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Scavenging of Cytosolic Octanoic Acid by Mutant LplA Lipoate Ligases Allows Growth of *Escherichia coli* Strains Lacking the LipB Octanoyltransferase of Lipoic Acid Synthesis▽

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The LipB octanoyltransferase catalyzes the first step of lipoic acid synthesis in *Escherichia coli*, transfer of the octanoyl moiety from octanoyl-acyl carrier protein to the lipoyl domains of the E2 subunits of the 2-oxoacid dehydrogenases of aerobic metabolism. Strains containing null mutations in *lipB* are auxotrophic for either lipoic acid or octanoic acid. We report the isolation of two spontaneously arising mutant strains that allow growth of *lipB* strains on glucose minimal medium; we determined that suppression was caused by single missense mutations within the coding sequence of the gene (*lplA*) that encodes lipoate-protein ligase. The LplA proteins encoded by the mutant genes have reduced K_m values for free octanoic acid and thus are able to scavenge cytosolic octanoic acid for octanoylation of lipoyl domains.

Escherichia coli has three lipoic acid-dependent enzyme systems: pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (OGDH), and the glycine cleavage system (GCV) (8). PDH catalyzes the oxidative decarboxylation of pyruvate to acetyl-coenzyme A (CoA), the tricarboxylic acid (TCA) cycle substrate and fatty acid building block. OGDH functions in the TCA cycle, where it catalyzes the decarboxylation of 2-oxoglutarate to succinyl-CoA, the precursor of several amino acids. GCV is involved in the breakdown of glycine into ammonia and C_1 units. Whereas GCV is expressed only in the presence of glycine, PDH and OGDH are required for aerobic growth. (During anaerobic growth, acetyl-CoA is synthesized by other enzymes and an OGDH-independent branched form of the TCA cycle forms succinyl-CoA from succinate.) The three enzyme systems contain subunits (the E2 subunits of PDH and OGDH and the H protein of GCV) which contain at least one lipoyl domain, a conserved structure of ca. 80 residues (8). Lipoic acid is attached in an amide bond to a specific lysine residue of these domains, where it functions as a classical "swinging arm," carrying reaction intermediates between the active sites of the lipoate-dependent systems (27).

Lipoic acid [*R*-5-(1,2-dithiolan-3-yl)pentanoic acid, also called 6,8-dithiooctanoic acid and thioctic acid] is composed of an eight-carbon fatty acid backbone to which two sulfur atoms are attached at carbons 6 and 8 (Fig. 1). In the oxidized state, the sulfur atoms are in a disulfide linkage forming a five-membered ring with three backbone carbons. The disulfide bond is reduced upon binding of the intermediates (an acetyl moiety in the case of PDH, a succinyl moiety in the case of OGDH, and an aminomethyl moiety in the case of GCV). Following release of the intermediates to form the products of the enzyme complexes, the reduced lipoyl moiety must be reoxidized before entering another catalytic cycle. Oxidation is catalyzed by lipoamide dehydrogenase, a subunit component of the three lipoic acid-dependent enzyme systems (8). *E. coli* strains defective in lipoic acid biosynthesis are unable to grow on aerobic glucose minimal media unless the media are supplemented with acetate and succinate to bypass the need for the two lipoic acid-dependent dehydrogenases (15, 32).

Studies in our laboratory and others have elucidated the lipoic acid synthesis pathway of *E. coli* (Fig. 1). The LipB octanoyl-[acyl carrier protein {ACP}]:protein *N*-octanoyltransferase (20, 33, 34) transfers the octanoyl moiety from octanoyl-ACP, a fatty acid biosynthetic intermediate, to lipoyl domains. This reaction proceeds through an acyl enzyme intermediate in which the octanoyl moiety is in thioester linkage to a conserved cysteine residue in the enzyme active site (22, 33). The thioester bond is then attacked by the ε-amino group of the target lipoyl domain lysine residue to give the amide-linked lipoate moiety. The product of this catalysis, an octanoyl domain, is the substrate of the LipA lipoate synthase, an S-adenosylmethionine radical enzyme which inserts sulfur atoms at carbons 6 and 8. In addition to the LipB-LipA pathway of lipoic acid synthesis, *E. coli* also contains an enzyme that scavenges lipoic acid from the growth medium, the LplA lipoate-protein ligase. LplA uses ATP to activate lipoic acid to lipoyl-adenylate, the mixed anhydride of which is attacked by the lipoyl domain lysine reside to give the lipoylated domain (Fig. 1). LplA is also active with octanoic acid and efficiently attaches exogenous octanoate to lipoyl domains both in vivo and in vitro (11, 25, 26, 34). *lplA* null mutants have no phenotype in strains having an intact lipoic acid synthesis pathway (26).

The subject of this report is the behavior of *lipB* null mutants, which (as expected from the above discussion) are lipoic acid auxotrophs (26, 32). Growth of *lipB* strains can also be supported by supplementation of the medium with octanoate (34). Upon plating of *lipB* null mutants on plates of minimal glucose medium, colonies arise that no longer require lipoic acid (26). These are suppressor mutations because the block in lipoic acid synthesis remains. Suppression in the strains studied

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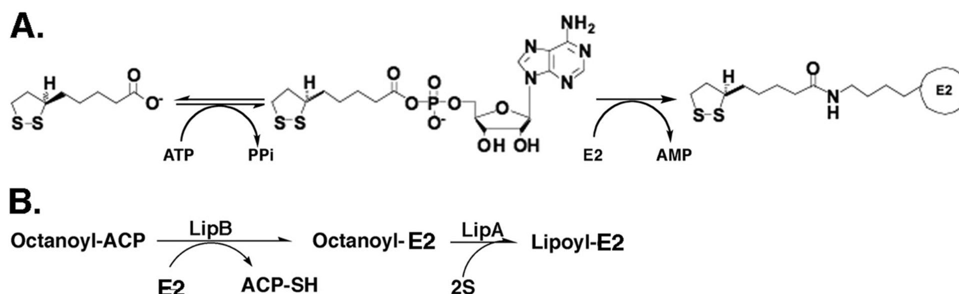

FIG. 1. Lipoic acid metabolism in *E. coli*. (A) LplA lipoate ligase reaction, in which lipoate reacts with ATP to form the activated intermediate, lipoyl-adenylate (lipoyl-AMP), which remains firmly bound within the active site. The lipoyl-adenylate mixed anhydride bond is then attacked by the ε-amino group of the target lysine residue of the acceptor lipoyl domain to form lipoylated protein. LplA also utilizes octanoic acid. (B) Lipoic acid synthesis in *E. coli*. LipB transfers an octanoyl moiety from the fatty acid biosynthetic intermediate, octanoyl-ACP, to the lipoyl domain of a lipoate-accepting protein (in this case the E2 subunit of a 2-oxoacid dehydrogenase). The octanoylated domain is the substrate of LipA, an *S*-adenosylmethionine radical enzyme that replaces one hydrogen atom on each of octanoate carbons 6 and 8 with sulfur atoms. For a review, see reference 8.

in this work maps to the *lplA* gene. The LplA proteins encoded by these suppressor mutants contain point mutations that greatly decrease the Michaelis constant for free octanoic acid and allow efficient scavenging of cytosolic octanoate.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains used in this study (Table 1) are derivatives of *E. coli* strains MG1655 and W3110. Transduction using phage P1*vir* and transformation were conducted following conventional methods (24). Strain FH6 was made by transducing strain MG1655 to kanamycin resistance with a P1 phage stock grown on strain KER184. Strains FH26 and FH27 were isolated by plating strain FH6 on glucose minimal plates, followed by incubation of the plates for 2 to 3 days at 37°C. The tetracycline resistance marker of transposon Tn*10* was introduced upstream of the *lplA* gene in strains FH26 and FH27 by transduction with a P1 phage stock grown on strain CAG18430 (30) to give strains FH66 and FH35, respectively. The *lplA* genes of FH66 and FH35 were transferred into strain ZX221 by P1 transduction, followed by screening for growth on glucose minimal plates in the absence of supplementation. The resulting strains were designated strains FH145 and FH146, respectively. Strains FH46 and FH47 were made by transducing the tetracycline resistance marker from TM135 into strains FH26 and FH27, respectively, with selection on LB plates supplemented with succinate and acetate. Strains FH57 and FH58 were obtained by transducing FH26 and FH27 with a P1 phage stock grown on KER310 with selection on LB plates supplemented with lipoic acid, kanamycin, and tetracycline.

Plasmids pFH1 and pFH2, which express the S221P and V19L LplAs, respectively, under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter, were made by site-directed mutagenesis of plasmid pYFJ16 by the QuikChange procedure (Stratagene).

Media and growth conditions. Luria-Bertani and minimal E media were prepared as described previously (24). Supplements were added at the following concentrations: glucose, 0.4%; glycerol, 0.4%; succinate, 5 mM; acetate, 5 mM; and lipoic acid, 7.5 ng/ml. Antibiotics were used at the following concentrations (in μg/ml): ampicillin, 100; spectinomycin, 100; tetracycline, 15; kanamycin, 50; and chloramphenicol, 20. The growth temperature was 37°C.

DNA extraction, PCR, and sequencing. DNA was extracted using the Promega Wizard genomic DNA purification kit. PCR was conducted using either *Taq* (New England Biolabs) or *Pfu* (Invitrogen) DNA polymerase and appropriately designed primers per the manufacturers' recommendations. DNA sequencing was conducted by the Core Sequencing Facility of the Carver Biotechnology Center of the University of Illinois, Urbana Champaign.

Assays of PDH and OGDH activities. A method similar to that described previously (12) was used. Cultures were grown to late log phase in 100 ml of glucose minimal medium supplemented with succinate and acetate. The cells were then collected by centrifugation, washed twice with 10 ml of 150 mM Tris-HCl buffer (pH 8.5), frozen in a dry ice-ethanol bath, and stored overnight at −20°C. The next day, the cell pellet was thawed on ice. The cells were suspended in 1 ml of 150 mM Tris-HCl buffer (pH 8.5) per mg wet cell weight and lysed by passage twice through a French press cell at 1,000 lb/in^2. The crude extract was collected on ice and cleared by centrifugation, and the protein concentration was determined by the Bio-Rad Bradford assay (3) using a standard curve of bovine serum albumen. Crude extract protein preparations (100 to 300 μg) were added directly to cuvettes containing the assay reaction mixture,

TABLE 1. Bacterial strains, plasmids, and primers used in this work

Strain, plasmid, or primer	Relevant characteristics or sequence	Reference
Strains		
CAG18430	*mdoB202*::Tn*10*	30
DK574	SJ16 carrying plasmids pMS421 and pMR19	7
FH6	*lipB*::Tn*1000* Km	This study
FH26	*lipB*::Tn*1000* Km *lplA10*	This study
FH27	*lipB*::Tn*1000* Km *lplA11*	This study
FH46	FH26 *lplA*::Tn*10*Tet	This study
FH47	FH27 *lplA*::Tn*10*Tet	This study
FH57	*lipA*::Tn*1000* Km *lipB*::Tn*10*Tet *lplA10*	This study
FH58	*lipA*::Tn*1000* Km *lipB*::Tn*10*Tet *lplA11*	This study
FH35	FH27 *mdoB202*::Tn*10*	This study
FH66	FH26 *mdoB202*::Tn*10*	This study
FH145	*lipB*::Cm *mdoB202*::Tn*10 lplA10*	This study
FH146	*lipB*::Cm *mdoB202*::Tn*10 lplA11*	This study
FH213	MG1655 carrying plasmids pMS421 and pJH2	This study
JK1	*rpsL*	26
KER184	*rpsL lipB*::Tn*1000 Km*	26
KER310	*rpsL lipA*::Tn*1000* Km *lipB*::Tn*10*Tet	26
QC146	*lipB lplA*	4a
TM135	*rpsL lplA*::Tn*10*Tet	26
YFJ239	BL21 carrying plasmid pYFJ84	17
ZX221	*rpsL lipB*::Cm	4a
Plasmids		
pFH1	*lplA11* on pQE2	This study
pFH5	*lplA10* on pQE2	This study
pJH2	Lysis plasmid	5
pMR19	Encodes *acpP*	7
pMS421	Expresses LacI^q	7
pGS331	Hybrid lipoyl domain on p*tac*	1
pTJ93	*acpS*	7
pYFJ16	*lplA* on pQE2	18
pYFJ84	*Vibrio harveyi aasS* on pET16b	17
Primers		
lplAfor	TGGCAATCGGTGTAGTGCTG	This study
lplArev	GCGCTTGGTTAACGGCGATC	This study

consisting of 150 mM Tris-HCl buffer (pH 8.5), 3 mM L-cysteine hydrochloride as a reducing agent, 0.1 mM CoA, and 5 mM thiamine pyrophosphate in a 500-μl volume. PDH assay mixtures also contained 5 mM $MgCl_2$. 3-Acetylpyridine adenine dinucleotide (Sigma-Aldrich) was then added to a final concentration of 2 mM, and the mixture was allowed to equilibrate at room temperature for 15 min. The reaction was started by adding either 5 mM pyruvate (PDH activity) or 5 mM 2-oxoglutarate (OGDH activity). Increased absorption at 366 nM, corresponding to reduction of 3-acetylpyridine adenine dinucleotide, was monitored for 5 min in a Beckman DU640 spectrophotometer. The activities of the complexes were calculated in the range of linearity between the initial reaction velocity and the protein concentration. The extinction coefficient of the reduced form of the cofactor used in the calculation was 7.0 nM^{-1} cm^{-1} (4a).

Detection of in vivo lipoylation by Western blotting. Strains carrying plasmid pGS331, which encodes a hybrid PDH lipoyl domain under the control of the *tac* promoter (1), were cultured to late log phase in 15 ml of glucose minimal medium supplemented with succinate, acetate, ampicillin, and IPTG (1 mM), with or without octanoic acid (50 μM). The cells were collected, washed twice with 1 ml of 20 mM Tris-HCl (pH 7.5), and stored at −20°C overnight. The following day, the pellet was thawed on ice and resuspended in 500 μl of the same buffer. The cells were lysed in a Misonix sonicator (two 5-min intervals with 1 min of cooling in between). The extract was cleared by centrifugation, and the protein concentration was determined as described above. A 20% native polyacrylamide gel was loaded with 100 μg of extract protein, and following electrophoresis, the proteins were then transferred to a polyvinylidene difluoride membrane using a standard protocol (2). Lipoylated domain was detected using rabbit antilipoyl protein primary antibodies from Calbiochem and goat antirabbit antibody from Roche.

Protein expression and purification. Hexahistidine-tagged (N-terminal) versions of the wild-type, V19L, and S221P LplAs were purified using Qiagen nickel nitrilotriacetic acid columns as recommended by the manufacturer. Contaminants which copurified with the proteins were removed using a modification of a prior procedure (11). The proteins were adsorbed to Vivapure Maxi H type Q columns equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 10% glycerol, and 0.1 mM phenylmethanesulfonylfluoride. The LplA proteins were eluted with the same buffer containing 120 mM ammonium sulfate. Protein concentrations were measured by absorbance at 280 nm using a molar extinction coefficient of 46,250 M^{-1} cm^{-1} (11).

Apo-lipoyl domain was expressed from pGS331 in strain QC146 and purified by a modification of the method of Ali and Guest (1). After titration of the pH of the crude extract to 4 and centrifugation, the pH of the supernatant was increased to 7 with 1 M NaOH. The extract was dialyzed overnight against 10 mM ammonium acetate buffer (pH 5) and then subjected to anion exchange chromatography on a Poros HQ column eluted with a 10 to 600 mM ammonium acetate gradient at pH 5. Protein concentrations were determined at 280 nm using a molar extinction coefficient of 2,020 M^{-1} cm^{-1} (1).

Holo-ACP was expressed and purified from strain DK574 carrying plasmid pJT93 as described by Cronan and Thomas (7). Histidine-tagged *Vibrio harveyi* acyl-ACP synthase was expressed from strain YFJ239 and purified using Qiagen nickel nitrilotriacetic acid columns as recommended by the manufacturer. Octanoyl-ACP was synthesized as described by Cronan and Thomas (7).

Gel shift assay of LplA activity. A 20-μl reaction mixture contained 10 mM sodium phosphate buffer (pH 7), 0.3 mM dithiothreitol, 30 μM apo-lipoyl domain, and 5 μM enzyme. When checking for ligase activity, 5 mM $MgCl_2$, 5 mM ATP, and various concentrations of lipoic acid or octanoic acid were added. Alternatively, to assay octanoyltransferase activity, octanoyl-ACP was added in place of ATP and octanoate/lipoate. The reactions were run for 2 h at 37°C, after which 10 μl was loaded on a 20% polyacrylamide gel containing 2.5 M urea and separated by electrophoresis.

Filter disk shift assay of LplA activity. The reaction mixture was the same as that used in the gel shift assay except that the assay contained various concentrations of [1-^{14}C]octanoic acid. After equilibration at 37°C, a sample was removed as a no-enzyme control and enzyme was added to a final concentration of 100 nM, which was determined to be within the range of linear activity with respect to the enzyme concentration under these conditions (data not shown). Samples (15 μl) were removed every 3 min for 15 min and spotted onto 2.3-mm-diameter Whatman filter disks which had been presoaked in 5% trichloroacetic acid and dried. The disks were then dried and washed three times (10 min for each wash) with a solvent mixture containing chloroform-methanol-acetic acid (3:6:1). After a final wash with absolute ethanol, the disks were dried, added to vials containing 4 ml of scintillation cocktail, and counted in a Beckman Coulter LS 6500 scintillation counter (18). The data were analyzed using the Prism analysis software program, and K_m and V_{max} values were determined by the Edie-Hofstee method (16).

Fatty acid extraction and butyl ester synthesis. Strain FH213 was grown in 1 liter of glycerol minimal medium containing spectinomycin and ampicillin. When the culture reached stationary phase, IPTG was added to a final concentration of 1 mM and induction was allowed to proceed for 3 h. The cells were then collected, lysed by freezing in a dry ice-ethanol bath, and then thawed at room temperature. Fatty acids were converted to their sodium salts by mixing the cell extract with 0.1 M sodium bicarbonate overnight at 4°C, after which the solution was acidified to pH 3 with glacial acetic acid and 3 ml hexane was added. After 1 h of stirring at 4°C, the fatty acid-containing organic phase was collected and transferred to a 5-ml glass vial with a plastic stopper. Heptanoic acid (1 μg) was added to serve as an internal standard. Butyl ester synthesis was carried out in a manner similar to the method of Hallmann et al. (14). Butanol-BF_3 (0.1 ml) and 1 g of anhydrous sodium sulfate were added, and the vial was sealed with Parafilm, followed by incubation in a 65°C heating block for 2 h. The reaction mixture was then cooled to room temperature. The solution was washed three times with 3 ml distilled water and concentrated under nitrogen gas to 100 μl, of which 5 μl was analyzed by gas chromatography-mass spectrometry (GC-MS). Samples (5 μl) were injected in splitless mode into the GC-MS system, consisting of an Agilent 6890N gas chromatograph, an Agilent 5973 mass selective detector, and an HP 7683B (Agilent Inc., Palo Alto, CA) autosampler. Injections were performed on a 30-m HP-5MS column with 0.25-mm inside diameter and 0.25-μm film thickness (Agilent Inc, Palo Alto, CA) with an injection port temperature of 250°C, the interface set to 250°C, and the ion source adjusted to 230°C. The helium carrier gas was set at a constant flow rate of 1 ml min^{-1}. The temperature program was 1 min of isothermal heating at 40°C, followed by an oven temperature increase of 5°C min^{-1} to 180°C for 1 min. The mass spectrometer was operated in positive electron impact mode at 69.9 eV ionization energy in an m/z 50 to 550 scan range. The spectra of all chromatogram peaks were evaluated using the HP Chemstation (Agilent, Palo Alto, CA) and AMDIS (NIST, Gaithersburg, MD) software programs.

Random mutagenesis of *lplA*. The *lplA* gene was PCR amplified from strain FH6 DNA with primers lplAfor and lplArev (Table 1), which are complementary to regions about 150 nucleotides upstream and downstream of the coding region, respectively. The PCR product was purified using the Qiagene PCR cleanup kit and then subjected to either 12 or 35 cycles of error-prone PCR (2). The mutagenized products were electroporated into strain FH6 expressing the λ Red recombinase system (9). The cells were allowed to recover in glucose minimal medium supplemented with succinate and acetate for 90 min at 37°C. The cells were collected, washed three times with minimal medium, and plated on glucose minimal plates.

RESULTS

Suppression in strains FH26 and FH27 requires *lipA* and is due to point mutations in the *lplA* coding region. Strain FH6 (a *lipB* knockout) is blocked in the first step in lipoyl protein synthesis, and thus, it lacks active PDH and OGDH complexes (26). Consequently, the strain is unable to grow aerobically on glucose minimal medium unless it is supplemented with either lipoic or octanoic acid, which LplA attaches to PDH and OGDH (26). Growth also occurs when the medium is supplemented with acetate and succinate, which bypass the enzymatic steps catalyzed by PDH and OGDH, respectively. Strains FH26 and FH27 were isolated as suppressors of the *lipB* null mutation in strain FH6, which grew on unsupplemented glucose minimal medium. Introduction of a *lipA* null mutation into strains FH26 and FH27 (to give strains FH57 and FH58, respectively) by transduction resulted in a loss of the ability to grow on glucose minimal medium. These results indicated that suppression was caused by a pathway that resulted in octanoylation of PDH and OGDH. Previous work suggested that in vitro LplA has weak octanoyl transferase activity (20) and showed that overproduction of LplA alleviates the growth defect of *lipB* null mutants (26). We therefore asked if the growth of strains FH26 and FH27 was mediated by LplA. Two observations supported this hypothesis. First, transductional introduction of an *lplA* null mutation into strains FH26 and FH27

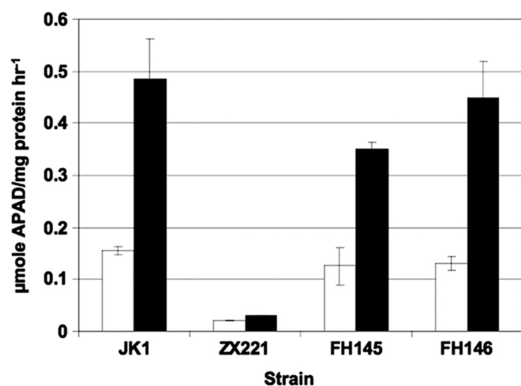

FIG. 2. PDH (solid bars) and OGDH (open bars) activities of various isogenic strains grown on glucose minimal medium supplemented with succinate and acetate. The values reported are the averages of three measurements. Strain JK1, wild type; strain ZX221, *lipB*; strain FH145, *lipB lplA10*; and strain FH146, *lipB lplA11*.

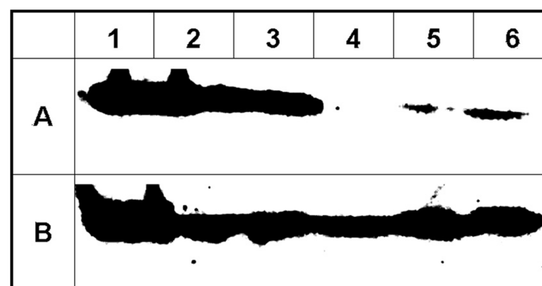

FIG. 3. Western blot analysis of protein lipoylation. All strains were isogenic and produced a hybrid lipoyl domain encoded by plasmid pGS331 (1). Equal amounts of total extract protein were loaded in each lane. In panel A, the cells were from cultures grown without octanoic acid supplementation, whereas in panel B, the cultures were grown with octanoic acid supplementation. Lane 1, lipoyl domain standard; lane 2, strain JK1 (wild type); lane 3, strain TM135 (*lplA*); lane 4, strain ZX221 (*lipB*); lane 5, strain FH145 (*lipB lplA10*); lane 6, strain FH146 (*lipB lplA11*).

(to give strains FH46 and FH47, respectively) resulted in a lack of growth on unsupplemented glucose minimal medium. Second, transductional backcrossing of the *lplA* genes (and linked regions) from strains FH26 and FH27 into a *lipB* knockout strain (to give strains FH145 and FH146, respectively) conferred the ability to grow on glucose minimal medium.

These data suggested two straightforward hypotheses for the observed octanoylation mediated by the mutant *lplA* alleles of strains FH26 and FH27. First, the *lplA*-linked mutations may result in overexpression of LplA, which is known to suppress the *lipB* phenotype (26). The second hypothesis was that the mutations were within the *lplA* coding region and resulted in mutant LplA proteins which catalyze octanylation more efficiently than does the wild-type protein. This could be due to an increase in the putative octanoyltransferase activity of LplA or to a heretofore-unrecognized pathway. To test these hypotheses, the *lplA* genes and upstream regions of the strain FH26 and FH27 chromosomes were PCR amplified, and the amplification products were sequenced. Both strains were found to carry a missense mutation within the *lplA* coding sequence. The *lplA* gene of strain FH26 contained a G58C point mutation, which results in a V19L amino acid substitution (referred to here as *lplA10*), whereas strain FH27 contained a T664C point mutation, which gives rise to an S221P amino acid substitution (referred to as *lplA11*).

The suppressor strains contain active lipoylated PDH and OGDH complexes. The PDH and OGDH complexes contain essential lipoate-dependent subunits, and thus, their activities indicate in vivo lipoylation. We assayed the strains carrying either the *lplA10* (strain FH145) or the *lplA11* (strain FH146) allele for PDH and OGDH activities in cells from cultures grown on glucose minimal medium supplemented with succinate and acetate. Both mutant strains had levels of PDH and OGDH activities similar to those of strain JK1, the wild-type parental strain, whereas the activities of the isogenic *lipB* null mutant strain ZX221 were near background levels (Fig. 2). These data indicated that the LplA proteins encoded by strains FH145 and FH146 were able to activate the PDH and OGDH complexes.

In a second approach, we expressed a PDH lipoyl domain in various strains grown on glucose minimal medium supplemented with succinate and acetate and examined whether or not the domain became lipoylated. Since all of the strains contained a functional LipA lipoyl synthase, any octanoyl domain formed would be converted to a lipoyl domain, which could be detected by Western blotting with antilipoate antibody (the antibody does not recognize octanoyl domains; Q. Christensen and J. E. Cronan, unpublished data). When the medium was supplemented with octanoic acid (Fig. 3B), the wild-type strain, a *lipB* null strain, and the two strains with mutant *lplA* genes (FH145 and FH146; lanes 5 and 6) accumulated the lipoylated domain because these strains contained active LplA, which catalyzed attachment of exogenous octanoic acid to the unmodified domain, where it was converted to lipoate by LipA. Likewise, an *lplA* strain also accumulated the lipoylated domain due to LipB function. In contrast, strains FH145 and FH146 grown in the absence of octanoic acid supplementation contained detectable levels of domain modified with lipoate, whereas the *lipB* strain did not (Fig. 3A). These data indicated that the suppressor strains were able to attach octanoate to the lipoyl domain in the absence of exogenous octanoic acid. (The modified domains of the wild-type and *lplA* strains resulted from LipB activity.)

Mutant LplA proteins efficiently scavenge octanoic acid. As stated earlier, previous work has suggested that LplA utilizes octanoyl-ACP (20). To test the utilization of octanoyl-ACP, we purified histidine-tagged versions of wild-type, V19L, and S221P LplA proteins and tested their octanoyltransferase activity using the gel shift assay. In this assay, an unmodified hybrid lipoyl domain is provided as a substrate along with octanoyl-ACP (33). Attachment of octanoic acid to the appropriate lysine residue on the lipoyl domain results in loss of a positive charge, which causes the domain to migrate more rapidly than the unmodified protein in native gel electrophoresis. In our initial experiments, we observed activity with V19L and S221P LplAs but not with wild-type LplA, using as a substrate octanoyl-ACP preparations purified by precipitation and washing of the precipitate. However, although an octanoylated domain was synthesized, we did not see the expected concomitant release of holo-ACP. We subsequently found that

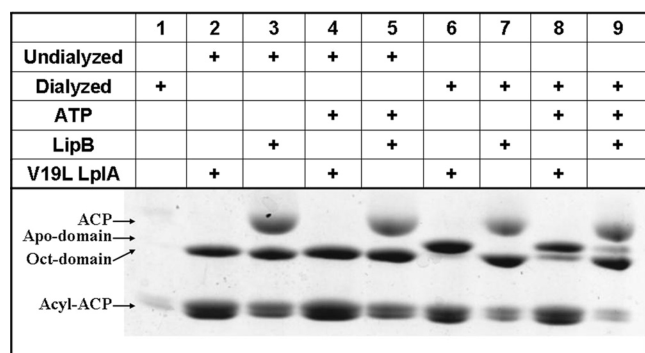

FIG. 4. Gel shift assay with V19L LplA and octanoyl-ACP as a substrate. Two preparations of octanoyl-ACP (undialyzed and dialyzed) were tested. Lanes 3, 5, 7, and 9 were controls for the integrity of the octanoyl-ACP thioester bond in both preparations because LipB cannot utilize free octanoic acid. In these four lanes, the modified domain migrates more rapidly than the unmodified domain. In the LipB reactions, a holo-ACP band is seen at the top of the gel. Lanes 2 and 4 show V19L LplA-catalyzed modification of the domain in the presence of an undialyzed octanoyl-ACP preparation. However, there was no accumulation of holo-ACP, indicating that octanoyl-ACP was not the source of octanoate. In lane 6, an extensively dialyzed octanoyl-ACP preparation was the substrate, and no modification of the domain was seen. In lane 8, ATP was added, which resulted in some modification of the domain, which may be due to hydrolysis of octanoyl-ACP to give free octanoate plus Mg^{2+} introduced with the octanoyl-ACP (ACP is known to avidly bind Mg^{2+}, which was present at a high concentration during octanoyl-ACP synthesis). The S221P LplA protein gave essentially identical results.

use of preparations of octanoyl-ACP that were extensively dialyzed following acyl-ACP synthetase-catalyzed synthesis of octanoyl-ACP to remove excess octanoic acid, ATP, and $MgCl_2$ gave no activity with the LplA proteins whereas the preparations were active with LipB (Fig. 4). These results indicated that the mutant LplAs did not utilize octanoyl-ACP but rather used the traces of free octanoic acid, ATP, and $MgCl_2$ that remained in the octanoyl-ACP preparations purified by precipitation and washing. The fact that we observed a reaction with the mutant LplA proteins but not with wild-type LplA was the first indication that the mutant LplA proteins may have reduced K_m values for one of the substrates, probably octanoate. This was confirmed by assaying [1-^{14}C]octanoic acid attachment to lipoyl domains by the filter disk assay (Materials and Methods) (Table 2). The two mutant LplA proteins had K_m values for octanoate that were 50-fold (the S221P mutant) or 20-fold (the V19L mutant) lower than that of the wild-type enzyme. The mutations also decreased the V_{max} values of the two enzymes, with the S221P protein showing a decrease of 10-fold.

***E. coli* contains free octanoic acid.** The multiple lines of evidence obtained both in vivo and in vitro indicated that the mutant LplA proteins must suppress *lipB* null mutants by utilizing cytosolic octanoic acid. This argued that there must be an intracellular pool of this fatty acid, although there were no data in the literature to support this premise. Intracellular pools of short-chain free fatty acids, such as octanoate, had not been found in *E. coli*, but this could readily be explained by the slight solubility of octanoic acid in water (ca. 0.7 g/liter) plus the volatility of the acid and especially of its methyl ester, the most commonly analyzed derivative. To test for the presence of octanoate, we first trapped the short-chain acids as their nonvolatile sodium salts and then converted the salts to butyl esters, which are much less volatile than the methyl esters.

Free fatty acids were extracted from wild-type cells grown in glycerol minimal medium carrying the lysis plasmid pJH2, used in order to allow gentle disruption of the cellular membrane (5). After lysis, the fatty acids were converted to their sodium salts and subsequently acidified to break down the salts and allow extraction of the acids into hexane. Heptanoic acid (1 μg) was added as an internal standard, and the fatty acids were esterified with butanol in the presence of acid (BF_3). The butyl esters were detected by GC-MS (Fig. 5). A total of 712 ng of octanoic acid was extracted from 1 liter of cells (about 1.18×10^{12} cells). Interestingly, decanoic acid was also detected (Fig. 5). Since quantitative extraction and derivatization could not be assumed, we determined the efficiency of our extraction by spiking an extract with [1-^{14}C]octanoic acid. One liter of cells was grown, collected, and lysed as described above. [1-^{14}C]Octanoic acid was added to the extract, and the usual extraction procedure was followed as described in Materials and Methods. After the final step (concentration under N_2), the extracted [1-^{14}C]octanoic acid radioactivity was determined by scintillation counting. Only 17% of the initial radioactivity was recovered. In a separate experiment, we determined the efficiency of the butyl ester formation and found it to be quantitative.

Other mutant LplA proteins that suppress a *lipB* null allele. The *lplA* gene was randomly mutagenized by error-prone PCR. The PCR products were transformed into strain FH6 expressing the λ Red recombinase system enzymes (9). After recovery, the cells were plated on glucose minimal medium without supplementation. A total of 13 colonies were isolated, and the *lplA* gene from each of these colonies was PCR amplified, cloned into the pCR2.1 TOPO cloning vector, and sequenced. Four isolates had the same point mutation as V19L LplA. Another isolate had the same point mutation as the S221P mutation, and two isolates had no mutations within the coding sequence. The remaining six isolates had *lplA* genes that contained multiple point mutations (Table 3).

DISCUSSION

In this work, we isolated and studied two spontaneously arising *lipB* suppressor strains and determined that suppres-

TABLE 2. Kinetic parameters for wild-type, V19L, and S221P LplA proteins

Mutation	K_m (μM)	V_{max} (nmol/min)	K_{cat} (mol/min/mol)	K_{cat}/K_m
None	214.3 ± 37.8	$2.4 \times 10^{-2} \pm 0.34 \times 10^{-2}$	2.4 ± 0.34	0.011
V19L	10.4 ± 3.0	$1.6 \times 10^{-2} \pm 0.18 \times 10^{-2}$	1.6 ± 0.18	0.058
S221P	4.0 ± 1.6	$0.25 \times 10^{-2} \pm 0.79 \times 10^{-4}$	$0.25 \pm 0.79 \times 10^{-2}$	0.063

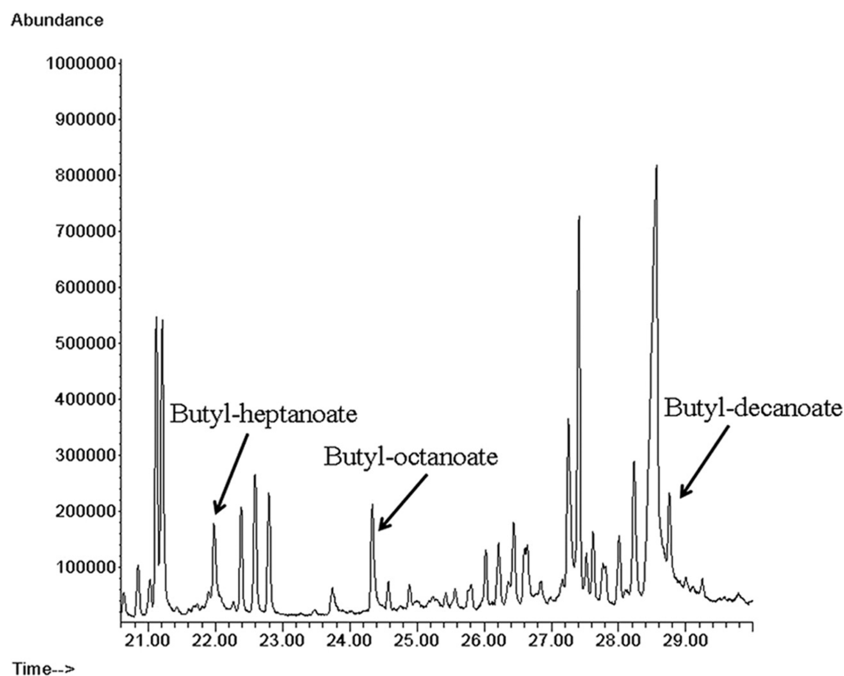

FIG. 5. Gas chromatogram of the butyl esters of free fatty acid from cell extracts. The internal-standard butyl-heptanoate peak represents 50 ng. The mass spectra of the butyl esters of heptanoate, octanoate, and decanoate were identical to those of authentic standards and the database entries.

sion was caused by single point mutations in the LplA protein which reduce the K_m for free octanoic acid. The V19L mutation resulted in a 20-fold decrease in the K_m for octanoic acid, whereas the S221P mutation resulted in a 50-fold decrease. Since the suppressor strains grew and formed lipoylated proteins in the absence of octanoic acid supplementation, an in vivo-synthesized pool of free octanoic acid must be present in *E. coli*. Indeed, we were able to extract 712 ng of free octanoic acid from the cells of a 1-liter culture. Taking the cell volume as 0.88×10^{-15}L (www.ecocyc.org) and correcting for the low efficiency with which we extracted octanoic acid from cell extracts (17%), this indicates an intracellular octanoate concentration of about 28.2 μM. This intracellular concentration is well above the K_m values of the V19L and S221P LplA enzymes but 7.6-fold lower than the K_m value of wild-type enzyme. Hence, *lipB* strains encoding mutant LplAs (either the V19L or the S221P proteins) are able to synthesize lipoylated proteins (Fig. 3) and thus active PDG and OGDH complexes (Fig. 2). It is important to note, however, that despite the benefit gained in these mutant LplAs in terms of reducing the K_m for octanoate, this was not without consequence; both enzymes had decreased V_{max} values. This could explain why these point mutations do not naturally occur in wild-type strains. Interestingly, these two residues are far removed from the active site predicted from the *E. coli* LplA-lipoic acid cocrystal structure of Fujiwara et al. (10). However, we believe the *E. coli* LplA-lipoic acid complex is an experimental artifact, because the lipoic acid molecules were heterogeneously bound and were poorly resolved (10). In one case, the lipoic acid carboxyl was hydrogen bonded to Ser-72, whereas in another case, Arg-140 was the hydrogen bond donor (10). Since enzymes rarely show such plasticity and lipoic acid is a hydrophobic molecule, it seemed possible that the observed association of the cofactor with a hydrophobic LplA surface within the interdomain cavity was artifactual. Moreover, in prior work, Reed and coworkers had isolated LplA mutants resistant to inhibition by an analogue of lipoic acid in which the sulfur atoms had been replaced with selenium (28). Since this is a very discrete modification of the LplA substrate, the mutant protein would be expected to have an alteration close to the pocket that binds the lipoic acid thiolane ring. However, the site of this mutation (Gly-76 to serine, [26]) was distal from the reported *E. coli* lipoate-binding sites. This dilemma was resolved by two lipoic acid-containing structures of an LplA homologue from the archaeon *Thermoplasma acidophilum* (21, 23) that can be readily superimposed on the *E. coli* LplA structure except that the *T. acidophilum* protein lacks the *E. coli* LplA C-terminal domain. In both *T. acidophilum* structures, the lipoate thiolane ring was adjacent to the glycine residue that corresponds to *E. coli* Gly-76, the residue giving resistance to the selenium analogue, and a plausible reorganization of the molecule to prevent binding of the slightly larger analogue was proposed (23). Moreover, addition of lipoic acid

TABLE 3. Results of random mutagenesis of *lplA* and selection for growth of a *lipB* null mutant

Isolate	LplA mutations[a]
12R3	E116A, E312K, L328F, US, DS
12R4	S8T, N63K, F78Y, A110T
12R7	R58L, H79N
35R3/35R5	F35L, V113I, 4US
35R4	F15S, T101A, S114I, 3US

[a] US, mutations upstream of the coding sequence; DS, mutations downstream of the coding sequence.

to a *T. acidophilum* LplA-ATP complex gave lipoyl-AMP, which demonstrated that the lipoic acid was bound in a physiologically meaningful manner (21). Residue 19, the LplA residue altered in suppressor strain FH145, is close to the lipoic acid binding pocket in the *Thermoplasma acidophilum* LplA/lipoyl-AMP cocrystal structure, although the side chain faces away from the lipoyl moiety. Given the importance of the two suppressor mutations we studied (as demonstrated by their reisolation by random mutagenesis), it is noteworthy that *E. coli* LplA residues 19 and 221 are not conserved in the LplA proteins of other organisms and neither are the residue substitutions found in the V19L and S221P mutants. The V19L mutation replaces a hydrophobic residue with one having a longer side chain. This could increase the affinity of the protein for octanoic acid by creating a more hydrophobic environment. The S221P mutation might open up the lipoic acid/octanoic acid binding pocket and thereby increase substrate access. Except for the F78Y and H79N substitutions, the residue changes of the other mutant LplA proteins isolated by random mutagenesis are not readily interpretable. The altered residues either lie far away from the active site or seem to be rather modest substitutions. However, a caveat is that although the V19L change is very modest in character (one branched-chain residue for another), the mutation has very marked effects on enzyme activity. Judging from the *T. acidophilum* crystal structures, residue F78 is located in the ATP binding pocket whereas H79 is located in the lipoic acid binding pocket. Both residues make direct interactions with the substrate. However, residue 78 is a threonine in the *T. acidophilum* protein.

The Regulon Data Base (http://regulondb.ccg.unam.mx/) predicts that *lplA* is cotranscribed with the upstream gene, *ytjB*. This prediction is consistent with the data of Morris et al. (26), who showed that insertion of a strong transcription terminator into *ytjB* results in a deficiency in lipoic acid utilization in a *lipA* background that is alleviated by *trans* expression of LplA but not of YtjB (then called Smp) (26). This is relevant to two puzzling *lplA* alleles that were isolated in our random mutagenesis of *lplA* with selection for *lipB* suppression (Table 3). Isolates 35R3 and 35R5 have point mutations in the *ytjB* termination codon. In both strains, the termination codon was changed from TGA to the more stringent TAA stop codon. Read-through of TGA codons has often been reported, and the nucleotide immediately downstream of the termination codon is known to have a major influence on the efficiency of termination (31). It seems noteworthy that TGAC, the sequence found at the end of *ytjB*, is the weakest of the four base translational stop signals (31). The *ytjB* termination codon is 27 nucleotides upstream of the *lplA* initiation codon, and thus, any read-through would add the *ytjB* coding sequence plus the nine codons of the intergenic region to the N terminus of LplA with possible deleterious effects on LplA activity. Indeed, if, as annotated, YtjB is a membrane protein, the fusion protein could also mislocalize LplA activity. Another possibility is that ribosomes transversing the intergenic region might interfere with *lplA* translational initiation, resulting in decreased LplA levels. The more-stringent TAA codon would more efficiently block translation of the intergenic region (31) and perhaps raise LplA expression to a level sufficient to allow effective utilization of the cytosolic octanoic acid pool. It could be argued that decreased read-through would be a minor effect. However, it should be noted that the physiological requirement for lipoic acid is very low (only hundreds of molecules/cell), and thus, slight overproduction of wild-type LplA (e.g., by vector copy number) is sufficient to allow growth of *lipB* null strains on glucose minimal medium. Moreover, not all of the available lipoyl domains need be lipoylated for optimal PDH and OGDH complex activities (13, 19).

It has long been thought that there are no cytosolic free fatty acids in *E. coli* because fatty acid biosynthesis is tightly coupled to phospholipid biosynthesis. Free fatty acids were detected only when phospholipid synthesis was blocked (6) and in strains overexpressing a cytosol-entrapped TesA thioesterase (4). In both cases, detection required that the strains were defective in ß-oxidation. Even under such conditions, Cho and Cronan (4) detected only traces of octanoic acid. In this study we employed an optimized fatty acid extraction and derivatization strategy and were able to detect substantial amounts of free cytosolic octanoic acid. The question remains as to the origin of this intracellular pool of free fatty acids. We constructed strains lacking the TesA and TesB thioesterases in our suppressor backgrounds, but these strains retained the ability to grow on glucose minimal medium (data not shown). However, there are five other thioesterases in *E. coli* of unassigned function which may contribute to the pool. Alternatively, the pool may be formed by spontaneous hydrolysis of acyl-ACPs or of intermediates bound to the fatty acid elongation enzymes (e.g., the 3-ketoacyl-ACP synthases). A final possibility is that the pool is generated by hydrolytic removal of acyl chains inappropriately incorporated by an acyltransferase. Evidence for such an editing reaction by the phospholipid acyltransferases has been reported (29).

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