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Lipoic Acid Synthesis: A New Family of Octanoyltransferases Generally Annotated as Lipoate Protein Ligases†

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ABSTRACT: *Bacillus subtilis* lacks a recognizable homologue of the LipB octanoyltransferase, an enzyme essential for lipoic acid synthesis in *Escherichia coli*. LipB transfers the octanoyl moiety from octanoyl-acyl carrier protein to the lipoyl domains of the 2-oxoacid dehydrogenases via a thioester-linked octanoyl-LipB intermediate. The octanoylated dehydrogenase is then converted to the enzymatically active lipoylated species by insertion of two sulfur atoms into the octanoyl moiety by the *S*-adenosyl-L-methionine radical enzyme, LipA (lipoate synthase). *B. subtilis* synthesizes lipoic acid and contains a LipA homologue that is fully functional in *E. coli*. Therefore, the lack of a LipB homologue presented the puzzle of how *B. subtilis* synthesizes the LipA substrate. We report that *B. subtilis* encodes an octanoyltransferase that has virtually no sequence resemblance to *E. coli* LipB but instead has a sequence that resembles that of the *E. coli* lipoate ligase, LplA. On the basis of this resemblance, these genes have generally been annotated as encoding a lipoate ligase, an enzyme that in *E. coli* scavenges lipoic acid from the environment but plays no role in de novo synthesis. We have named the *B. subtilis* octanoyltransferase LipM and find that, like LipB, the LipM reaction proceeds through a thioester-linked acyl enzyme intermediate. The LipM active site nucleophile was identified as C150 by the finding that this thiol becomes modified when LipM is expressed in *E. coli*. The level of the octanoyl-LipM intermediate can be significantly decreased by blocking fatty acid synthesis during LipM expression, and C150 was confirmed as an essential active site residue by site-directed mutagenesis. LipM homologues seem the sole type of octanoyltransferase present in the firmicutes and are also present in the cyanobacteria. LipM type octanoyltransferases represent a new clade of the PF03099 protein family, suggesting that octanoyl transfer activity has evolved at least twice within this superfamily.

Lipoic acid is a covalently bound enzyme cofactor required for energy-conserved decarboxylation of 2-oxoacids in aerobic metabolism as well as for glycine cleavage in all three domains of life. In each lipoic acid-dependent enzyme, a specific subunit is modified by attachment of lipoic acid to a specific lysine residue located within a highly conserved domain or domains (called lipoyl domains or LDs).[1] An amide linkage is formed between the carboxyl group of lipoic acid and the ε-amino group of the specific lysine residue. During catalysis, the protein-bound lipoamide moieties serve as carriers of reaction intermediates among the multiple active sites of these multienzyme complexes.

There are four known 2-oxoacid dehydrogenases. These are pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, branched chain dehydrogenase, and acetoin dehydrogenase (*1*), although many organisms have only a subset of these enzymes. 2-Oxoglutarate dehydrogenase is a component of the citric acid cycle, whereas pyruvate dehydrogenase is required for the entry of carbon into the cycle; thus, both enzymes are required for aerobic respiration. The branched chain dehydrogenase is required for branched chain fatty acid production in bacteria and also for degradation of branched chain amino acids. Acetoin dehydrogenase degrades the fermentative product, acetoin (3-hydroxybutanone), to acetyl-CoA (*2*).

Most of our knowledge of lipoic acid biosynthesis comes from studies in *Escherichia coli* (*3*) (Figure 1). In the first step of the pathway, an octanoyl moiety is transferred from the fatty acid synthetic intermediate, the octanoyl thioester of acyl carrier protein (ACP). To a specific lysine moiety of the LD by an enzyme called LipB or octanoyltransferase (systematic name, octanoyl-[acyl-carrier-protein]-protein *N*-octanoyltransferase; (EC 2.3.1.181). The LipB reaction proceeds via an octanoyl-thioester enzyme intermediate (*4*). Once the lipoyl-dependent complex has been octanoylated, the LipA lipoyl synthase (EC 2.8.1.8) inserts two sulfur atoms, the first at octanoate carbon 6 and then at carbon 8, to yield the dihydrolipoyl-LD that is oxidized to lipoyl-LD (*5*). LipA is an *S*-adenosylmethionine radical enzyme, and the source of the sulfur atoms is a LipA iron−sulfur cluster (*6*). Thus, in this unusual pathway, lipoic acid is synthesized on its cognate enzymes. *E. coli* also has an enzyme called LplA that functions to scavenge lipoic acid from the environment (*7*, *8*). LplA is a lipoate protein ligase (EC 2.7.7.63) that first activates lipoate with ATP to give enzyme-bound lipoyl-adenylate (*9*). This intermediate is then attacked by the ε-amino group of the LD lysine residue to give the lipoylated protein. *E. coli* LplA is also active with octanoic acid (*10*) and can

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[1]Abbreviations: LD, lipoyl domain; AasS, acyl-ACP synthetase soluble; ACP, octanoyl-*S*-acyl carrier protein; FabA, 3-hydroxydecanoyl-ACP dehydratase:*trans*-2-decenoyl-ACP isomerase; TCEP, tris(2-carboxyethyl)phosphine; DTT, dithiothreitol; GcvH, glycine cleavage system H protein.

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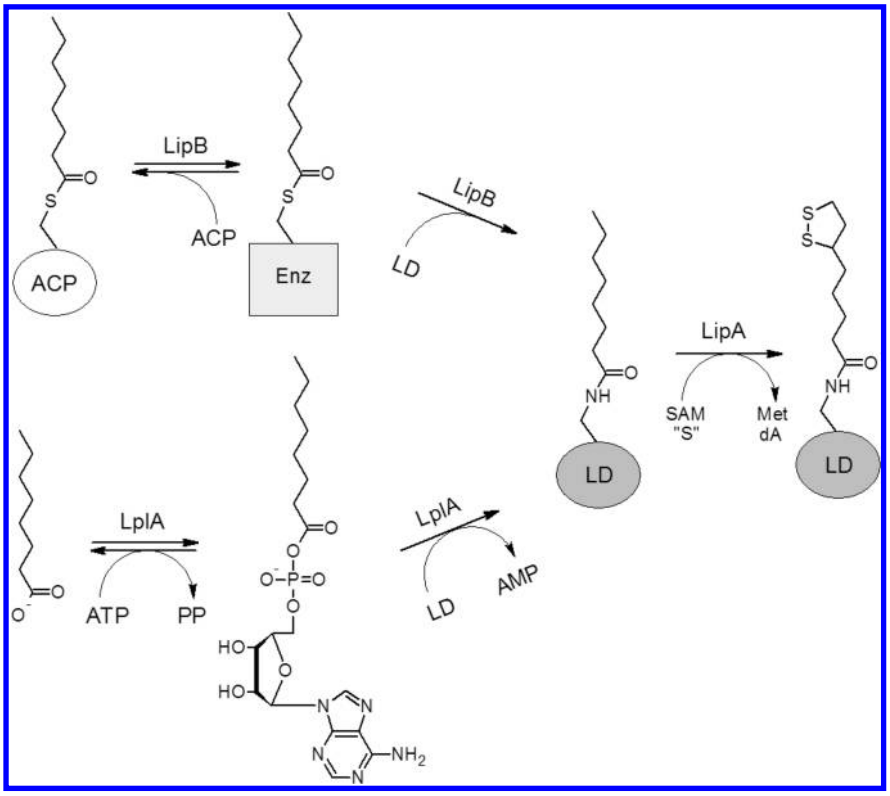

FIGURE 1: Octanoylation of lipoyl domains. The model for octanoyl-LD synthesis by either octanoyl transfer or octanoyl ligation is shown. The reactions of the characterized octanoyltransferases proceed via an octanoyl-enzyme thioester intermediate, whereas the characterized lipoyl ligases (which also function with octanoate) proceed via an enzyme-bound acyl-adenylate. Octanoyl-LD is converted to lipoyl-LD by lipoyl synthase.

bypass the LipB step of the biosynthetic pathway in the presence of exogenous or endogenous octanoic acid (*11*, *12*). Despite the lack of sequence similarity and the fact that they catalyze reactions that proceed by markedly different chemistries, LipB and LplA have similar three-dimensional structures (*13*) as first predicted by Reche (*14*). *E. coli* LplA and *Mycobacterium tuberculosis* LipB can be aligned over the length of LipB (the smaller protein) with a Cα value of 2.5 Å. Together with the biotin protein ligases, these enzymes form the highly divergent PFAM 03099 protein family. Despite the sequence divergence, the cofactor ligands of the LipB, LplA, and *E. coli* BirA biotin protein ligase crystal structures are in register, indicating that the substrate binding geometries are similar (*13*).

Although *Bacilli* have an unusually large number of lipoic acid-dependent enzymes (all of the enzymes listed above), the annotations of these genomes argue that these bacteria have an incomplete lipoic acid synthesis pathway. Although *Bacillus subtilis* encodes a readily recognized and functional LipA homologue (*15*), as well as multiple putative LplA homologues, no LipB homologue can be recognized. Hence, it was unclear how this organism produces the octanoyl-LD substrates required for the insertion of sulfur by LipA. A similar situation was found in the other firmicute bacteria that include other important organisms such as the *Staphylococci* and *Clostridia*. We provide evidence that the octanoyltransferase of *B. subtilis* is one of the proteins previously annotated as a lipoate protein ligase, and thus, this protein, LipM, defines a new class of octanoyltransferases.

EXPERIMENTAL PROCEDURES

Media and Chemicals. *E. coli* K-12 strains were grown on LB or M9 minimal medium (*16*). Antibiotics were used at the following concentrations: 100 μg/mL sodium ampicillin, 50 μg/mL kanamycin sulfate, 12 μg/mL chloramphenicol, 25 μg/mL gentamicin sulfate, and 12 μg/mL tetracycline hydrochloride. All chemicals were obtained from Sigma unless otherwise indicated. Difco Vitamin-Assay Casamino Acids was obtained from Becton-Dickenson. [1-^{14}C]Octanoic acid and [1-^{14}C]octanoyl-CoA were purchased from Moravek. American Radiolabeled Chemicals provided *n*-[2,2′,3,3′-^{3}H]octanoic acid.

Bacterial Strains and Plasmids Constructed. *E. coli* strains, plasmids, and primers used are listed in Tables 1 and 2. *B. subtilis* strain 168 was from the *Bacillus* Genetic Stock Center. Bacterial cultures were grown in shake flasks at 37 °C, and growth was measured by the absorbance at 600 nm using a Beckman DU600 spectrophotometer unless otherwise indicated. Standard techniques (*16*) for DNA manipulation and cloning were employed unless otherwise indicated. Polymerase chain reaction (PCR) amplification was performed using either Taq (New England Biolabs) or Pfu (Stratagene) polymerases according to the manufacturer's recommendations except with the addition of 5% DMSO to the Pfu reaction mixtures. The *lipB* and *lplA* derivatives of strain EPI300 (Epicenter) were constructed by P1 phage transductions of the mutant alleles from strains QC144 and ZX221 to give strains QC067 and QC068. A functional *recA* gene was provided by temperature-sensitive plasmid pEK2. FLP recombinase-mediated removal of the antibiotic resistance cassettes was conducted using temperature-sensitive plasmid pCP20 to yield strain QC069. To obtain a strain deficient in unsaturated fatty acid biosynthesis, a previously described (*17*) null mutant-fusion derivative of *E. coli fabA* tagged with chloramphenicol resistance was transduced from strain MH120 to strain W3110 to yield strain QC134. The strain was maintained in LB medium supplemented with 0.5 mM sodium oleate and 0.1% Tergitol

Table 1: Strains, Plasmids, and Primers Used

strain	relevant characteristics	source
MG1655	*rph-1*	CGSC[a]
W3110	*rph-1* IN(*rrnD-rrnE*)	CGSC
JK1	*rpsL8*	*7*
168	*trpC2*	BGSC[a]
EPI300	recA1 endA1 araD139 Δ(ara, leu)7697 λ- rpsL (StrR) trfA dhfr	Epicenter
MC1061	recA1 araD139 Δ(araA-leu)7697 Δ(codB-lacI)3 λ- e14- rpsL150(Str) hsdR2	
TM136	*rpsL lipB*::Tn1000 *lplA*::Tn10	*7*
TM131	*rpsL lipA*::Tn1000 *lplA*::Tn10	*7*
Acella DE3	*ompT hsdS*B(rB- mB-) *gal dcm* (DE3) Δ*endA* Δ*recA*	EdgeBio
MFH120	JC7623 *lacZ*ΔM15 Δ(*fabA-lacZ*)1(Hyb)*cat fadAB poxB*::pMFH23(*fabA*$^+$)	*17*
ZX221	*rpsL8* Δ*lipB*::FRT::*cat*	*29*
QC144	*rph-1* Δ*lplA*::FRT::*aph*	*29*
QC146	*rph-1* Δ*lplA*::FRT Δ*lipB*::FRT	*29*
QC038	*rpsL lipA*::Tn1000 *lplA*::Tn10/pBAD322G	*29*
QC035	*rpsL*/pBAD322G	*29*
EMM99	*E. coli* BL21(DE3)/pEM88	*26*
QC032	*rpsL lipB::Tn1000 lplA::Tn10*/pQC004	this study
QC034	*rpsL lipB::Tn1000 lplA::Tn10*/pQC006	this study
QC036	*rpsL lipB::Tn1000 lplA::Tn10*/pQC007	this study
QC057	*rpsL lipB::Tn1000 lplA::Tn10*/pBAD322G	this study
QC067	EPI300 Δ*lplA*::FRT::*aph*/pEAK2	this study
QC068	EPI300 Δ*lplA*::FRT::*aph*Δ*lipB*::FRT::*cat*	this study
QC069	EPI300 Δ*lplA*::FRT Δ*lipB*::FRT	this study
QC087	*rpsL lipA::Tn1000 lplA::Tn10*/pQC004	this study
QC088	*rpsL lipA::Tn1000 lplA::Tn10*/pQC006	this study
QC089	*rpsL lipA::Tn1000 lplA::Tn10*/pQC007	this study
QC097	Acella DE3/pQC015	this study
QC101	EPI300 Δ*lplA*::FRT Δ*lipB*::FRT/pQC039	this study
QC111	EPI300/pCC1FOS	this study
QC112	EPI300 Δ*lplA*::FRT Δ*lipB*::FRT/pCC1FOS	this study
QC134	*rph-1* IN(*rrnD-rrnE*)1 DE(*fabA-lacZ*)1(Hyb)*cat*	this study
QC142	*rph-1* IN(*rrnD-rrnE*)1 DE(*fabA-lacZ*)1(Hyb)*cat*/pCY598, pQC015	this study
QC161	*rph-1* Δ*lplA*::FRT Δ*lipB*::FRT/pQC057, pTARA	this study

plasmid	relevant characteristics	source
pBAD322G	arabinose inducible expression vector	*18*
pTARA	pACYC origin, arabinose inducible T7 polymerase	*48*
pCCFos	cosmid cloning vector	Epicenter
pCY598	RSF origin, arabinose inducible T7 polymerase	*49*
pET30b+	Kn, T7 promoter expression vector	Novagen
pET101TOPO	T7 promoter expression vector	Invitrogen
pCR2.1	TOPO TA cloning vector	Invitrogen
pEAK2	Ts *recA* expression plasmid	*50*
pSJ112	*E. coli lipB* expression plasmid	*19*
pNRD136	*sfp* expression plasmid	*51*
pMM88	*B. subtilis acpP* expression plasmid	*26*
pQC004	*B. subtilis* 168 *yqhM*	this study
pQC006	*E. coli lipB*	this study
pQC007	*E. coli lplA*	this study
pQC015	*B. subtilis* 168 N-terminally hexahistidine-tagged *yqhM*	this study
pQC036	*B. subtilis gcvH* C-terminally hexahistidine tagged	this study
pQC039	cosmid carrying *B. subtilis* 168 (2538341..2570341) containing *yqhM*	this study
pQC057	*B. subtilis* 168 *gcvH* C-terminally hexahistidine-tagged	this study

[a]BGSC and CGSC denote the *Bacillus* and *E. coli* Genetic Stock Centers, respectively.

NP-40. The *fabA* allele was also transduced into strain TM131 to yield strain QC168. This strain was maintained on LB medium adjusted to pH 7.0 after supplementation with 5 mM acetate, 5 mM succinate, 0.5 mM oleate, and 0.1% Tergitol NP-40.

For complementation analysis, candidate genes were amplified from genomic DNA by PCR and inserted into pBAD322G (*18*). The *B. subtilis* 168 *lipM* (*yqhM*) coding sequence was amplified using primers Q005 and Q006 and inserted into the EcoRI and XbaI sites of pBAD322G to give pQC004. *E. coli* MG1655 *lplA* was amplified with primers Q017 and Q018 and inserted into the EcoRI and XbaI sites of pBAD322G to give pQC007. The *E. coli* MG1655 *lipB* coding sequence was obtained by NcoI and HindIII digestion of pSJ112 (*19*). The *lipB* fragment was then inserted into the same sites of pBAD322G to give pQC008. These manipulations placed each of these genes under the control of the arabinose inducible *araBAD* promoter.

For purification of the *lipM* protein product, the gene was amplified with primers Q022 and Q023, which added a sequence encoding an N-terminal hexahistidine tag. The PCR product was cloned into pET101 using the TOPO cloning kit (Invitrogen).

Table 2: Oligonucleotide Primers Used

primer	sequence
Q005	TAAGAATTCACCATGCAAAAAGAAACTTGGCG
Q006	TACGTCTAGAATCTTCCATACTTGGTGTTGTC
Q017	AGCGAGAAAAAAGAGTGACCCATTACTACAAGAAAGGAAATCGTTGTGTAGGCTGGAGCTGCTTC
Q018	AAAATCCGGCAAATCGAAGAGAAAGTTGCCCGCATGGGCGGGTAACATATGAATATCCTCCTTAG
Q022	CACCATGCATCATCATCATCATCATATGCAAAAAGAAACTTGGCG
Q023	CCATTACAAGTTTACACTAATGAACTTG
Q047	CATATGAGCATACCAAAAGATTTGCG
Q048	TTAATGATGATGATGATGATGGTCTTCTTGTGTCATCTCTTCGTATTG
Q051	CACCTAAATAGCTTGGCG
Q053	TGCAGGTCGACTCTAGAG
Q133	GGTTGAGGGGCGCGCGGTGGCGGGAAGC
Q134	GCTTCCCGCCACCGCGCGCCCCTCAACC
Q135	GTGGTTGAGGGGCGCCGTGTGGCGGGAAGCGCG
Q136	CGCGCTTCCCGCCACACGGCGCCCCTCAACCAC
Q137	TCCGCGGTCATCTGTTGCTTTTGACGCGCCTTCG
Q138	CGAAGGCGCGTCAAAAGCAACAGATGACCGCGGA
Q139	CGCGGTCATCTGTTTCTTTTGACGCGCCTTC
Q140	GAAGGCGCGTCAAAAGAAACAGATGACCGCG

Point mutations within *lipM* were introduced using the QuikChange II site-directed mutagenesis kit (Stratagene) with primers Q133−Q140. The gene encoding the *B. subtilis* 168 glycine cleavage H protein (*gcvH* or *yusH*) was PCR amplified with primers Q047 and Q048, which added a C-terminal hexahistidine tag to the gene product. This PCR product was inserted into pCR2.1 using the TA cloning kit (Invitrogen). The *gcvH* insert was subsequently inserted into the NdeI and SacI sites of pET30a+ to give pQC057. In each of these final constructs, the genes were placed under the control of a promoter dependent on phage T7 polymerase (*20*).

Cosmid Library Selection for Octanoyltransferases. Selection of a complementing cosmid was conducted using the CopyControl Cosmid Library system (Epicenter) as described. Briefly, genomic DNA from *B. subtilis* 168 was mechanically sheared by repeated pipetting, and fragments of ~40 kb were extracted from an agarose gel run overnight at 15 V and gel purified. Size-selected DNA was then end repaired and ligated into the predigested pCC1FOS vector. The ligation mix was packaged in vitro into λ phage particles, and the particles were used to infect strain QC069. After being washed three times, the cells were titered on LB containing 0.4% glucose, 5 mM acetate, 5 mM succinate, and 24 μg/mL chloramphenicol. To select for lipoic acid prototrophy, cells were plated on M9 minimal medium containing 0.4% glycerol, 12 μg/mL chloramphenicol, and isoleucine, leucine, and valine (each at 0.1 mM). The plates were incubated at 37 °C for 4 days, and eight colonies were obtained from ~580 clones tested. These colonies were reisolated on the same medium to verify their phenotypes, and their cosmids were isolated and transformed into strain QC069 to confirm their complementation abilities. The end sequences of the *B. subtilis* chromosomal fragments were determined by sequencing into the inserts from the flanking vector sequences by the Keck Biotechnology Center using primers purchased from Epicenter.

The growth phenotype of strain QC069 retransformed with the COS1 cosmid was measured. Strains were grown on M9 minimal agar plates of the same composition as that given above and then subcultured overnight in liquid medium of the same composition. The cells were washed thrice with medium lacking acetate and succinate and then diluted to an OD_{600} of 0.05. Growth of the cultures (0.4 mL per well) was followed by the absorbance at 600 nm using a Bioscreen C instrument with continuous and robust shaking. Growth was measured every 15 min.

Complementation of E. coli Lipoate Auxotrophs. Genes were expressed in *E. coli* from plasmids with an arabinose inducible promoter. The strains were first grown on M9 minimal agar and then overnight in liquid with 0.2% arabinose, 0.1% Vitamin-Assay Casamino Acids, 5 mM acetate, 5 mM succinate, and gentamicin. Complementation of *lipB lplA* strain TM136 was tested using M9 minimal medium containing 0.2% arabinose and gentamicin. Complementation of *lipA lplA* strain TM131 was tested using M9 minimal medium containing 0.2% arabinose, gentamicin, and 5 μg/mL sodium lipoate. Overnight cultures were washed thrice before being subcultured to an OD_{600} of 0.1. Growth curves were obtained using the Bioscreen C instrument as described above for cosmid complementation.

Phylogenetic Analysis and Bioinformatics. Comparison of genes homologous to characterized lipoic acid metabolic genes of different bacteria was conducted using the SEED database subsystems tool (*21*). SEED was also used to compare genome contexts in different organisms and to predict functional coupling.

The phylogeny of the LipB_LplA_BirA family (PF03099) was determined with sequences selected from the Pfam database (*22*). An unweighted alignment of protein sequences was performed using T-Coffee (*23*) at the European Bioinformatics Institute Web site (http://www.ebi.ac.uk) using the default settings. The edges of the alignment were trimmed using Jalview (*24*) so only the catalytic domain remained. A minimum evolution tree was constructed with bootstrap analysis of 1000 replicates using Mega4 (*25*) with the default settings.

Purification of B. subtilis AcpP. The acyl carrier protein of fatty acid biosynthesis was purified from strain EMM99 by a modification of the method previously described (*26*). All protein steps were conducted at 4 °C unless otherwise indicated. Following nickel affinity chromatography, the hexahistidine tag was cleaved from approximately 30 mg of ACP with 500 units of AcTEV protease (Invitrogen). The reaction was conducted for 24 h at room temperature during dialysis against the reaction buffer and then subjected to subtractive nickel affinity chromatography as described previously (*26*). The modification state of AcpP was verified using conformationally sensitive native PAGE using 20% acrylamide, 2.5 mM urea gels buffered with Tris-glycine (*27*).

The TEV protease-digested AcpP migrated much slower than the undigested tagged protein. Fractions containing the TEV-cleaved apo and holo AcpP were pooled, concentrated with a Vivaspin concentrator (GE Healthcare), and flash-frozen for storage at −80 °C. Quantitative conversion of the mixture to holo-ACP was achieved using the *B. subtilis* Sfp phosphopantetheinyl transferase as previously described (*28*). Strain QC120 was grown in LB with kanamycin and induced for 2 h with 1 mM isopropyl β-D-1-thiogalactopyranoside at an OD_{600} of 0.6. Sfp was purified from strain QC120 extracts by nickel affinity chromatography as described for LplA (*29*). Pooled fractions containing Sfp were precipitated with ammonium sulfate (35−55% of saturation) as described previously (*28*) and dialyzed overnight against 50 mM sodium phosphate (pH 7.0), 10 mM $MgCl_2$, 10% glycerol, and 1 mM DTT. The protein was concentrated with a Vivaspin concentrator (GE Healthcare) and flash-frozen for storage at −80 °C. Conversion to holo-ACP was verified using conformationally sensitive gel electrophoresis as described previously (*27*). The AcpP modification state was also assayed by electrospray ionization mass spectrometry performed by the University of Illinois School of Chemical Sciences Mass Spectrometry Facility. The sample was prepared by dialysis overnight against 10 mM ammonium bicarbonate, and then the solvent was removed by evaporation under a stream of nitrogen. The mass was determined by electrospray ionization mass spectrometry in positive ion mode using a Micromass Quattro II instrument with 100 pmol/mL protein in 50% aqueous acetonitrile containing 0.1% formic acid.

Preparation of [^{3}H]Octanoyl-ACP. Octanoyl-ACP was prepared using *Vibrio harveyi* acyl-ACP synthetase (AasS) purified by nickel affinity and ion exchange chromatography as previously described (*30*). The 100 μL reaction mixture contained 10 mM Tris-HCl (pH 8.0), 10.1 mM $MgCl_2$, 10 mM ATP, 1 mM TCEP, 5 mM sodium [^{3}H]octanoate, 0.5 mM holo-ACP, and 5 μM AasS. The reaction was allowed to proceed for 2 h at 37 °C. The product was precipitated at 4 °C overnight after addition of an equal volume of acetone. The pellet was resuspended in 100 mM Tris-HCl (pH 8.0) and dialyzed overnight against 10 mM sodium 2-(*N*-morpholino)ethanesulfonic acid (pH 6.1). The protein was found to be >95% octanoyl-ACP by gel electrophoresis using conformationally sensitive native PAGE (*27*). Proteins were quantified by use of extinction coefficients calculated using PROTPARAM on the EXPASY website (*31*). Octanoyl-ACP was quantified at 280 nm using a calculated extinction coefficient of 1490 M^{-1} cm^{-1}.

Purification of B. subtilis GcvH. To purify hexahistidine-tagged *B. subtilis* GcvH, we used lipoic acid auxotrophic strain QC146. The strain was grown in LB with 0.1% glucose, 5 mM acetate, and 5 mM succinate (pH 7.0) to an absorbance of 0.5 at 600 nm. Expression was induced with 0.2% arabinose and the culture incubated for an additional 4 h before the cells were pelleted by centrifugation and frozen at −80 °C. The protein was purified by nickel affinity and anion exchange chromatographic steps and analyzed by mass spectroscopy as described above for AcpP. GcvH was quantified at 280 nm using a calculated extinction coefficient of 16960 M^{-1} cm^{-1}.

Purification of LipM. LipM was initially purified from strain QC103 by nickel affinity and anion exchange chromatographic steps as previously described for *Thermoplasma acidophilum* LplA (*29*) except that the reducing agent was 5 mM TCEP instead of DTT. Unmodified LipM was obtained using *fabA* strain QC142. The strain was grown in LB with 0.1% glucose, 0.5 mM sodium oleate, 0.1% Tergitol NP-40, ampicillin, and kanamycin to an OD_{600} of 0.5. At that point, T7 polymerase expression was induced by addition of 0.2% arabinose for 1.5 h. Then fatty acid biosynthesis was inhibited by addition of 0.1 μg/mL triclosan for 40 min. Cells were harvested by centrifugation and frozen at −80 °C. Lysis and purification were performed as described for the initial purification. A calculated extinction coefficient of 45380 M^{-1} cm^{-1} at 280 nm was used for LipM. The purified protein was analyzed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry at the University of Illinois School of Chemical Sciences Mass Spectrometry Facility. The sample was prepared by dialysis overnight in 10 mM ammonium acetate, dried under a stream of nitrogen, and subjected to MALDI performed using a Voyager-DE STR mass spectrometer (Applied Biosystems) equipped with a UV laser (337 nm N_2 laser). All measurements were taken using the linear mode, and the positive ion was recorded. We prepared samples by mixing 2 μL of the sample and 10 μL of the matrix, α-cyano-4-hydroxycinnamic acid.

Liquid Chromatography−Tandem Mass Spectrometry Analysis of LipM. LipM was purified from strain QC103 as described for LplA (*29*) and was subjected to size exclusion chromatography using a Sephadex 200 column developed with 10 mM sodium phosphate (pH 7), containing 100 mM sodium chloride and 1 mM DTT. Fractions containing pure LipM as judged by SDS−PAGE were pooled, dialyzed against 10 mM ammonium acetate, and dried under a stream of nitrogen. The protein was then dissolved in 100 mM Tris-HCl (pH 8.5) containing 1 M urea and 20 mM methylamine and digested with one-tenth mass of modified sequencing grade trypsin (Roche) for 16 h at 37 °C. Liquid chromatography−tandem mass spectrometric analysis was performed by the University of Illinois Biotechnology Center Protein Sciences Facility on a Waters Q-Tof API-US Quad-ToF mass spectrometer with a nanoAcquity UPLC system. The columns used were Waters nanoAcquity UPLC (75 μm × 150 mm, 3 μm, Atlantis dC18) and Atlantis dC18 5 μm Nanoease trap columns. A 60 min linear gradient from 1 to 60% acetonitrile in 0.1% formic acid was used to elute the peptides from the columns. Tandem mass spectrometric data were collected using the Data Directed Analysis method in MassLynx to fragment the top four ions in each survey scan. ProteinLynx (Waters) was used to process the mass spectral data into peak list files for analysis via Mascot (Matrix Science). Database searches were performed against the NCBI nonredundant database.

Assay of Enzyme-Bound Fatty Acids. LipM was purified from 2 L cultures with and without addition of triclosan by nickel affinity chromatography as described above. The eluates were twice dialyzed against 10 mM ammonium acetate first overnight and then again for 4 h. The dialysate was evaporated under a stream of nitrogen while being heated to 42 °C. A modification of previously described syntheses of fatty acid butyl esters was then conducted (*11*, *32*). To each glass vial were added 500 μL of hexanes, 500 μL of butanol-BF_3, and 100 μg of anhydrous magnesium sulfate. Heptanoic acid (10 μg) was added as an internal standard. Transesterification was conducted at 65 °C for 2 h. Salts and butanol were removed by three extractions with water. Butyl esters were analyzed by gas chromatography and mass spectrometry (GC−MS) by a modification of the method of Hermes and Cronan (*11*).

Samples (5 μL) were injected in split-less mode into the GC−MS system that consisted of an Agilent (Palo Alto, CA) 7890A gas chromatograph, an Agilent 5975 mass selective detector,

and an Agilent 7683B autosampler. Injections were performed on a 30 m ZB-WAX column with a 0.32 mm inside diameter and a 0.25 mm film thickness (Phenomenex) with an injection port temperature of 230 °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 3 mL/min. The temperature program consisted of isothermal heating at 90 °C for 5 min followed by an oven temperature increase of 5 °C/min to 260 °C for 10 min. The mass spectrometer was operated in positive electron impact mode (EI) at a 69.9 eV ionization energy in the m/z 30−800 scan range. The spectra of all chromatogram peaks were evaluated using HP Chemstation (Agilent) and AMDIS [National Institute of Standards and Technology (NIST), Gaithersburg, MD]. The spectra of all chromatogram peaks were compared with electron impact mass spectrum libraries NIST08 (NIST) and WILEY08 (Palisade Corp.).

To determine the efficiency of esterification, we created octanoyl-LipM in vitro. AasS was used to generate octanoyl-ACP in the LipM reaction mixture. The 200 μL reaction mixtures contained 100 mM sodium phosphate (pH 7.0), 50 mM NaCl, 5 mM TCEP, 10 mM ATP, 10 mM $MgCl_2$, 10 mM [^{14}C]-octanoate, 50 μM holo-ACP, 5 μM AasS, and 5 μM LipM. After incubation at 37 °C for 2 h, the reaction mixture and the control reaction mixture without LipM were dialyzed. The proteins were dried, esterified, and analyzed by GC−MS as described above.

Assay of LipM-Catalyzed Octanoyl Transfer. The transfer of octanoate from octanoyl-ACP to GcvH was assayed using a coupled system in which AasS provided the octanoyl-ACP substrate. The 25 μL reaction mixtures contained 100 mM sodium phosphate (pH 7.0), 50 mM NaCl, 5 mM TCEP, 1 mM ATP, 1 mM $MgCl_2$, 250 μM [^{14}C]octanoate, 50 μM holo-ACP, 20 μM GcvH, 2 μM AasS, and 1 μM LipM. When formation of the acyl-enzyme intermediate was studied, the concentrations of wild type and mutant LipM proteins were increased to 10 μM. To test octanoyl-CoA as a LipM substrate, a coupled assay was also used. The 25 μL reaction mixtures contained 100 mM sodium phosphate (pH 7.0), 50 mM NaCl, 5 mM TCEP, 1 mM ATP, 1 mM $MgCl_2$, 1 mM [^{14}C]octanoate, 50 μM trilithium CoA, 20 μM GcvH, 0.1 unit of acyl-CoA synthetase (*Pseudomonas* sp. from Sigma), and 1 μM LipM. The reaction mixtures were incubated at 37 °C for 1 h and subjected to SDS−PAGE using a loading buffer that lacked reducing agents and 4 to 20% Tris-glycine gels. The gels were stained with Coomassie R-250, soaked in Amplify fluorographic reagent (GE Healthcare), and dried onto Whatman filter paper. Dried gels were exposed to Biomax XAR film (Kodak) flashed with an Amersham Sensitize Preflash Unit (GE Healthcare) (*33*). The exposure period was 16−24 h at −80 °C.

Isolation and Transfer of the Octanoyl Moiety of the Octanoyl-LipM Intermediate. Synthesis of the octanoyl-LipM intermediate was conducted in a 500 μL assay containing 50 mM sodium phosphate (pH 7.0), 5 mM TCEP, 10 mM $MgCl_2$, 10 mM ATP, 1 mM [^{14}C]octanoate, 0.1 mM holo-ACP, 2 μM AasS, and 10 μM LipM. The reaction mixture was incubated at 37 °C for 1 h. The product was diluted 10-fold in 25 mM Tris-HCl (pH 8.0) and purified by anion exchange chromatography as described for LplA (*29*). LipM eluted at ∼110 mM sodium chloride, the same point as apo-LipM. Fractions containing pure LipM were pooled, concentrated, and exchanged into 10 mM sodium phosphate (pH 7.0) using a Vivaspin concentrator (GE Healthcare). The mixture for the transfer from octanoyl-LipM to GcvH or holo-ACP contained 100 mM sodium phosphate (pH 7.0), 5 mM TCEP, 15 μM LipM, and either 20 μM GcvH or 20 μM holo-ACP. The reaction mixtures were incubated at 37 °C for 1 h and visualized by SDS−PAGE and radiography as previously described for the LipM assay.

Table 3: *B. subtilis* 168 Genome Segments in the Complementing Cosmids[a]

cosmid	coordinates	size	orientation
COS1	2570129...2539139	39129	+
COS2	2573410...2536688	44861	+
COS3	2582822...2540810	50151	+
COS4	2575523...2539485	44177	+
COS5	2540006...2572838	40971	−
COS6	2567917...2534304	41752	+
COS7	2535189...2575816	48766	−
COS8	2575100...2536131	47108	+

[a]The cosmid clones that complement the *E. coli lipB* mutation contain the *B. subtilis* genome segments indicated by the nucleotide coordinates of the genome sequence (GenBank entry AL009126). The insert size is given in base pairs for each cosmid. The *lipM* (*yqhM*) open reading frame runs from base pair 2543968 to 2544804 of the genome. Positive (+) and negative (−) orientation indicates the orientation of the insert with respect to the origin of replication of the cosmid vector.

Competition Assay Using CoA and ACP Octanoyl Thioesters. LipM was assayed for its ability to utilize a mixture of octanoyl-ACP and octanoyl-CoA in a competition assay similar to that described previously (*11*). The 100 μL reaction mixtures contained 10 mM sodium phosphate (pH 7.0), 100 mM sodium chloride, 5 mM TCEP, 250 μM [^{14}C]octanoyl-CoA, 50 μM [^{3}H]octanoyl-ACP, 100 μM GcvH, 2 μM AasS, and 1 μM LipM. The reaction mixture was preincubated at 37 °C for 3 min before addition of LipM. After 30 min at 37 °C, the assay was stopped via addition of guanidine HCl to a final concentration of 5 M. The entire reaction mixture was applied to a Ni-NTA spin column (Qiagen) pre-equilibrated with 50 mM Tris-HCl (pH 8.0) containing 4 M urea. The column was washed four times with 600 μL of the same buffer. Hexahistidine-tagged LipM and GcvH were eluted with 400 μL of the same buffer containing 500 mM imidazole. The eluate was diluted to 1 mL with water and mixed with 4 mL of Biosafe II scintillation cocktail. The mixture was subjected to scintillation counting using a Beckman LS 6500 instrument. The ^{3}H and ^{14}C isotopes were differentially counted using channels 0−400 and 0−670, respectively. The ^{3}H counts were adjusted by subtraction of 67% of the ^{14}C counts for that sample, which was experimentally determined from six different amounts of [^{14}C]octanoyl-CoA in the assay elution buffer.

RESULTS

Genetic Complementation of an E. coli lipB Strain. Although amino acid homology is a useful tool for predicting function, the *B. subtilis* genome encoded no recognizable homologue of LipB. Therefore, to find the gene(s) responsible for the octanoyl transfer step of the *B. subtilis* lipoic acid synthetic pathway, we constructed a library of *B. subtilis* chromosomal fragments in a cosmid vector and used the resulting phage particles to transfect an *E. coli lipB lplA* double mutant strain. The *lplA* mutation was introduced into this strain to prevent possible bypass of the *lipB* mutation by LplA-catalyzed ligation of traces of intracellular octanoate or lipoate (*11*). Glycerol was used as the carbon source to preclude bypass of succinate with acetate-dependent growth by fermentative metabolism. The *B. subtilis* genomic library contained a sufficient number of clones to cover 99.6% of the genome as calculated from the equation of Clarke and Carbon (*34*). The eight cosmid clones that allowed growth of

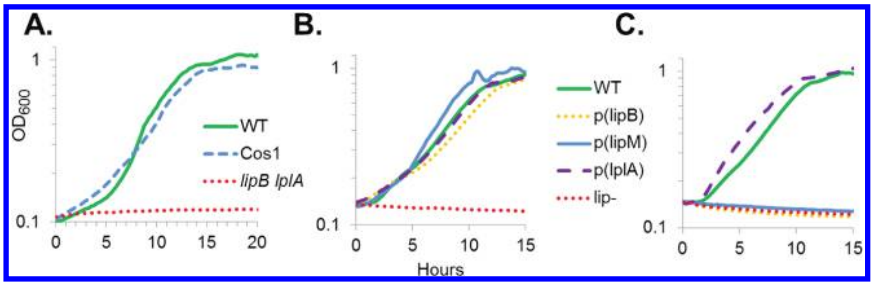

FIGURE 2: Complementation of *E. coli* lipoic acid auxotrophs. Growth curves of lipoic acid auxotrophs carrying various plasmids are shown. (A) Growth tests for *lipB* complementation in minimal glycerol medium. Growth curves of the wild type (WT) and *lipB lplA* strains with the empty pCC1FOS vector are shown. Cosmid isolate 1 (Cos1) or pQC039 was also tested in the *lipB lplA* strain. (B) Growth tests for *lipB* complementation in minimal arabinose medium. Growth curves for the WT and *lipB lplA* strains carrying empty vector pBAD322G are shown. Plasmids carrying the indicated genes were tested in a *lipB lplA* strain for their abilities to support growth. (C) Growth tests for *lplA* complementation in minimal arabinose medium containing lipoic acid. The WT and *lipA lplA* strains carrying empty vector pBAD322G are shown. Plasmids carrying the indicated genes were tested in a *lipA lplA* strain for their abilities to support growth. Growth was measured by optical density. Panels B and C have the same color code.

the *lipB lplA* strain carried overlapping fragments of the *B. subtilis* genome (Table 3) that contained a common region in which only a single candidate gene related to lipoic acid metabolism was found, the *yqhM* gene annotated as encoding a lipoate ligase. To test if *yqhM* was the gene responsible for complementation by the cosmid clones, the minimal gene was expressed from an arabinose inducible promoter. Expression of *yqhM* (or *E. coli lipB* or *E. coli lplA*) allowed growth of the *lipB lplA* double mutant strain, TM136 (Figure 2). Because the growth observed could be caused by either octanoyltransferase activity or high-level lipoyl ligase activity, we also expressed *lipM* in strain TM131, a *lipA lplA* strain, in the presence of lipoic acid. Expression of *yqhM* (or *E. coli lipB*) failed to allow growth of this strain, whereas expression of the *E. coli lplA* gene resulted in robust growth. Therefore, despite its sequence similarity to *E. coli* LplA (Figures 8 and 9), YqhM lacks lipoate ligase activity and instead has octanoyltransferase activity (Figure 2). Therefore, we have renamed *yqhM* as *lipM*.

Purification of GcvH. To provide a substrate for in vitro studies of LipM, we purified the putative glycine cleavage H protein (GcvH) of *B. subtilis* 168 in its apo form from an *E. coli* strain deficient in lipoic acid biosynthesis. The pure protein formed a doublet when analyzed by SDS−PAGE (Figure 3A), but electrospray mass spectrometry gave a major peak at 14944.2 amu (Figure 3B). This is 1.2 amu lower than that theoretically predicted, which we attribute to deamidation of the protein (which results in a negligible mass difference) perhaps at N73 because it is followed by a glycine residue should be unusually labile (*35*). Deamidation of other LDs has been observed and does not affect modification (*36*). Note that GcvH migrates aberrantly during SDS−PAGE analysis (Figure 3A). It runs as though it is twice its actual size of 14.1 kDa. Such slow migration rates are often seen with small, very acidic proteins (GcvH has a calculated pI of 3.9) and can be attributed to an abnormally low level of SDS binding.

Modification of LipM and Purification of the Apoprotein. To directly test LipM for octanoyltransferase activity, we purified the protein obtained after high-level expression in *E. coli*, and it appeared homogeneous by SDS−PAGE (Figure 3A) and size exclusion chromatography, where it eluted as a 14 kDa protein mainly in the monomeric form with a variable small secondary peak that may be a multimer (Figure 3D). However, the protein obtained in the initial purifications of LipM was inactive and had an average molecular weight of 33338 ± 32 amu (five measurements), a value 143 amu greater than the expected value, which suggested that the protein had been post-

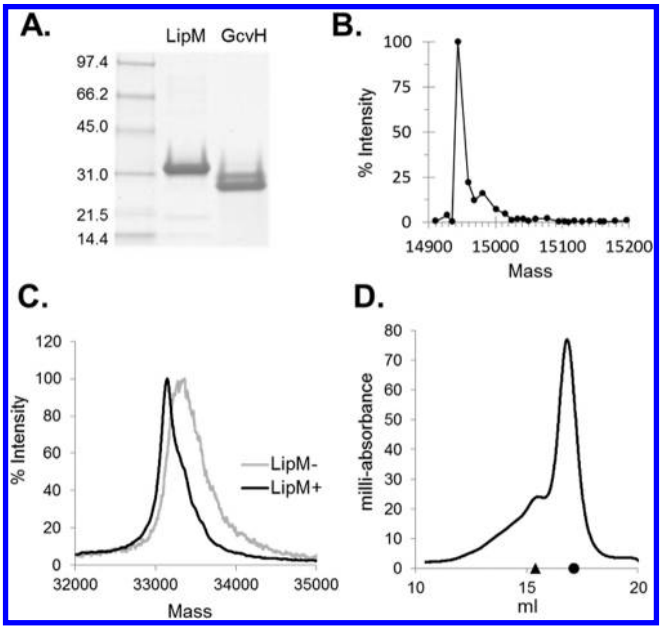

FIGURE 3: Analysis of purified proteins. (A) SDS−PAGE of 0.2 μmol of purified LipM and 0.2 μmol of purified GcvH. Molecular mass standards are indicated in kilodaltons. (B) Electrospray ionization mass spectrum of GcvH. Calculated masses are represented by black circles. (C) MALDI mass spectra of purified LipM preparations. LipM− denotes the enzyme purified from cells of a wild type strain grown without triclosan or oleate, whereas LipM+ denotes the enzyme purified from cells of a Δ*fabA* strain grown with triclosan and oleate. The peak mass values for the LipM− and LipM+ proteins were 33300 and 33179, respectively. (D) Size exclusion chromatogram of LipM (the absorbance at 280 nm is plotted). The elution positions of chymotrypsinogen and ribonuclease A are designated by a triangle and a circle, respectively.

translationally modified [the initiator methionine was retained as expected from the specificity of *E. coli* methionine aminopeptidase (*37*, *38*)]. However, LipM gave rather imprecise MALDI mass spectra that may have resulted from a combination of a heterogeneous set of modifications and the smoothed and averaged data generated by the spectrometer (Figure 3C). To determine if the protein was post-translationally modified, we digested LipM with trypsin and subjected the resulting peptides to liquid chromatography−tandem mass spectrometry analysis. We found evidence of a series of modified peptides that had overlapping sequences and all contained residue C150. In some LipM molecules, the predicted C150 modification was an octanoyl thioester, whereas in others, a decanoyl adduct was present

A.

Peptide Sequence	ΔMass	Modification	Ion score
SSV**C**FDAPSWYELVVEGRK	126	Octanoylation	23
SSV**C**FDAPSWYELVVEGRK	171	Decanoyl-Adduct	43

B.

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1   MQKETWRFID SGNASPAFNM ALDEALLYWH SEKKIPPVIR FYGWNPATLS
51  VGYFQNIKKE INFEAVHKYN LGFVRRPTGG RGVLHDQELT YSVIVSEEHP
101 EMPATVTEAY RVISEGILQG FRNLGLDAYF AIPRTEKEKE SLKNPRSSVC
151 FDAPSWYELV VEGRKVAGSA QTRQKGVILQ HGSILLDLDE DKLFDLFLYP
201 SERVRERMQR NFKNKAVAIN ELIEKRVTMD EARKAFKEGF ETGLNIHLEP
251 YELSQEELDF VHHLAETKYA SDEWNYKR
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FIGURE 4: Liquid chromatography−tandem mass spectrometric analysis of LipM tryptic peptides. (A) Modification states of C150 detected. The theoretical peptides are shown with C150 in bold and underlined. The difference in mass from the modification is listed. The ion score is equal to $-10 \log(P)$, where P is the probability the result is random chance. (B) Sequence coverage of LipM. The LipM peptide sequences detected are shown in bold.

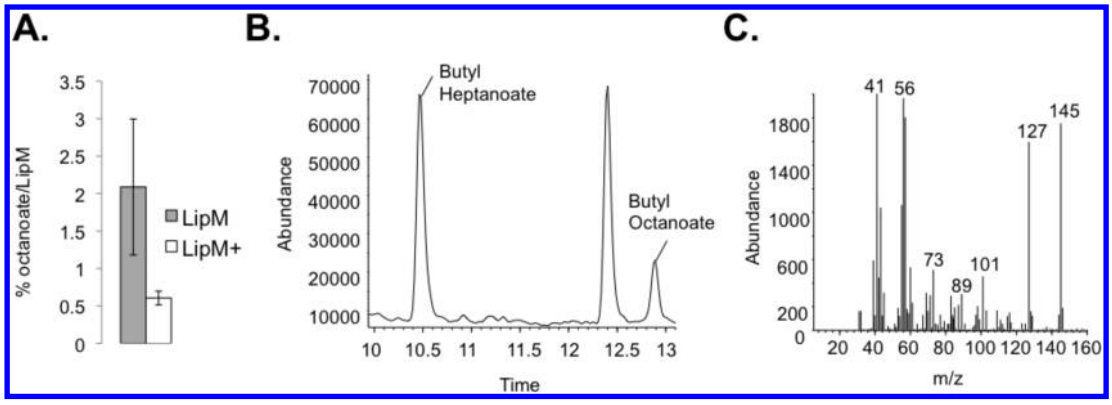

FIGURE 5: Gas chromatography−mass spectrometric analysis of LipM-bound octanoate. LipM-bound octanoyl moieties were assayed by transesterification to the butyl esters followed by GC−MS analysis as described in Experimental Procedures. (A) The values are the molar percentages of octanoic acid per LipM preparation. The gray bar is for cultures without triclosan added (LipM), whereas the white bar is for cultures with triclosan added (LipM+). The error bars represent one standard deviation for LipM preparations from three independent cultures. Both purifications were from a Δ*fabA* strain. (B) Representative gas chromatogram of a LipM preparation. The butyl heptanoate internal standard and the analyte, butyl octanoate, are indicated. (C) Mass spectrum of the butyl octanoate from a LipM preparation.

(Figure 4). By analogy to LipB, these data suggested that C150 is the LipM active site nucleophile and the octanoyl thioester modification is the acyl-enzyme intermediate (*4*).

The decanoyl adduct was previously found as a modification of *M. tuberculosis* LipB when the protein was expressed in *E. coli*, but not when expressed in a mycobacterium (*13*). In that work, it was proposed (albeit, not proven) that the *M. tuberculosis* LipB decanoic acid adduct arose by binding *cis*-3-decenoyl-ACP, a key intermediate in *E. coli* fatty acid biosynthesis by LipB, followed by Michael addition of the active site cysteine thiol to the double bond resulting in a thioether link to C-3 of the acyl chain. Because like *M. tuberculosis*, the *B. subtilis* unsaturated fatty acid synthetic pathway differs from that of *E. coli*, it seemed likely that in both cases the octanoyltransferase would accidently bind *cis*-3-decenoyl-ACP and the decanoyl adduct would form. To prevent the formation of a decanoyl adduct, we expressed LipM in an *E. coli fabA* mutant strain. This strain lacked the ability to make *cis*-3-decenoyl-ACP (hence unsaturated fatty acids), and therefore, growth of the strain required supplementation of the medium with an unsaturated fatty acid such as oleic acid (*17*).

To reduce the level of the octanoyl-enzyme intermediate, we added triclosan, an inhibitor of fatty acid biosynthesis (*39*), to the medium during LipM expression. We determined the levels of LipM with bound octanoate by GC−MS of butyl esters using an octanoyl-LipM standard prepared in vitro. Using this standard, a recovery efficiency of 93% based on complete conversion of LipM to octanoyl-LipM was found, which is within the expected efficiency for esterification (*32*). This method showed that the addition of triclosan resulted in a significant reduction in the level of the octanoyl-enzyme intermediate (Figure 5), and thus by addition of triclosan and use of the *fabA* strain, we obtained LipM in a largely unmodified and active form.

In Vitro Demonstration of LipM Activity. We employed the AasS acyl-ACP synthetase to generate acyl-ACPs in the LipM assay mixture and found that the transfer of the octanoyl group from octanoyl-ACP to *B. subtilis* GcvH occurred in a LipM-dependent manner (Figure 6). In the absence of the GcvH acceptor protein, LipB was converted to the octanoyl-enzyme intermediate. The octanoyl-LipM species was purified and shown to transfer the octanoyl moiety to either GcvH in the forward reaction or ACP in the reverse reaction, as expected of a catalytically competent intermediate. Because of the lengthy time of incubation, we expect that the relative levels of transfer in the forward and reverse directions reflect the equilibria of the transfer reactions. We also tested octanoyl-CoA as an octanoyl donor by using acyl-CoA synthetase to generate the substrate in the reaction mix. This system was used to demonstrate that LipM also uses octanoyl-CoA as a substrate, albeit poorly (Figure 7). This is not unexpected as CoA is a substrate mimic of ACP. To determine which of the substrates LipM preferred, we performed a double-label experiment with equal concentrations of octanoyl-CoA and octanoyl-ACP differentially labeled in the octanoyl moiety. Octanoyl-ACP was much the preferred substrate for

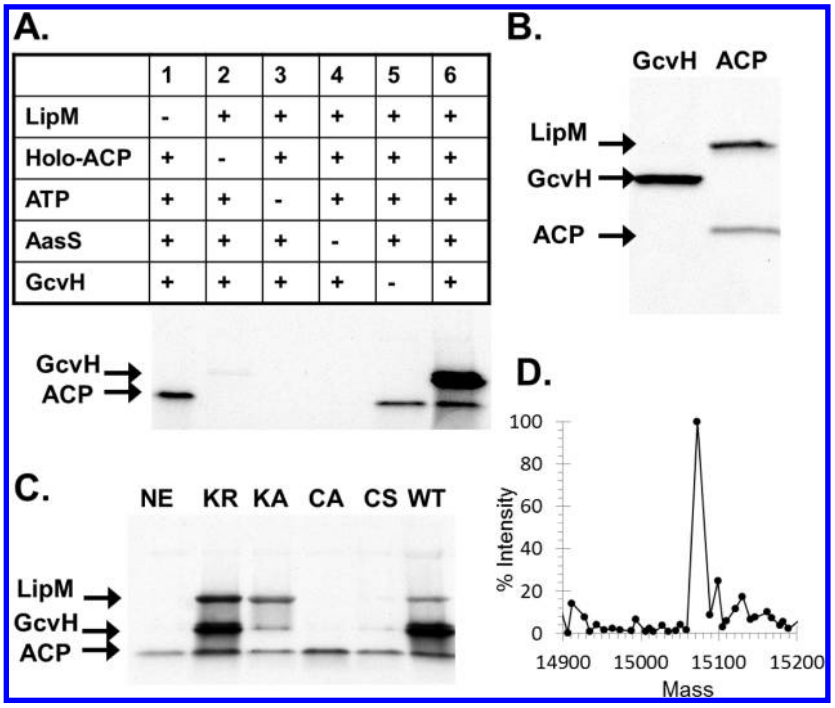

FIGURE 6: Octanoyltransferase activities of wild type and mutant LipM proteins. Assay of the transfer of the octanoyl group from octanoyl-ACP to *B. subtilis* GcvH using either [1-^{14}C]octanoyl-ACP or octanoyl-ACP as the substrate as described in Experimental Procedures. Autoradiograms of dried SDS−PAGE gels are shown. (A) The [1-^{14}C]octanoyl-ACP was synthesized from [1-^{14}C]octanoate via AasS added to the reaction mixture (ATP was also added as an AasS substrate). Synthesis of octanoyl-ACP required AasS, ATP, and holo-ACP, whereas formation of octanoyl-GcvH required apo-GcvH, LipM, and octanoyl-ACP. (B) Transfer of octanoate from purified [1-^{14}C]octanoyl-LipM to an equimolar amount of either GcvH or holo-ACP as indicated. (C) Same assay mixture from panel A with 10-fold more LipM or mutant LipM proteins added to allow detection of the octanoyl-LipM intermediate: lane NE, no enzyme; lane KR, LipM K165R; lane KA, LipM K165A; lane CA, LipM C150A; lane CS, LipM C150S; and lane WT, wild type LipM. (D) Reaction as in lane 6 of panel A conducted with octanoate in place of [1-^{14}C]octanoate. After incubation, GcvH was recovered from the reaction mixture by Ni^{2+} cheleate chromatography and analyzed by electrospray ionization mass spectrometry. The mass obtained was 15072.4 amu, a value 128.2 amu greater than that of the apoprotein (Figure 3) and in good agreement with the octanoyl moiety mass (126.2 amu).

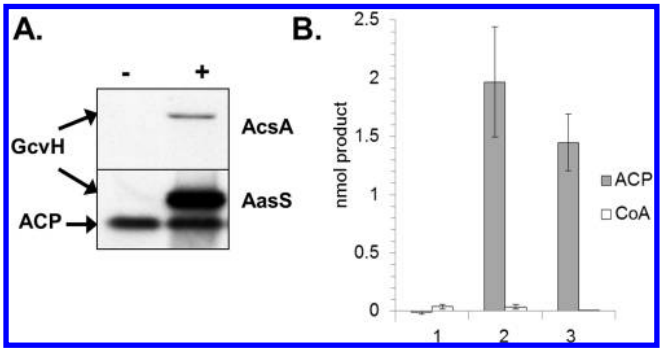

FIGURE 7: Comparison of the octanoyl thioesters of ACP and CoA as LipM substrates. Assays of the transfer of the octanoyl group to *B. subtilis* GcvH using either octanoyl-CoA or octanoyl-ACP as described in Experimental Procedures. (A) Autoradiograms of dried SDS−PAGE gels are shown. [1-^{14}C]Octanoate was converted to [1-^{14}C]octanoyl-ACP or [1-^{14}C]octanoyl-CoA by AasS or acyl-CoA synthetase (AcsA), respectively. The presence (+) or absence (−) of LipM is indicated. (B) Double-label experiment with a mixture of purified [1-^{14}C]octanoate-CoA and purified [^{3}H]octanoyl-ACP. The substrates were [1-^{14}C]octanoate-CoA (column 1), an equimolar mixture of both substrates (column 2), and [^{3}H]octanoyl-ACP (column 3). The error bars represent one standard deviation from six independent assays.

modification of GcvH, although some modification caused by transfer from octanoyl-CoA was observed (Figure 7).

C150 and K165 Are Key LipM Catalytic Residues. We constructed point mutations in *lipM* to test the importance of the predicted active site residues. The residues mutated were C150 and the conserved lysine, K165 in LipM, which is present in all members of PF03099 (*14*). Mutation C150A or C150S resulted in loss of the overall catalytic activity and an inability to form the acyl-enzyme intermediate; the K165A mutant had weakened catalytic ability, and the K165R mutant remained active under these conditions (Figure 6C).

Bioinformatic Analysis of LipM. Phylogenetic analysis of LipM and proteins with a high degree of sequence similarity revealed that these proteins form a clade distinct from other functional groups of related enzymes, as well as being distinct from LipB type octanoyltransferases (Figures 8 and 9). This suggests that other members of the LipM clade are also octanoyltransferases. The LipM clade groups with lipoate protein ligases with 73% bootstrap support, which is considered significant (*40*). This means that LipMs are closely related to LplAs and likely share a more recent ancestor with LplAs than with any other group of proteins, consistent with the current incorrect annotation of LipM as a lipoate protein ligase.

Close homologues of LipM are found in firmicutes and cyanobacteria (Figure 9). Although relative to LipB the cyanobacterial LipMs form a separate clade from firmicute LipMs, they have a similar insertion that contains the active site cysteine as in *B. subtilis* LipM. This and the high degree of amino acid sequence similarity (~50%) suggest that the cyanobacterial LipMs are octanoyltransferases. The cyanobacteria also encode a putative LipB type octanoyltransferase that (if both enzymes are active) would give these bacteria a pair of octanoyltransferase isozymes. The presence of two isozymes in cyanobacteria suggests that there may be a physiological difference between the two enzymes within at least these organisms. Further work is

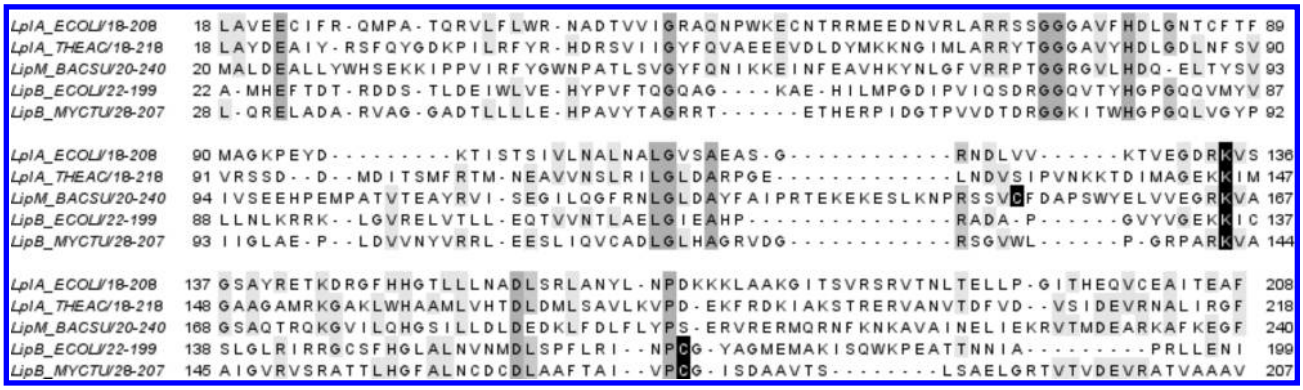

FIGURE 8: Alignment of LipM with enzymatically characterized LipB and LplA proteins. Alignments were performed as described in Experimental Procedures and displayed using Jalview (*24*) . The sequence name indicates the enzyme type; the Uniprot code indicates the organism of origin, and the numbers indicate the amino acid residues displayed. Positions that are ≥50% similar are highlighted in gray. The catalytic cysteine residues of the octanoyltransferases are boxed and highlighted in black, as is the conserved PF03099 lysine residue.

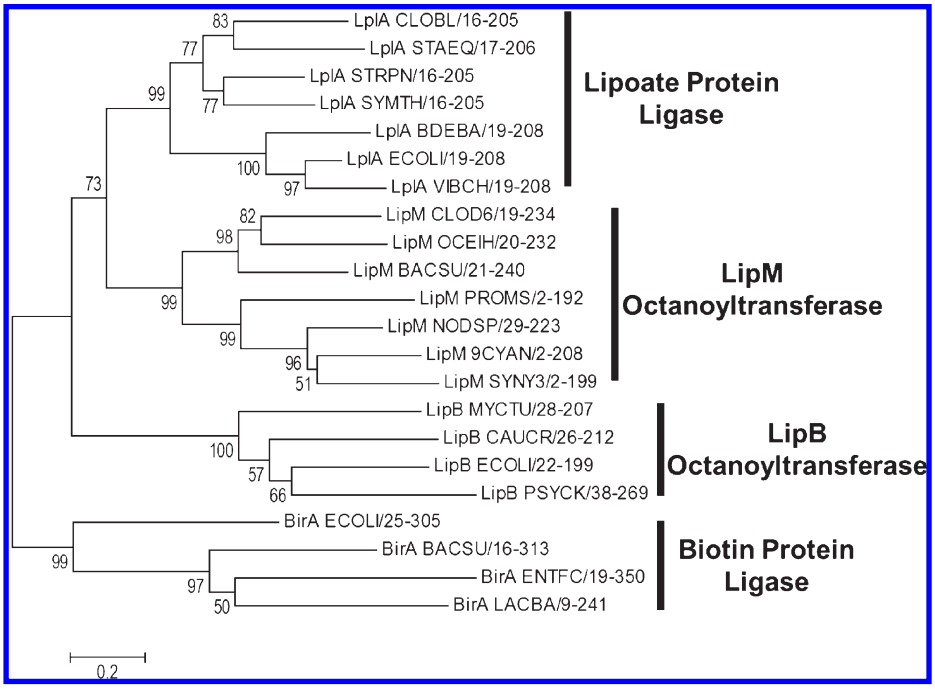

FIGURE 9: Phylogeny of LipM. The minimum evolution tree of selected PF03099 protein sequences with bootstrap percentage confidence values shown for each branch is given. Phylogenetic analyses were conducted as described in Experimental Procedures. The scale bar corresponds to a 20% difference in compared residues, on average, per branch length. Biotin protein ligase sequences were used as a related out-group to compare lipoate protein ligases, LipB octanoyltransferases, and LipM octanoyltransferases.

necessary to determine if there are physiologically relevant differences between the LipB and LipM types of octanoyltransferase.

Using the SEED subsystem database (*21*), we found that firmicutes and cyanobacteria also encode putative lipoyl synthases, and thus, the sulfur insertion enzyme seems to be present. Moreover, the SEED database shows that putative firmicute *lipM* genes are functionally coupled to the glycine cleavage system; *lipM* is very often found adjacent to genes encoding putative glycine cleavage system P and T proteins. The covariance with putative LipA homologues and the functional coupling with the glycine cleavage system provide further indications that these LipM homologues are involved in lipoic acid biosynthesis.

DISCUSSION

Using an approach unbiased by bioinformatics, we have identified a *B. subtilis* gene encoding a new type of octanoyltransferase. Our isolation of *lipM* on eight different cosmid clones suggests that it is the only *B. subtilis* 168 gene able to complement *E. coli lipB*, although this suggestion must be tested by genetic analyses. A *B. subtilis* strain lacking LipM activity should be a lipoate auxotroph assuming that the *yhfJ* gene encodes a lipoate ligase (Figure 1) (*11*). We and others have found that overproduction of *E. coli lplA* complements *lipB* null mutants (Figure 2) (*7*, *11*), and thus, we would expect to have also isolated the *yhfJ* gene. However, this was not the case. The most straightforward explanation for this result was that the level of *yhfJ* expression was insufficient to restore growth under our experimental conditions. Either high-level expression of wild type *lplA* or normal expression of an LplA mutant protein having increased octanoate affinity is required for *lipB* complementation (Figure 2) (*11*). Other possibilities are that our library is biased and lacked *yhfJ* clones and that *yhfJ* does not encode a lipoate ligase. We favor the biased library explanation because this would be consistent with the unexpectedly frequent isolation of

LipM clones. Library bias due to gene toxicity is a known problem with *B. subtilis* genomic DNA libraries in *E. coli* and was even seen with a chromosomally integrated library (*41*). Although we used a vector with a single-copy F factor replication origin vector, basal expression from the second *trfA*-dependent replication origin (*42*) may also have been an issue. Indeed, growth of most isolates of our cosmid library was much better on glycerol or glucose minimal medium than on medium containing arabinose (data not shown), which would induce *trfA* expression resulting in greatly increased plasmid copy numbers (*42*).

Upon purification of LipM, we found that the average mass determined by MALDI was greater than that calculated from the amino acid sequence, and this, and the precedent of the *M. tuberculosis* LipB decanoic acid adduct (*13*), strongly suggested that the protein was modified. Analysis of the LipM tryptic peptides indicated that both the decanoic acid adduct and the octanoyl-LipM intermediate were present in these preparations. The level of octanoyl-enzyme intermediate was significantly decreased by addition of the fatty acid synthesis inhibitor, triclosan (which inhibits FabI, the enzyme responsible for the last step of the fatty acid synthesis cycle), during LipM expression. The uncertainties of peptide ionization efficiencies precluded quantitation of the levels of octanoylation by analysis of tryptic peptides, and thus, we developed a method for directly detecting the octanoyl groups as their butyl esters, a derivative that allows efficient recovery of short chain volatile acids such as octanoate (*11*). To the best of our knowledge, this is the first time this method has been adapted for quantification of a protein modification.

Despite their marked differences in sequence, the LipM and LipB octanoyltransferases share the same general catalytic mechanism. The octanoyl moiety is transferred from octanoyl-ACP to the enzyme active site cysteine thiol to give the octanoyl-enzyme intermediate, the thioester bond of which is attacked by the ε-amino group of the LD lysine residue to give octanoyl-LD. The octanoyl moiety can also be transferred from the acyl enzyme intermediate back to ACP. However, the results of mutagenesis studies distinguish the detailed LipM mechanism from that of LipB. Although loss of the active site thiol by substitution of the cysteine residue with alanine or serine results in inactivation of both enzymes, the *E. coli* LipB C169A protein performs dead-end acylation of the enzyme (*4*), whereas LipM C150A does not. Moreover, the LipB C169S protein forms a dead-end octanoyl ester (*4*), whereas LipM C150S does not. This may simply be a result of the different environments of the active site nucleophile, or it may reflect a physiologically relevant mechanism for imparting resistance against self-inactivation to LipM. The members of the PF03099 enzyme family have a strictly conserved lysine residue, the only totally conserved residue of the protein family (*14*). Loss of the charge of the conserved lysine side chain inactivates *M. tuberculosis* LipB (*13*), whereas the LipM mutant protein retained some activity (Figure 6). From the available structures of LplA and LipB proteins, it appears that this residue is near the reactive carbonyl of the substrate. This suggests it is important for stabilization of the oxyanion, as is the case in the mononucleotide binding fold (*43*). Other positively charged lysine and arginine side chains are in the vicinity of K165, which may explain why the LipM K165A mutant retains trace activity. The activity of the LipM K165R protein supports this notion because substitution of the longer positively charged side chain results in a protein with activity similar to that of the wild type protein.

It is interesting that LipM and LipB, together with the LplA-LplB bipartite lipoate ligase (*11*), share the property of anomalous migration in size exclusion chromatography. The three proteins all migrate as though they are approximately half of their known sizes. LipM elutes as a 14 kDa protein, whereas *E. coli* LipB elutes as though it were a 10 kDa protein (*29*, *44*). The most straightforward explanation of this unusual behavior is that these proteins interact with the chromatographic matrix within the bead and thus emerge from the matrix more slowly than expected [this behavior is independent of the salt concentration of the eluate (*44*)]. Given these data, it seems likely that both LipM and LipB are monomeric proteins, although confirmatory data obtained by another method (e.g., analytical ultracentrifugation) are clearly needed.

As discussed above, LipB octanoyltransferases and LplA lipoate protein ligases have similar structures and together with the biotin protein ligases make up the divergent enzyme family PF03099. The LipM type of octanoyltransferase provides a bridge between the LipB and LplA proteins, in that LipM has the activity of LipB but an amino acid sequence that resembles that of LplA. Although LipM is homologous with *E. coli* LplA in terms of sequence, it is a considerably shorter protein. The characterized LipB octanoyltransferases are composed of a single domain, whereas canonical lipoate protein ligases possess a catalytic domain of a size similar to that of LipB with a C-terminal accessory domain. Because nearly half of the sequences in the LplA subfamily contain only a single catalytic domain (*29*), it is tempting to designate all of these "short LplAs" as octanoyltransferases. However, the accessory domain can also be a separate protein as recently demonstrated for the *Thermoplasma acidophilum* lipoate ligase (*29*, *45*). Although the absence of an accessory domain indicates that the short proteins annotated as lipoate ligases lack lipoyl ligase activity (*29*), for any given protein it remains possible that the protein is inactive because of the loss of its accessory domain protein. In the known case of a bipartite lipoate ligase, the accessory protein is encoded by a gene adjacent to the LplA homologue (*29*). However, there is no reason that this must be the case (although it could simplify stoichiometric expression of the subunits). Indeed, there are putative bipartite ligases encoded by genes located far apart in the genome, such as in *Pyrococcus furiosus* (*29*).

The distinct phylogeny of LipM proteins should allow more correct prediction and annotation of genes currently annotated as encoding short lipoyl ligases. Because the octanoyltransferases and lipoate ligases perform different chemistry, octanoyltransferases and ligases could be distinguished by the octanoyltransferase active site cysteine and the neighboring residues. However, the residues that flank the active site cysteine residues of LipM and LipB show no similarity. Indeed, relative to LipB, the C150 region of LipM seems to reside on an inserted loop (Figure 8). As such, they are different octanoyltransferase types and must be considered separately.

The LipM and LipB octanoyltransferases can be added to the list of enzymes that have undergone convergent evolution functionally and mechanistically (*46*, *47*). As previously stated, these enzymes come from the same family and have a similar protein fold. Despite this, they have distinct primary structures indicating divergence at the sequence level. Therefore, it appears that octanoyltransferase activity has evolved from the same protein scaffold by two different paths. This highlights the multitude of evolutionary outcomes that are possible, which explains the rarity of true convergent evolution at the sequence level (*46*).

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