	*rpsL lipA150*::Tn*1000*dKn	pKR56, pMS421	This study
TM177	*rpsL lipA150*::Tn*1000*dKn	pMR16, pMS421	This study
TM178	*rpsL lipA150*::Tn*1000*dKn *lipB175*Tn*10*dTc	pKR56, pMS421	This study
TM179	*rpsL lipA150*::Tn*1000*dKn *lipB175*Tn*10*dTc	pMR16, pMS421	This study
TM199	*rpsL lipA150*::Tn*1000*dKn *lplA148*::Tn*10*dTc *fadE*	pTM69, pMS421	This study
TM229	*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* Δ*smp*::ΩCm		This study
TM231	*rpsL lipA150*::Tn*1000*dKn Δ*smp*::ΩCm		This study
TM232	*rpsL* Δ*smp*::ΩCm		This study
TM233	*rpsL lipA150*::Tn*1000*dKn Δ*smp*::ΩCm Hfr(PO 45) *recA56 srl*::Tn*10*		This study
TM245	*rpsL*	pGS331	This study
TM246	*rpsL lipA150*::Tn*1000*dKn	pGS331	This study
TM247	*rpsL lipB182*::Tn*1000*dKn	pGS331	This study
TM248	*rpsL lplA148*::Tn*10*dTc	pGS331	This study
TM249	*rpsL lipA150*::Tn*1000*dKn *lplA148*::Tn*10*dTc *fadE*	pGS331	This study
TM250	*rpsL lipB182*::Tn*1000*dKn *lplA148*::Tn*10*dTc	pGS331	This study
TM251	*rpsL lipA150*::Tn*1000*dKn *lipB175*Tn*10*dTc	pGS331	This study
TM254	*rpsL lipB182*::Tn*1000*dKn	pTM61-4	This study
TM255	*rpsL lipA150*::Tn*1000*dKn *lplA148*::Tn*10*dTc *fadE*	pKR112	This study
TM274	*rpsL lipA150*::Tn*1000*dKn *lplA148*::Tn*10*dTc *fadE*	pKR123, pMS421	This study
TM300	*serA25 lipA150*::Tn*1000*dKn *lysA thi-1 lplA148*::Tn*10*dTc		This study
148	*rpsL lipA150*::Tn*1000*dKn *lplA148*::Tn*10*dTc		20
329	*rpsL lipA150*::Tn*1000*dKn *lplA329*::Tn*10*dTc		20

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

protein-bound lipoyl groups (30). Thus, *lipB* appeared to be involved in the ligation of lipoyl groups to apoproteins, but the partial nature of the *lipB* defect suggested that some other gene(s) must encode a lipoyl ligase enzyme. Indeed, the *lplA* gene encoding such a ligase has been cloned and used to overexpress and purify the LplA lipoyl ligase enzyme (20). Characterization of the nutritional and biochemical defects of strains with *lplA* null mutations has subsequently revealed an unexpected redundancy between the functions of the *lplA* and *lipB* genes. This conclusion was confirmed by measurements of lipoate transport, lipoate utilization, and lipoyl-protein synthesis in various mutant strains. We propose a two-pathway-plus-two-ligase model of lipoate metabolism to explain these results.

MATERIALS AND METHODS

Bacterial strains and media. Most strains used in this work (Table 1) were derived from strain JK1, a prototrophic strain of *E. coli* K-12 from the laboratory of J. Konisky. Strain constructions utilizing bacteriophage P1*vir* transductional crosses were carried out by conventional methods (18). References for previously described strains are given in Table 1. Novel strains were constructed as follows. Strain KER176 was transduced to tetracycline resistance by a P1 lysate of LS7072, and transductants were screened for growth on oleate and acetate as carbon sources. One candidate which grew on acetate but failed to grow on oleate was saved as strain TM125 (*fadD zea*::Tn*10*). The tetracycline resistance markers of strains 148 and 329 were transduced into strain KER184 to give strains TM136 and TM137, respectively. Strain KER184 was also transformed with the appropriate plasmids (as indicated in Table 1) to give strains TM174, TM175, TM247, and TM254. Strain KER176 was transformed with the appropriate plasmids to give strains TM176, TM177, and TM246. Strain KER310 was transformed with the appropriate plasmids to give strains TM178, TM179, and

TM251. Strain TM131 was transformed with the appropriate plasmids to give strains TM199, TM249, TM255, and TM274. Strains JK1, TM134, and TM136 were transformed with plasmid pGS331 to give strains TM245, TM248, and TM250, respectively.

To generate a chromosomal disruption of the *smp* gene, plasmid pTM77 was linearized with *Pst*I and used to transform strain JC7623 to chloramphenicol resistance. An ampicillin-sensitive and chloramphenicol-resistant transformant was saved as strain TM229 (Δ*smp*::ΩCm). A P1 lysate of strain TM229 was then used to transfer the Δ*smp*::ΩCm allele into strains KER176 and JK1 to yield strains TM231 and TM232, respectively. Strain TM231 was then mated with JC10240, and exconjugants were selected on rich broth plates supplemented with acetate, succinate, tetracycline, chloramphenicol, and kanamycin. These exconjugants were screened for sensitivity to UV light, and one sensitive isolate was saved as strain TM233 (*recA srl*::Tn*10*). Strain KER332 was transduced to tetracycline resistance by a P1 lysate of strain TM134 to yield TM300. A P1 lysate of strain LS5283 was used to transduce strain KER176 to tetracycline resistance, thus yielding strain TVB129 (*fadL*::Tn*10*). A P1 lysate of strain CAG18442 (34) was used to donate tetracycline resistance to strain KER264, and the resulting strain (KER282) was then transduced to kanamycin resistance by a lysate from strain GP150 to form the *recA* derivative KER316.

The culture medium was rich broth (5) or minimal salts medium E (4) supplemented with 0.4% glucose and, as needed, DL-α-lipoic acid (0.5 to 50,000 ng/ml; Sigma), 5 mM sodium acetate, 5 mM sodium succinate, 0.1% vitamin-free casein hydrolysate (Difco), lysine (100 μg/ml), glycine (100 μg/ml), serine (100 μg/ml), thiamine (10 μg/ml), streptomycin (30 μg/ml), kanamycin (25 μg/ml), tetracycline (3 to 10 μg/ml), ampicillin (100 μg/ml), spectinomycin (30 μg/ml), and chloramphenicol (30 μg/ml). When used as primary carbon sources, acetate and succinate were present in minimal E medium at 50 mM, while oleate was present at 0.1% (wt/vol). Selenolipoic acid (1,2-diselenolane-3-pentanoic acid) was synthesized as previously described (31) and added to minimal E glucose medium at 10 to 4,000 ng/ml. Solid media contained 1.5% agar. All cultures were grown at 37°C. Anaerobic growth experiments were carried out in GasPak anaerobic jars (Becton Dickinson).

Plasmids and plasmid constructions. Plasmids pTM59, pTM61-1, pTM61-4, pTM69, pKR56, pMS421, and pKK223-3 were described previously (20). Plasmid pKR112 carries the *lipB* gene under control of the *tac* promoter (30). Plasmids pKR123 and pKR124 carry the *lplA1* gene from the chromosome of strain KER264 as a 1.25-kb *Hpa*I fragment inserted into the *Stu*I site of pMTL20 (3), thus placing the *lplA1* gene under control of the *lac* promoter (pKR123) or in the opposite orientation (pKR124). Plasmid pGS331 expresses an 85-residue recombinant lipoate-accepting domain under control of the *tac* promoter (1). Plasmid pMS421 carries the *lacI*^{q} allele (8) and was used to modulate transcription of various coresident plasmids as indicated in Table 1. A 2.6-kb *Bst*YI-*Acc*I fragment of pTM59 (carrying intact *smp* and *lplA* genes) was inserted between the *Bam*HI and *Acc*I sites of pMTL22 (3) to form pTM74. A 371-bp *Hpa*I internal fragment of the *smp* gene was deleted from pTM74 and replaced with a blunt-ended 3.7-kb *Bam*HI fragment from pHP45ΩCm (27) to form pTM77. Although the *smp* gene of pTM77 was thus disrupted as a result of this selectable (chloramphenicol resistance) replacement, the adjacent *lplA* gene remained intact. This insertion into *smp* carried flanking transcription and translation termination signals, and the disrupted allele was designated Δ*smp*::ΩCm (Table 1). A 1.65-kb *Acc*I-*Bss*HII fragment from pTM74 (carrying the intact *smp* gene) was inserted into pMTL21 (3) to form plasmid pTM79.

Recombinant DNA methods. Plasmid DNA was isolated and manipulated by standard procedures (33). DNA sequence was generated by automated sequencing using *Taq* polymerase and fluorescent dye-labeled terminators (28). Sequencing templates were double-stranded plasmids pKR123 and pKR124 and *Eco*RI-*Bss*HII, *Eco*RI-*Nru*I, *Nru*I-*Hin*dIII, and *Bss*HII-*Hin*dIII deletion derivatives of pKR123. Oligonucleotides used for sequencing were the universal primer, reverse primer, primer 4 (5′-CCGGCATTCTCGCATCTG-3′), primer 5 (5′-GGCCTCGGTTATGGCCTC-3′), and primer 6 (5′-AGCATATCTGCGCGGTAC-3′). In experiments designed to detect any chromosomal genes homologous to *lplA*, the 1.25-kb *Hpa*I fragment of plasmid pTM59 (containing the entire *lplA* coding sequence) was ^{32}P labeled with a Multiprime kit (Amersham) and used to probe the Kohara et al. (14) λ miniset. This set of ordered phage λ clones spanning the entire *E. coli* chromosome was immobilized on a nitrocellulose membrane (TaKaRa Biochemical). After hybridizations at low-stringency temperatures (42, 40, and 37°C), positive clones were detected by autoradiography.

Bioassays of lipoyl-protein content. Bioassays of lipoyl-protein were similar to previous assays (30). Briefly, cells were cultured in minimal E glucose medium without lipoate and disrupted in a French press, and cleared lysates equivalent to 1 mg of protein were treated with 10% trichloroacetic acid. The resulting acid-insoluble pellets were extracted twice with ethanol-ether (1:1, [vol/vol]) to remove residual noncovalently bound lipoate. Protein-bound lipoyl groups were then released by acid-catalyzed hydrolysis. Duplicate dilutions of each acid hydrolysate were then assayed for lipoate by culturing the indicator strain KER176 in minimal E succinate medium (containing 0.1% vitamin-free casein hydrolysate) versus lipoate standards. The reported values are averaged from measurements of at least two independent cultures for each strain assayed.

Lipoate-protein ligase assay. Lipoate-protein ligase activity was assayed in vitro as described previously (20), using a heat-treated extract of TM178 as a source of apoprotein for the incorporation of [^{35}S]lipoic acid.

Immunoblotting experiments. Strains to be analyzed by immunoblotting were initially cultured in minimal E glucose medium supplemented with vitamin-free casein hydrolysate, acetate plus succinate, and 0.5 ng of lipoate per ml. Each culture was diluted 1:75 into two flasks of fresh medium containing acetate plus succinate and either no lipoate or 5,000 ng of lipoate per ml and then grown overnight to saturation. Washed cells were disrupted by sonication in 20 mM Tris-HCl (pH 7.5) and centrifuged at 10,000 × *g* to remove solid material. Volumes of each extract equivalent to 50 or 63 μg of total protein were separated on sodium dodecyl sulfate (SDS)–10% acrylamide gels and electroblotted to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). After washing and blocking, membranes were incubated with a 1:250 dilution of reconstituted human serum from patients with primary biliary cirrhosis (PBC) (7). Cross-reactive bands corresponding to the E2 subunits of the α-keto acid dehydrogenases were detected by incubation with a 1:500 dilution of goat anti-human immunoglobulin G conjugated to horseradish peroxidase (F_c specific; Sigma) and reaction with the chromogenic peroxidase substrate 4-chloro-1-naphthol (Pierce Chemical Co.).

In vitro octanoylation and lipoylation reactions. E2 apoproteins from a cleared extract of strain TM250 were octanoylated or lipoylated by treatment with purified LplA lipoate-protein ligase (20). Reaction mixtures contained 100 μg of TM250 extract, 20 mM Tris-HCl (pH 7.5), 1.5 mM ATP, 1.5 mM $MgCl_2$, 0.3 mM dithiothreitol, 1.5 mM unlabeled lipoate or octanoate, and 1.3 μg of LplA. After 30 min at 37°C, reactions were stopped by the addition of SDS loading buffer, and 50 μg from each reaction was then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting as described above.

Lipoate and octanoate uptake assays. Strains to be assayed were cultured, washed, resuspended, and assayed for total [^{35}S]lipoate uptake as described previously (20). For assays of strains carrying plasmid pKR56 or pMR16, overproduction of acyl carrier protein (ACP)-E2p and ACP, respectively, was induced by the addition of 1 mM isopropylthiogalactopyranoside (IPTG) for 1.5 h prior to harvesting of cells for assay. As needed, the amount of [^{35}S]lipoate converted to [^{35}S]lipoyl-protein was determined by pipetting samples from selected time points directly into ice-cold 10% trichloroacetic acid. The resulting protein pellets were washed twice with 3 ml of ethanol-ether (1:1 [vol/vol]) to remove free lipoic acid and then suspended in 0.2 ml of 10% SDS. The detergent-dissolved pellets were boiled and counted in 4 ml of Biosafe counting scintillant (Amersham). Assays of octanoate uptake were carried out as described for lipoate, using [1-^{14}C]octanoic acid (55 mCi/mmol; American Radiolabeled Chemicals, St. Louis, Mo.) at a concentration of 750 nM.

Radiolabeling of cultures. Strains were cultured overnight in minimal E glucose medium with acetate plus succinate and diluted 1:100 into 1 ml of fresh medium containing 0.03 to 0.7 μCi of [^{35}S]lipoate (55 to 440 mCi/mmol) or 3 to 5 μCi of [1-^{14}C]octanoate (55 mCi/mmol). After growth to saturation (12 to 18 h), cells were precipitated with 10% trichloroacetic acid and washed with 1% trichloroacetic or 100% acetone, and the resulting samples were boiled in denaturing SDS buffer and separated on SDS–10% acrylamide gels. Specifically labeled [^{35}S]lipoyl-proteins and [^{14}C]octanoyl-proteins were visualized by fluoroautoradiography. [^{35}S]lipoate was synthesized and purified as described previously (31).

RESULTS

***lplA* and *lipB* define separate protein lipoylation pathways.** During the characterization of *lplA* null mutants, we found compelling evidence for a second protein lipoylation pathway which did not require the *lplA* gene product. First, we noted that previous in vivo labeling experiments with [^{35}S]lipoic acid and [^{14}C]octanoic acid showed a low but reproducible accumulation of specifically radiolabeled lipoylated and octanoylated E2 subunits by *lplA* null mutants (20). Also, when independently derived *lplA* null alleles (which were isolated in a *lipA* strain [20]) were transduced into wild-type strains, the resulting mutants demonstrated no growth defects on minimal E glucose medium (Table 2), suggesting that these strains possessed functional (therefore lipoylated) α-keto acid dehydrogenases. This was confirmed directly via bioassay of the lipoyl-protein content of strain TM134 (*lplA*) and its wild-type parent strain JK1. In four independent experiments, the average protein-bound lipoate content of JK1 was 20.8 ng/mg of soluble protein, whereas that of TM134 was 25.6 ng/mg of soluble protein. Thus, it was clear that TM134 contained an alternative lipoyl ligase activity which functioned well even in the absence of the *lplA* gene product. In contrast to wild-type strains, however, *lplA* null mutants TM134 and TM135 were totally resistant (Table 2) to the growth-inhibiting effects of selenolipoic acid, a selenium-containing analog of lipoic acid

TABLE 2. Lipoate-related growth phenotypes of *lplA*, *lipA*, and *lipB* mutants[a]

Null mutation(s) (strain)	Multi-copy gene	Phenotype		
		No supplement added to minimal E glucose media	Lipoate (5–50,000 ng/ml)	Selenolipoate (10–40,000 ng/ml)
None (JK1)		++	++	−
lplA (TM134-135)		++	++	++
lipA (KER176)		−	++	−
lipB (KER184)		+/−	++	−
lplA lipA (TM131-132)		−	−	−
lplA lipB (TM136-137)		−	−	−
lipB (TM254)	*lplA*$^+$	++	++	−
lplA lipA (TM255)	*lipB*$^+$	−	−	−

[a] Strains were cultured in minimal E glucose medium supplemented with acetate plus succinate, and then dilute inocula were transferred to fresh media containing supplements as indicated. Growth phenotypes were scored as ++ for the rapid growth (~70-min doubling time) and high culture densities (optical density at 600 nm of ~3.3) observed for strains with functional lipoylated enzymes, +/− for the very slow but detectable growth of *lipB* null mutants on unsupplemented solid media, and − for no detectable growth. Strain TM254 expressed the wild-type *lplA* gene under control of the *tac* promoter from plasmid pTM61-4, and strain TM255 expressed the wild-type *lipB* gene under control of the *tac* promoter from plasmid pKR112.

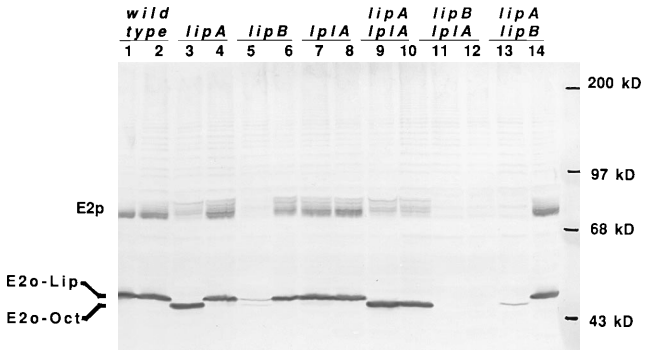

FIG. 1. Immunoblotting of E2p and E2o proteins with anti-PBC antiserum. Extracts were from strains TM245 (lanes 1 and 2), TM246 (lanes 3 and 4), TM247 (lanes 5 and 6), TM248 (lanes 7 and 8), TM249 (lanes 9 and 10), TM250 (lanes 11 and 12), and TM251 (lanes 13 and 14). Lanes 1, 3, 5, 7, 9, 12, and 13 were loaded were extracts from cells grown without lipoate, whereas lanes 2, 4, 6, 8, 10, 12, and 14 contained extracts from the same strains grown in the presence of a saturating level (5,000 ng/ml) of lipoate. Cross-reacting proteins were detected by incubation with secondary antibody (goat anti-human immunoglobulin G coupled to horseradish peroxidase) as described in Materials and Methods. The octanoylated and lipoylated forms of the α-ketoglutarate dehydrogenase E2o protein are indicated as E2o-Oct and E2o-Lip, respectively, whereas both forms of the pyruvate dehydrogenase protein are indicated simply as E2p.

(31). Moreover, we determined that *lplA* null mutations caused growth defects only when transduced into strains which were already mutant in either the *lipA* or *lipB* gene. That is, wild-type *lplA* function was required for the utilization of exogenous lipoic acid only by strains which were also defective in the endogenous synthesis of lipoylated enzymes (Table 2).

We further observed that the leaky growth phenotype of the *lipB* null strain KER184 in the absence of lipoate supplementation was converted to a very tight growth phenotype in the derivative *lipB lplA* double mutants, TM136 and TM137 (Table 2). Subsequent bioassays of strains KER184 (*lipB*) and TM136 (*lipB lplA*) demonstrated that the low lipoyl-protein content of the *lipB* null mutant (3.2 ng/mg of protein) was further depressed to undetectable levels (less than 0.1 ng/mg of protein) in the *lipB lplA* double mutant. Thus, both the growth phenotypes and lipoyl-protein assays of these strains suggested a possible overlap in the functions of the *lplA* and *lipB* genes. This hypothesis was addressed by testing if expression of each of these wild-type genes from multicopy plasmids could suppress the growth defects conferred by null mutations in the other gene. We expressed the *lplA* gene from the inducible *tac* promoter of pTM61-4 in a *lipB* mutant host (KER184) and found that the resulting strain (TM254) no longer displayed any growth defects on minimal glucose medium. However, expression of the *lipB* gene from the *tac* promoter of plasmid pKR112 in a *lplA lipA* host failed to suppress the lipoate utilization phenotype in the resulting strain, TM255, even in the presence of extremely high concentrations (50,000 ng/ml) of lipoate (Table 2).

We then tested if multicopy expression of *lipB* from pKR112 would allow detection of the *lplA*-independent lipoylation activity, using in vitro assays. Accordingly, plasmid pKR112 was moved into the *lplA* null strain TM134, which had previously been shown to express no detectable lipoylation activity (19, 20). However, even upon IPTG induction of *lipB* expression, we were unable to detect any incorporation of [^{35}S]lipoate by freshly prepared and concentrated extracts of the plasmid-bearing strain. Since these data suggested that some other gene(s) may have been required for enhanced expression of the *lipB*-dependent lipoylation activity, the Kohara et al. (14) λ miniset of the *E. coli* chromosome was probed to detect any λ clones with sequences homologous to the *lplA* gene. However, hybridizations with a 1.25-kb ^{32}P-labeled *Hpa*I fragment of pTM59 (carrying the entire *lplA* coding sequence) conducted at extremely low stringencies (42 to 37°C) detected only the Kohara phages (λ674 and λ675) known to carry the *lplA* gene (20).

Detection of the *lipB* dependent octanoylation-lipoylation pathway. Several groups have shown that *E. coli* strains which are rendered lipoate deficient (either by mutations in *lipA* or by overexpression of lipoate-accepting apoproteins) accumulate significant levels of aberrantly modified octanoyl-proteins in place of properly modified lipoyl-proteins (2, 6, 11). Since our results had revealed the existence of two genetically distinct lipoylation pathways, we sought to determine which of these pathways was responsible for this unusual modification in lipoate-deficient strains. Accordingly, we used immunoblotting with anti-lipoyl domain antibody to assess the modification state of lipoate-accepting protein domains from strains bearing various combinations of mutations in lipoate-related genes.

This assay was based on two previous observations. First, sera from human patients with the autoimmune disease PBC contain anti-PBC antibodies that cross-react strongly with lipoylated and octanoylated E2 proteins of *E. coli* but fail to react with unmodified E2 apoproteins (7). Second, the E2o subunit of the α-ketoglutarate dehydrogenase reproducibly migrates faster upon SDS-PAGE when octanoylated than when lipoylated (7, 20, 30). Thus, the banding pattern produced by immunoblotting SDS-polyacrylamide gels of *E. coli* extracts allowed us to assess the presence of lipoylated E2o protein (as the slower-migrating species), octanoylated E2o protein (as the faster-migrating species), and unmodified E2o apoprotein (as the lack of cross-reactive E2o species). We extended this analysis by determining the modification state of E2o from extracts of cells grown either in the presence or in the absence of saturating levels (5,000 ng/ml) of lipoate (Fig. 1). (It should be noted that no exogenous octanoate was present in the growth media, and thus the octanoyl groups attached to E2 proteins in these extracts were derived from endogenous synthesis.)

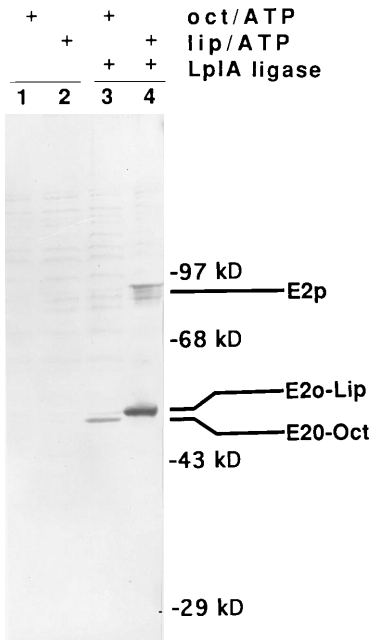

FIG. 2. In vitro lipoylation and octanoylation of E2 apoproteins. An E2 apoprotein-containing extract of strain TM250 was incubated with octanoate (oct) plus ATP (lanes 1 and 3) or lipoate (lip) plus ATP (lanes 2 and 4) and purified LplA enzyme (lanes 3 and 4) as described in Materials and Methods. Following electrophoresis, the cross-reacting lipoylated and octanoylated E2 proteins were detected by immunoblotting with anti-PBC serum as shown in Fig. 1.

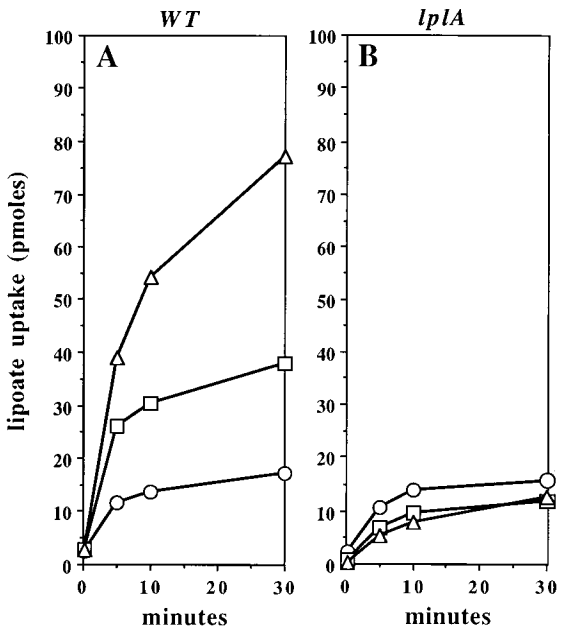

FIG. 3. Lipoic acid uptake in wild-type and *lplA* strains. Lipoate was present at 750 nM. Total [^{35}S]lipoate uptake is plotted as picomoles per 0.2 ml of washed cells (optical density at 600 nm of 3.0). (A) Total uptake by strains JK1 (wild type; circles), KER176 (*lipA*; squares), and KER184 (*lipB*; triangles). (B) Total uptake by strains TM134 (*lplA*; circles), TM131 (*lplA lipA*; squares), and TM136 (*lplA lipB*; triangles).

As expected, strains TM245 (wild type) and TM248 (*lplA*) accumulated exclusively lipoylated E2o regardless of the presence or absence of lipoate in the medium (Fig. 1, lanes 1, 2, 7, and 8), whereas strain TM246 (*lipA*) accumulated octanoylated E2o unless supplemented with lipoate, which gave fully lipoylated E2o (lanes 3 and 4). Strain TM247 (*lipB*) accumulated only slight amounts of octanoylated and lipoylated E2o in the absence of lipoate but accumulated wild-type levels of lipoylated E2o when grown with excess lipoate (lanes 5 and 6). The low but detectable accumulation of lipoylated E2o by the *lipB* mutant in the absence of exogenous lipoate was consistent with previous measurements of lipoyl-protein content in *lipB* strains (30). We further observed that TM249 (*lipA lplA*) accumulated no detectable lipoylated E2o and substantial octanoylated E2o regardless of lipoate supplementation (lanes 9 and 10). In contrast, strain TM250 (*lipB lplA*) accumulated only unmodified apoproteins, as indicated by the lack of cross-reactive E2 proteins in cells grown with or without lipoate (lanes 11 and 12). When grown in the absence of lipoate, strain TM251 (*lipA lipB*) accumulated a low level of octanoylated E2o, whereas only lipoylated E2o was detected in cells grown with lipoate (lanes 13 and 14).

To confirm that E2 apoproteins failed to cross-react with the anti-PBC serum, purified LplA enzyme was used to attach octanoate and lipoate to the apoproteins present in an extract of strain TM250 (*lipB lplA*). After the in vitro modification reactions, the resulting proteins were again analyzed by immunoblotting with anti-PBC serum. These results demonstrated that nonreactive E2 apoproteins present in the TM250 extract (Fig. 2, lanes 1 and 2) could be converted to the cross-reacting octanoylated and lipoylated forms, respectively (Fig. 2, lanes 3 and 4). This experiment also verified the altered migration of octanoylated versus lipoylated E2o subunits upon SDS-PAGE.

An alternative method was used to corroborate the results of Fig. 1. Ali and Guest (1) showed that octanoylation or lipoylation of a heat-stable 85-residue recombinant lipoate-accepting protein (expressed from plasmid pGS331) causes altered migration of the recombinant protein in nondenaturing polyacrylamide gels. Since each of the extracts analyzed in Fig. 1 also contained pGS331, heat-treated samples of these extracts were also analyzed by 15% native PAGE. Coomassie blue and silver stains of these native gels (data not shown) demonstrated that the proportion of modified to unmodified recombinant 85-residue protein in these extracts was similar to the proportions shown in Fig. 1 for the nonrecombinant E2o subunit.

Thus, the octanoylation of lipoate-accepting proteins by endogenously synthesized octanoyl groups was primarily mediated by the *lipB*-dependent pathway, although the LplA enzyme generated a low level of octanoylated protein when both lipoate and a functional *lipB* gene were absent (Fig. 1, lane 5). Furthermore, the lack of cross-reactive E2 proteins in the *lipB lplA* strain TM250 (Fig. 1, lanes 11 and 12), as well as the absence of any octanoylated or lipoylated recombinant 85-residue protein in this strain (not shown), indicated that neither octanoylation nor lipoylation occurred when both the *lipB*-dependent and *lplA*-dependent pathways were inactivated by null mutations.

Coupling of lipoate uptake to the *lplA* lipoylation path. To better define the pathway for utilization of exogenous lipoic acid, we examined the kinetics of [^{35}S]lipoate uptake by various mutant strains. Previous direct comparisons of lipoate uptake by strains JK1 (wild type) and its congenic derivative TM134 (*lplA*) demonstrated that uptake by whole cells did not require the *lplA* gene product (20). However, when strains bearing different combinations of wild-type, *lipA* null, *lipB* null, and *lplA* null alleles were tested, it became clear that the *lplA* gene product did play a role in enhancing the level of lipoate uptake, but only in lipoate-deficient cells. As shown in Fig. 3, the

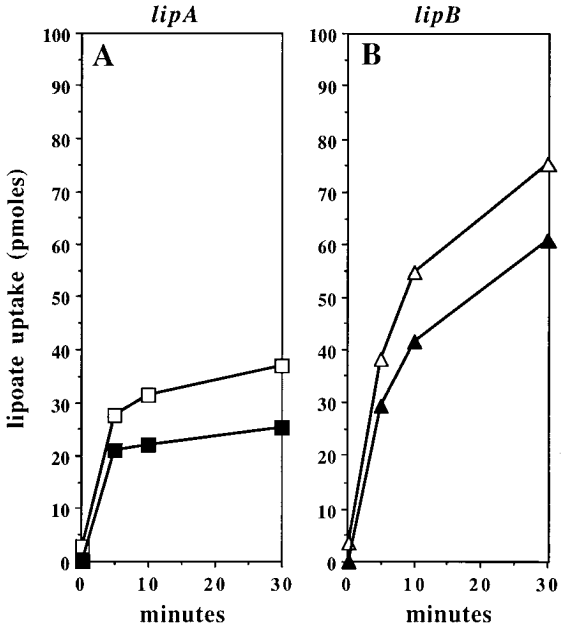

FIG. 4. Total versus protein-bound [^{35}S]lipoate incorporation by *lipA* and *lipB* null mutants. Uptake is plotted as picomoles per 0.2 ml of washed cells. Lipoate was present at 750 nM. (A) Total (open squares) and protein-bound (closed squares) incorporation by strain KER176 (*lipA*). (B) Total (open triangles) and protein-bound (closed triangles) incorporation by strain KER184 (*lipB*).

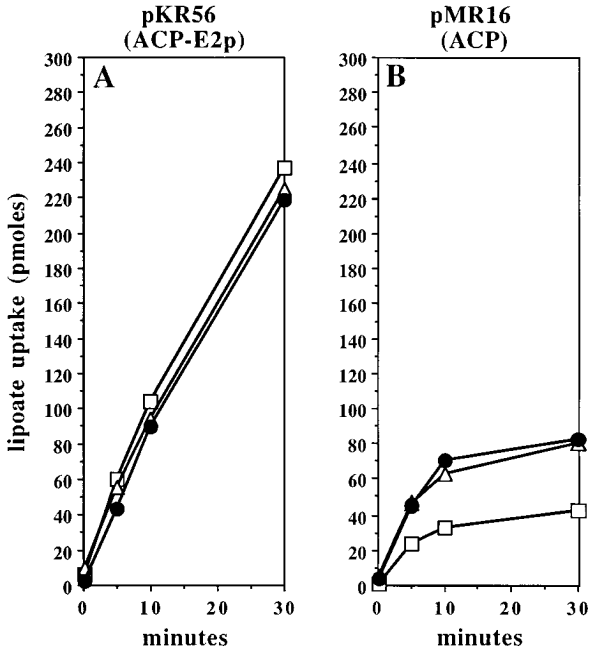

FIG. 5. Effect of overexpressed lipoate-accepting fusion protein upon lipoate uptake. Uptake is plotted as picomoles per 0.2 ml of washed cells. Lipoate was present at 3.75 μM. (A) Total uptake by cells expressing the lipoate-accepting ACP-E2p fusion protein from plasmid pKR56. Samples were from *lipA* strain TM176 (squares), *lipB* strain TM174 (triangles), and *lipA lipB* strain TM178 (circles). (B) Total uptake by strains expressing native (therefore non-lipoate-accepting) ACP from the control plasmid pMR16. Samples were from *lipA* strain TM177 (squares), *lipB* strain TM175 (triangles), and *lipA lipB* strain TM179 (circles).

lipoate-sufficient strains JK1 (wild type) and TM134 (*lplA*) took up very similar and low levels of labeled lipoate. Trichloroacetic acid precipitation of selected samples from JK1 and TM134 then demonstrated that all of the [^{35}S]lipoate was present in the free (non-protein-bound) form (data not shown). In contrast, the lipoate-deficient strains KER176 (*lipA*) and KER184 (*lipB*) incorporated two- and fourfold-higher levels of lipoate, respectively, than the Lip$^+$ strains JK1 and TM134 (Fig. 3A). This enhanced incorporation was not observed when a *lipA* or *lipB* mutation was combined with the *lplA* null allele (Fig. 3B), suggesting that the enhanced uptake was due to the prior accumulation of unmodified lipoate-accepting apoproteins in *lipA* and *lipB* cells.

This hypothesis was tested by comparing [^{35}S]lipoate incorporation into total cell-bound radioactivity versus trichloroacetic acid-precipitable radioactivity. As shown in Fig. 4, virtually all of the additional [^{35}S]lipoate transported by *lipA* and *lipB* null mutants was converted to covalently bound [^{35}S]lipoyl-protein. Because enhanced lipoate incorporation required the wild-type *lplA* gene (Fig. 3), this observation confirmed that the LplA enzyme also participated in coupling the transport of lipoate to its subsequent attachment to apoprotein. An analogous coupling hypothesis has been proposed in biotin metabolism to explain the observation that *birA* biotin ligase mutants often exhibit decreased rates of biotin transport (2a).

This coupling effect was further explored by testing if overexpression of a recombinant lipoate-accepting apoprotein would similarly enhance the uptake of lipoic acid. Accordingly, *lipA*, *lipB*, and *lipA lipB* null strains were transformed with plasmids pKR56 (encoding a lipoate-accepting ACP-E2P fusion protein [20]) or the control plasmid pMR16 (encoding native ACP [29]). As shown in Fig. 5, assays of the resulting strains showed a further large increase in the level of lipoate transport that was dependent upon the presence of the E2p lipoate-accepting domain (note that the vertical axis is expanded threefold relative to Fig. 3 and 4). Indeed, the rate of lipoate uptake by the ACP-E2p-expressing strains declined only slightly over the entire assay period, even at the highest lipoate concentration tested (3.75 μM). The uptake data of Fig. 5B also confirmed that *lipA* and *lipB* null mutants accumulated considerable levels of unmodified E2 apoproteins even without overexpression of recombinant lipoate acceptors. Moreover, the relative amounts of lipoate incorporation shown in Fig. 5B are consistent with the results of Fig. 1 regarding the relative levels of apoproteins in *lipA*, *lipB*, and *lipA lipB* null strains. That is, since *lipB* strains synthesized little or no octanoylated proteins (Fig. 1, lanes 5 and 13), such strains accumulated correspondingly higher levels of unmodified E2 apoproteins. This relative increase in apoprotein resulted in a twofold enhancement of lipoate uptake by *lipB* and *lipB lipA* strains compared with *lipA* strains (Fig. 4 and Fig. 5A).

Incorporation of exogenous octanoic acid. Since the *lplA* gene product also acts in the incorporation of exogenous octanoic acid (20), we examined the ACP-E2p-overexpressing strain TM140 for enhanced uptake of [^{14}C]octanoate. Although the lipoate-accepting fusion did increase octanoate uptake approximately 2-fold relative to the control strain TM141, the rate of octanoate incorporation was 30- to 40-fold slower than the rate of lipoate uptake for both strains. Furthermore, a 333-fold molar excess of unlabeled octanoate resulted in an 8% decrease in the rate of [^{35}S]lipoate uptake by strain TM140, suggesting that octanoate acted as only a weak inhibitor of lipoate incorporation.

Lipoate utilization by *fadD* and *fadL* mutants. Because lipoic acid is structurally similar to fatty acids, we determined if two genes involved in fatty acid uptake also participate in

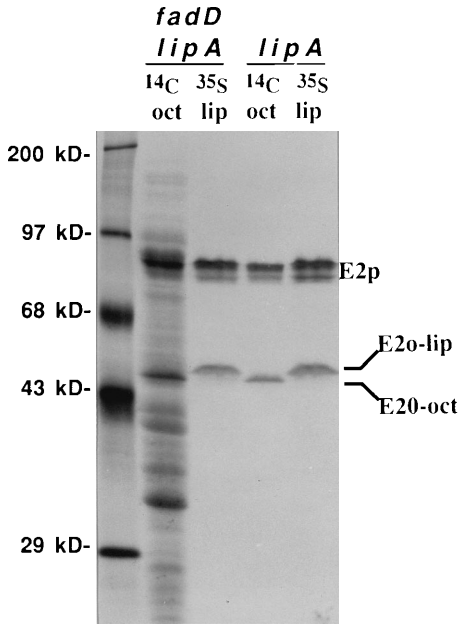

FIG. 6. In vivo radiolabeling of *lipA* and *lipA fadD* mutants with [^{35}S]lipoate and [^{14}C]octanoate. Strains KER176 (*lipA*) and TM125 (*lipA fadD*) were cultured in the presence of radiolabeled octanoate (oct) or lipoate (lip), and samples were analyzed by SDS-PAGE. Specifically radiolabeled proteins are the E2p subunit from pyruvate dehydrogenase as well as the octanoylated (E2o-oct) and lipoylated (E2o-lip) forms of the E2o α-ketoglutarate dehydrogenase subunit.

lipoate uptake. Thus, *fadD* and *fadL* mutant alleles were transduced into the lipoate-requiring strain KER176 (*lipA*), and the resulting double mutants TM125 (*lipA fadD*) and TVB129 (*lipA fadL*) were compared with the parent strain. When tested on solid minimal E glucose medium, all three strains exhibited identical responses to filter disks saturated with lipoate and/or the growth-inhibiting analog selenolipoic acid. In liquid minimal E succinate medium supplemented with 0.1% vitamin-free casein hydrolysate, all three strains achieved 50% maximal growth at 0.35 ng of lipoic acid per ml, demonstrating that neither *fadD* (fatty acyl coenzyme A synthetase) or *fadL* (outer membrane channel) mutations altered lipoate utilization. Radiolabeling of KER176 and TM125 cultures with [^{14}C]octanoate and [^{35}S]lipoate verified that the *fadD* gene was not required for the efficient incorporation of exogenous lipoate or octanoate onto E2 apoproteins (Fig. 6).

Lipoate utilization by Δ*smp*::ΩCm null mutants. Previous results (20) had revealed that the *lplA* gene is located immediately downstream of the *smp* gene, which encodes a membrane protein of unknown function (21, 22). Since this arrangement suggested that the *smp* and *lplA* genes may be cotranscribed, a null allele of the *smp* gene was constructed (see Materials and Methods) and tested for its effects on lipoate utilization in various genetic backgrounds. Although no growth defects were observed for the single mutant TM232 (*smp*), the double mutant TM231 (*smp lipA*) was clearly defective in the utilization of lipoate for growth on solid minimal E glucose and minimal E succinate media. While the *lipA* parent strain KER176 required less than 0.5 ng of lipoate per ml to achieve wild-type levels of growth, strains TM231 (*smp lipA*) and TM233 (*smp lipA recA*) failed to form observable colonies after 16 h on minimal E glucose plates with 0.5 to 50 ng of lipoate per ml. Even at very high lipoate levels (50,000 ng/ml), *smp lipA* double mutants formed markedly smaller colonies than did *lipA* single mutants. However, *smp lipA* strains routinely generated large revertant colonies when streaked for isolation in the presence of lipoate. Because these suppressors appeared at such a high frequency, we were unable to use liquid medium growth experiments to measure the extent of the lipoate utilization defect conferred by the *smp* null allele. By streaking for isolation on solid media, however, various plasmid subclones of the *smp* and *lplA* genes were tested for complementation of the defect in strain TM233 (*smp lipA recA*). These qualitative experiments revealed that plasmid pTM79 (carrying only the wild-type *smp* gene) failed to restore lipoate utilization, whereas plasmids pTM61-1 and pTM61-4 (carrying only the downstream *lplA* gene) restored growth to wild-type levels when lipoate was provided in the medium. These results suggested that *smp* was not required for lipoate utilization and that disruption of the *smp* gene may have exerted a polar effect upon expression of the adjacent *lplA* gene. Consistent with this interpretation, *lplA lipA* double mutants (having an intact *smp* gene adjacent to the disrupted *lplA* gene) displayed a stable lipoate utilization defect when grown on either solid or liquid media.

Selenolipoic acid resistance in *lplA* mutants. Recently, we reported the isolation of a spontaneous point mutation (designated *slr1*) which conferred resistance to growth inhibition caused by the selenium-containing analog selenolipoic acid (31). Because this mutation mapped to 99.6 min and displayed lipoate and selenolipoate incorporation defects which were qualitatively similar to those of *lplA* null mutants, we examined the possibility that *slr1* was allelic to *lplA*. Initially, we observed that plasmid pTM69 (carrying the wild-type *lplA* gene as a 1.25-kb *Hpa*I fragment) restored selenolipoate sensitivity to KER316 (*slr1 recA*). Subsequently, the corresponding 1.25-kb *Hpa*I fragment from the chromosome of KER264 (*slr1*) was cloned as plasmid pKR123. By complementation analysis of strains TVB57 (*recA*) and KER316 (*recA slr1*) transformed with pKR123 or pTM69, we found that both host strains were approximately 100-fold less sensitive to selenolipoate when they carried pKR123 than when they carried pTM69. Since this result indicated that the *slr1* mutation was carried by pKR123, the entire *lplA* gene from this plasmid was sequenced on both strands. We located a single GC-to-AT transition in the *lplA* structural gene (at nucleotide 302 of the sequence reported in reference 20), which resulted in the conversion of glycine 76 to serine in the deduced LplA sequence. Thus, we have redesignated the *slr1* mutation as *lplA1*. The finding that the *lplA1* point mutation conferred selenolipoic acid resistance was also consistent with our previous observations that *lplA* null mutants incorporated no detectable [^{75}Se]selenolipoic acid (20) and that transduction of independently derived *lplA* null mutations into strain JK1 was sufficient to confer complete resistance to selenolipoate by the resulting strains TM134 and TM135 (Table 2).

As shown in Fig. 7, the glycine residue that is altered by the *lplA1* mutation is conserved in alignments of known (or suspected) lipoate-protein and biotin-protein ligases. This conservation suggests that glycine 76 may serve important structural and/or functional roles in these enzymes. Since earlier work showed that the *lplA1* allele caused a fourfold drop in lipoate accumulation in vivo (31), we sought to determine if this mutation also altered the substrate specificity of the lipoate ligase enzyme. Accordingly, plasmids pTM69 (wild-type *lplA*) and pKR123 (*lplA1*) were moved into the host strain TM131, which carried null mutations in *lipA* and *lplA*. The resulting plasmid-bearing strains were thus forced to utilize only exogenous lipoate (as a result of the *lipA* null mutation) and expressed only the *lplA* alleles carried by recombinant plasmids (as a result of the *lplA* null mutation present on the chromosome). A series of

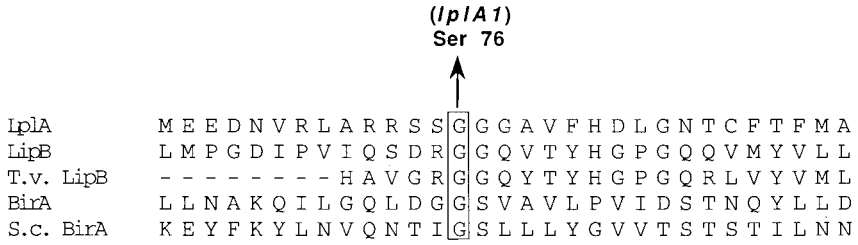

FIG. 7. Sequence alignments of known and candidate cofactor-apoprotein ligases. Sequences are aligned around the conserved glycine residue which is altered by the *lplA1* (*slr1*) missense mutation. LplA is the *E. coli* lipoate-protein ligase A (20); LipB is a candidate *E. coli* lipoyl ligase (30); T.v. LipB is a partial sequence of a putative LipB homolog from *Thiobacillus versutus* (37); BirA is the *E. coli* biotin ligase (12); and S.c. BirA is the biotin ligase of *S. cerevisiae* (3a).

growth experiments then determined the amount of selenolipoate which resulted in 50% growth inhibition of each strain at five different concentrations of lipoate. These results were then plotted as shown in Fig. 8. By measuring the slope of each plot, we found that strain TM199 (expressing the wild-type *lplA* gene) achieved 50% growth in the presence of a 5:1 molar ratio of selenolipoate to lipoate. In contrast strain TM274 (expressing the *lplA1* allele) achieved 50% growth in the presence of a 15:1 molar ratio of selenolipoate to lipoate. Thus, the *lplA1* strain was threefold better than the wild-type strain at discriminating against selenolipoate in favor of lipoate.

Lipoylation of the glycine cleavage enzyme by LplA. Although we had previously shown that the LplA ligase lipoylates both the pyruvate and α-ketoglutarate dehydrogenase complexes (20), radiolabeling experiments yielded inconclusive results regarding the ability of LplA to lipoylate the glycine cleavage enzyme complex. Therefore, an alternative approach was used to answer this question. Because the glycine cleavage system provides the pathway for the synthesis of serine from glycine, mutants blocked in serine biosynthesis (e.g., *serA* mutants) require the glycine cleavage enzyme in order to use glycine as a serine replacement. Consequently, *serA lipA* double mutants require both exogenous lipoate and glycine if serine is not provided (39). Thus, the growth of strain KER332 (*serA lipA*) was compared with that of strain TM300 (*serA lipA lplA*) by streaking cells on minimal E glucose medium supplemented with thiamine, methionine, lysine, acetate, succinate, and either serine (100 μg/ml) or glycine (100 μg/ml), with or without 5,000 ng of lipoic acid per ml. While both strains grew well on serine, only strain KER322 was able to use glycine plus lipoate as a serine replacement, indicating that the *lplA* null mutation in strain TM300 prevented lipoylation of the glycine cleavage enzyme complex. We also performed this experiment under anaerobic growth conditions (except that acetate and succinate were not added to the media since anaerobic metabolism provides these metabolites even in the absence of lipoate) and observed the same results. Thus, lipoylation of the glycine cleavage enzyme with exogenous lipoate required the wild-type *lplA* gene under both aerobic and anaerobic growth conditions.

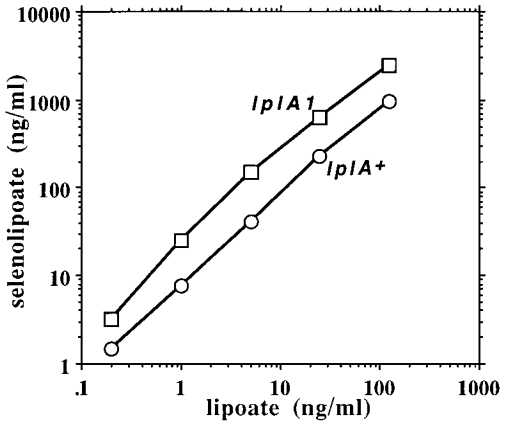

FIG. 8. In vivo substrate specificity of the wild-type and *lplA1*-encoded lipoate-protein ligases. Strains TM199 (expressing the *lplA1* allele; squares) and TM274 (expressing the wild-type *lplA* allele; circles) were cultured in minimal E succinate medium supplemented with 1 ng of lipoate per ml and 0.1% vitamin-free casein hydrolysate. After overnight growth, cells were washed and resuspended at an optical density at 600 nm of 0.05 in fresh medium containing either 0.2, 1, 5, 25, or 125 ng of lipoate per ml as well as serial twofold dilutions of selenolipoate (from 0.2 to 3,280 ng/ml). Cultures were then shaken at 37°C for 40 h, and final culture densities were measured. The ratio of selenolipoate to lipoate which resulted in 50% of maximal growth at each concentration of lipoate was then plotted. Each point was averaged from duplicate samples of at least two independent experiments.

DISCUSSION

Our data reveal several unexpected features of lipoate metabolism in *E. coli*. Most significantly, it is apparent that this bacterium expresses two genetically distinct ligase enzymes for the attachment of lipoyl groups to the lipoate-dependent proteins. While the function of LplA in the activation and ligation of exogenously provided lipoate and lipoate analogs is clear, the mechanism by which the *lplA*-independent ligase operates remains unknown. Nevertheless, we have shown that this second, as yet undefined enzyme is dependent instead on the *lipB* gene, which was initially identified during studies designed to isolate the genes involved in lipoate biogenesis (39). The role of *lipB* in lipoate synthesis per se was not clear, however, and null mutations in *lipB* were unexpectedly leaky in growth and the synthesis of functional (therefore lipoylated) α-keto acid dehydrogenases (30). The partial defect of *lipB* null mutants can now be understood as a result of the overlapping roles of *lipB* and *lplA*. The attenuated but still detectable accumulation of protein-bound lipoate by *lipB* null mutants is entirely due to the action of the *lplA* gene product. Upon introduction of an *lplA* null mutation into a *lipB* strain, the resulting double mutants were totally defective for the accumulation of lipoylated dehydrogenases, as determined by direct bioassay of lipoyl-protein content, the total lack of detectable lipoylated E2o species upon immunoblotting, and the very tight growth phenotypes of such strains. Moreover, the leaky growth phenotype of *lipB* single mutants was suppressed by simply expressing the *lplA* gene from multicopy plasmids, thus clearly demonstrating the redundant roles of these two genes. In contrast, however, analogous multicopy expression of the *lipB* gene failed to suppress the lipoylation defect of *lplA* null strains, as assessed in vivo (by growth studies) or in vitro (by lipoate-protein ligase

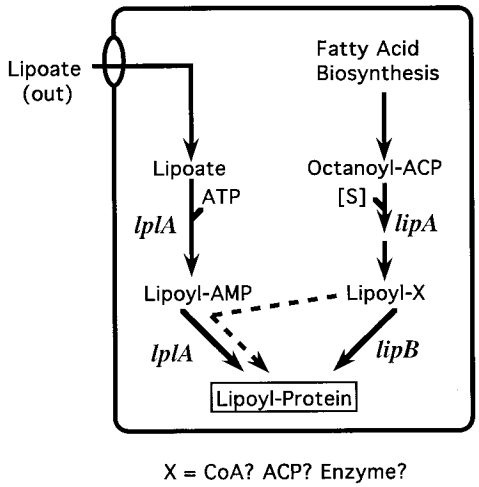

FIG. 9. Proposed two-pathway model of protein lipoylation in *E. coli*. CoA, coenzyme A.

activity assays). The observation that apoprotein accumulation causes markedly enhanced lipoate uptake provided an alternative method for demonstrating the overlapping functions of *lplA* and *lipB*. Thus, uptake assays of *lipB* null mutants confirmed that such strains accumulate very high levels of apoprotein when starved for exogenous lipoate and that subsequent lipoylation of apoprotein in *lipB* strains requires the wild-type *lplA* gene.

We propose that *E. coli* has evolved two different pathways to utilize the two different forms of lipoate that available to the cell (Fig. 9). When presented with free lipoic acid in the medium, wild-type prototrophic strains preferentially incorporate extracellular lipoate and apparently downregulate lipoate synthesis (10, 30). This scavenging pathway requires the LplA enzyme and utilizes ATP to generate the activated lipoyl donor in the form of lipoyl-AMP. When lipoate is not present in the medium, an alternative pathway generates lipoyl groups by de novo synthesis. We suggest that the biosynthetic pathway generates a lipoyl group which is largely unavailable to the LplA protein, presumably by being bound to an alternative (i.e., nonadenylate) carrier. Such an alternative carrier could be provided by the biosynthetic enzyme(s), the lipoate-accepting proteins themselves, coenzyme A, or even ACP, which must serve as the original carrier of the octanoyl carbon skeleton of lipoate during biosynthesis (40, 41). Indeed, Reed et al. (32) reported that the *Streptococcus faecalis* lipoylation enzyme required a protein cofactor with properties (unusually high temperature stability, acid resistance, and solubility in high concentrations of ammonium sulfate) that are typical of ACPs (16). It is this type of as yet unidentified species (lipoyl-X in Fig. 9) which we propose interacts specifically with the *lipB*-dependent lipoylation enzyme. This model thus explains why *lplA* null mutants are phenotypically Lip^+ unless also combined with mutations in the *lipA lipB* biosynthetic pathway. The dashed arrow in Fig. 9 indicates the largely one-way redundancy that we observed between *lipB* and *lplA* function. That is, LplA utilizes the lipoyl-X donor with low but detectable efficiency when expressed from a single-copy gene on the chromosome. Moreover, overexpression of LplA from multicopy plasmids increases this rare scavenging of lipoyl-X to levels which permit growth on lipoate-free media. In contrast, our results indicate that the *lipB*-dependent lipoylation system operates very poorly with exogenous lipoic acid as a lipoyl donor, even when lipoate is provided to *lipB*-overexpressing strains at very high concentrations.

We have also shown that the aberrant octanoylation of E2 apoproteins with endogenously derived octanoyl groups is primarily mediated by the *lipB*-dependent enzyme. Since free fatty acids such as octanoate are not present in *E. coli* cytoplasm (13), this observation is also consistent with the existence of the postulated lipoyl-X intermediate. When lipoyl-X is unavailable, the *lipB*-dependent enzyme apparently utilizes octanoyl-X (e.g., octanoyl-ACP) as an alternative substrate. This model further explains the inability (to date) to detect any non-*lplA*-encoded activity in cell extracts. If correct, then the model predicts that addition of the true in vivo lipoyl donor (e.g., lipoyl-enzyme, lipoyl coenzyme A, or lipoyl-ACP) should allow detection and study of the *lipB*-dependent enzyme in vitro. Until the *lipB*-dependent activity can be demonstrated in vitro, it will be difficult to determine the precise function of the *lipB* gene product in the endogenous synthesis and transfer of lipoyl groups to the lipoate-requiring apoenzymes.

The previously characterized *slr1* allele (31) corresponds to a single missense mutation within the *lplA* gene (now called *lplA1*) that alters a conserved glycine residue. This mutation results in a fourfold decrease in lipoyl-protein incorporation (31) and a threefold increase in the ability to preferentially incorporate lipoate instead of the growth-inhibiting analog selenolipoate. This mutation therefore seems to alter the portion of the enzyme active site that recognizes the thiophane ring of lipoic acid. Since selenium atoms are slightly larger than sulfur atoms, it seems possible that the *lplA1* mutation decreases the volume of the portion of the active site that contacts the thiophane ring. Since *lplA* null mutants are completely resistant to selenolipoate, it is now clear that this analog must be converted to the protein-bound form in order to cause growth inhibition, presumably by generating nonfunctional selenolipoylated dehydrogenase complexes (31). Although the present study provides a preliminary understanding of lipoyl-protein synthesis, further work will be needed to extend our knowledge of the genes and gene products which participate in the two pathways of lipoate metabolism in *E. coli.*

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REFERENCES

1. **Ali, S. T., and J. R. Guest.** 1990. Isolation and characterization of lipoylated and unlipoylated domains of the E2p subunit of the pyruvate dehydrogenase complex of *Escherichia coli*. Biochem. J. **271:**139–145.
2. **Ali, S. T., A. G. J. Moir, P. R. Ashton, P. C. Engel, and J. R. Guest.** 1990. Octanoylation of the lipoyl domains of the pyruvate dehydrogenase complex in a lipoyl-deficient strain of *Escherichia coli*. Mol. Microbiol. **4:**943–950.

2a. **Barker, D. F., and A. M. Campbell.** 1980. Use of *bio-lac* fusion strains to study regulation of biotin biosynthesis in *Escherichia coli*. J. Bacteriol. **143:** 789–800.

3. **Chambers, S. P., S. E. Prior, D. A. Barstow, and N. P. Minton.** 1988. The pMTL *nic*$^-$ cloning vectors. I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. Gene **68:**139–149.

3a. **Cronan, J., and J. Wallace.** Unpublished data.

4. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics: a manual for genetic engineering. Advanced Bacterial Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Davis, T. N., E. D. Muller, and J. E. Cronan, Jr.** 1982. The virion of the lipid-containing bacteriophage PR4. Virology **120:**287–306.
6. **Fujiwara, K., K. Okamura-Ikeda, and Y. Motokawa.** 1992. Expression of mature bovine H-protein of the glycine cleavage system in *Escherichia coli*

and in vitro lipoylation of the apoform. J. Biol. Chem. **267:**20011–20016.
7. **Fussey, S. P. M., S. T. Ali, J. R. Guest, O. F. W. James, M. F. Bassendine, and S. J. Yeaman.** 1990. Reactivity of primary biliary cirrhosis sera with *Escherichia coli* dihydrolipoamide acetyltransferase (E2p): characterization of the main immunogenic region. Proc. Natl. Acad. Sci. USA **87:**3987–3991.
8. **Grana, D., T. Gardella, and M. M. Susskind.** 1988. The effects of mutations in the *ant* promoter of phage P22 depend on context. Genetics **120:**319–327.
9. **Guest, J. R., and G. C. Russell.** 1992. Complexes and complexities of the citric acid cycle in *Escherichia coli*. Curr. Top. Cell. Regul. **33:**231–247.
10. **Herbert, A. A., and J. R. Guest.** 1975. Lipoic acid content of *Escherichia coli* and other microorganisms. Arch. Microbiol. **106:**259–266.
11. **Hipps, D., and R. N. Perham.** 1992. Expression in *Escherichia coli* of a subgene encoding the lipoyl and peripheral subunit-binding domains of the dihydrolipoamide acetyl transferase component of the pyruvate dehydrogenase complex of *Bacillus stearothermophilus*. Biochem. J. **283:**665–671.
12. **Howard, P. K., J. Shaw, and A. J. Otsuka.** 1985. Nucleotide sequence of the *birA* gene encoding the biotin operon repressor and biotin holoenzyme synthetase functions of *Escherichia coli*. Gene **35:**321–331.
13. **Jiang, P., and J. E. Cronan, Jr.** 1994. Inhibition of fatty acid synthesis in *Escherichia coli* in the absence of phospholipid synthesis and release of inhibition by thioesterase action. J. Bacteriol. **176:**2814–2821.
14. **Kohara, Y., K. Akiyama, and K. Isono.** 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50:**495–508.
15. **Kushner, S. R., H. Nagaishi, A. Templin, and A. J. Clark.** 1971. Genetic recombination in *Escherichia coli*: the role of exonuclease I. Proc. Natl. Acad. Sci. USA **68:**824–827.
16. **Magnuson, K. S., S. Jackowski, C. O. Rock, and J. E. Cronan, Jr.** 1993. Regulation of fatty acid biosynthesis in *Escherichia coli*. Microbiol. Rev. **57:** 522–542.
17. **Maloy, S. R., C. L. Ginsburgh, R. W. Simons, and W. P. Nunn.** 1981. Transport of long and medium chain fatty acids by *Escherichia coli* K-12. J. Biol. Chem. **256:**3735–3742.
18. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Morris, T. W., D. E. Brookfield, J. E. Cronan, Jr., and J. R. Guest.** Unpublished data.
20. **Morris, T. W., K. E. Reed, and J. E. Cronan, Jr.** 1994. Identification of the gene encoding lipoate-protein ligase A of *Escherichia coli*: molecular cloning and characterization of the *lplA* gene and gene product. J. Biol. Chem. **269:** 16091–16100.
21. **Neuwald, A. F., and G. V. Stauffer.** 1985. DNA sequence and characterization of the *Escherichia coli serB* gene. Nucleic Acids Res. **13:**7025–7039.
22. **Neuwald, A. F., and G. V. Stauffer.** 1989. An *Escherichia coli* membrane protein with a unique signal sequence. Gene **82:**219–4321.
23. **Ohsawa, I., D. Speck, T. Kisou, K. Hayakawa, M. Zinsius, R. Gloeckler, Y. Lemoine, and K. Kamogawa.** 1989. Cloning of the biotin synthase gene from *Bacillus sphaericus* and expression in *Escherichia coli* and *Bacilli*. Gene **80:** 39–48.
24. **Oppermann, F. B., and A. Steinbuchel.** 1994. Identification and molecular characterization of the *aco* genes encoding the *Pelobacter carbinolicus* acetoin dehydrogenase enzyme system. J. Bacteriol. **176:**469–485.
25. **Otsuka, A. J., M. R. Buoncristiani, P. K. Howard, J. Flamm, C. Johnson, R. Yamamoto, K. Uchida, C. Cook, J. Ruppert, and J. Matsuzaki.** 1988. The *Escherichia coli* biotin biosynthetic enzyme sequences predicted from the nucleotide sequences of the *bio* operon. J. Biol. Chem. **263:**19577–19585.
26. **Perham, R. N.** 1991. Domains, motifs, and linkers in 2-oxo acid dehydrogenase multienzyme complexes: a paradigm in the design of a multifunctional protein. Biochemistry **30:**8501–8512.
27. **Prentki, P., and H. M. Kirsch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
28. **Prober, J. M., G. L. Trainor, R. J. Dam, F. W. Hobbs, G. W. Robertson, R. J. Zagursky, A. J. Cocuzza, M. A. Jensen, and K. Baumeister.** 1987. A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. Science **238:**336–341.
29. **Rawlings, M., and J. E. Cronan, Jr.** 1988. *Escherichia coli* acyl carrier protein gene. FASEB J. **2:**A1539.
30. **Reed, K. E., and J. E. Cronan, Jr.** 1993. Lipoic acid metabolism in *Escherichia coli*: sequencing and functional characterization of the *lipA* and *lipB* genes. J. Bacteriol. **175:**1325–1336.
31. **Reed, K. E., T. W. Morris, and J. E. Cronan, Jr.** 1994. Mutants of *Escherichia coli* K-12 that are resistant to a selenium analog of lipoic acid identify unknown genes in lipoate metabolism. Proc. Natl. Acad. Sci. USA **91:**3720–3724.
32. **Reed, L. J., F. R. Leach, and M. Koike.** 1958. Studies on a lipoic acid activating system. J. Biol. Chem. **232:**123–142.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Singer, M., T. A. Baker, G. Schnitzler, S. M. Deischel, M. Goel, W. Dove, K. T. Jaacks, A. D. Grossman, J. W. Erickson, and C. A. Gross.** 1989. A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. Microbiol. Rev. **53:** 1–24.
35. **Steiert, P. S., L. T. Stauffer, and G. V. Stauffer.** 1990. The *lpd* gene product functions as the L protein in the *Escherichia coli* glycine cleavage enzyme system. J. Bacteriol. **172:**6142–6144.
36. **Sulo, P., and N. Martin.** 1993. Isolation and characterization of *LIP5*: a lipoate biosynthetic locus of *Saccharomyces cerevisiae*. J. Biol. Chem. **268:**176 34–17639.
37. **Ubbink, M., J. Van Beeumen, and G. W. Canters.** 1992. Cytochrome c_{550} from *Thiobacillus versutus*: cloning, expression in *Escherichia coli*, and purification of the heterologous holoprotein. J. Bacteriol. **174:**3707–3714.
38. **Vanden Boom, T. J., and J. E. Cronan, Jr.** 1990. Nonsense mutants defining seven new genes of the lipid-containing bacteriophage PR4. Virology **177:** 12–22.
39. **Vanden Boom, T. J., K. E. Reed, and J. E. Cronan, Jr.** 1991. 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. J. Bacteriol. **173:**6412–6420.
40. **White, R. H.** 1980. Stable isotope studies on the biosynthesis of lipoic acid in *Escherichia coli*. Biochemistry **19:**15–19.
41. **White, R. H.** 1980. Biosynthesis of lipoic acid: extent of incorporation of deuterated hydroxy- and thiooctanoic acids into lipoic acid. J. Am. Chem. Soc. **102:**6605–6607.
42. **Zhang, S., I. Sanyal, G. H. Bulboaca, A. Rich, and D. H. Flint.** 1994. The gene for biotin synthase from *Saccharomyces cerevisiae*: cloning, sequencing, and complementation of *Escherichia coli* strains lacking biotin synthase. Arch. Biochem. Biophys. **309:**29–35.