ENZYMATIC DIVERSITY IN LIPOIC ACID MODIFICATION OF PROTEINS

BY

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DISSERTATION

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ABSTRACT

Escherichia coli is one of a few organisms with a well characterized and complete metabolic model for lipoic acid metabolism. In this thesis I make discoveries through analysis of the diversity of lipoic acid metabolic genes in other organisms. By examining the distribution and phylogeny of the lipoate ligase protein family, I and others have found gaps and inconsistencies in our knowledge of lipoic acid metabolic pathways. I examine select proteins through heterologous expression in *E. coli*, allowing complementation studies and biochemical analysis. This enables prediction of lipoic acid metabolism in various organisms and reveals novel pathways and enzymology.

In the second chapter I investigate the lipoyl ligase from *Thermoplasma acidophilum*. Half of the members of the LplA family detected *in silico*, such as *Thermoplasma* LplA, have only the large catalytic domain. Two X-ray structures of the *T. acidophilum* LplA have been reported, although the protein was reported to lack ligase activity. McManus *et al.* hypothesized that the product of an adjacent gene was also required for ligase activity. I have shown this to be the case and have named the second protein LplB. I found that complementation of *E. coli* strains lacking lipoate ligase with *T. acidophilum* LplA was possible only when LplB was also present. LplA had no detectable ligase activity in *vitro in* the absence of LplB. Moreover LplA and LplB were shown to interact and were purified as a heterodimer. LplB was required for lipoyl-adenylate formation but was not required for transfer of the lipoyl moiety of lipoyl-adenylate to acceptor proteins. This provides a function for a previously cryptic protein family. Surveys of sequenced genomes show that most lipoyl ligases of the kingdom Archaea are heterodimeric. I propose that the presence of an accessory domain provides a diagnostic to distinguish lipoyl ligase homologues from other members of the lipoate/biotin attachment enzyme family. A survey of the lipoate protein ligase family reveals that half of the members lack a detectable accessory domain and therefore are likely to be catalyzing other reactions.

In the third chapter I find a new class of octanoyltransferase, LipM. LipM lack an accessory domain and is a non-homologous isozyme catalyzing the same reaction as

LipB. LipB is essential for lipoic acid biosynthesis in *E. coli*, but no obvious orthologue could be found in *Bacillus subtilis*. Using a cosmid library approach, I isolate 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. I 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 are the only known type of octanoyltransferase present in 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.

In the fourth chapter we characterize three lipoyl ligase homologues from *B. subtilis*, one of them being LipM. I performed this in collaboration with a fellow graduate student Natalia Martin from the laboratory of Diege de Mendoza, who performed molecular biology work in *B. subtilis*. The *B. subtilis* genome encodes three lipoyl ligase homologues: *yhfJ, yqhM,* and *ywfL,* which we have renamed *lplJ, lipM,* and *lipL*, respectively. We show that *lplJ* encodes the sole lipoyl ligase of this bacterium. Physiological and biochemical characterization of a Δ*lipM* strain showed that LipM is absolutely required for the endogenous lipoylation of all lipoate-dependent proteins, confirming its role as the *B. subtilis* octanoyltransferase. However, we also report that in contrast to *E. coli, B. subtilis* requires a third protein for lipoic acid assembly, LipL. *B. subtilis ΔlipL* strains are unable to synthesize lipoic acid despite the presence of LipM and LipA, which based on the *E. coli* model should suffice for lipoic acid biosynthesis. LipM is only required for the endogenous lipoylation pathway, whereas LipL also plays a

role in lipoic acid scavenging. Expression of *E. coli lipB* allows growth of *B. subtilis* Δ*lipL* or Δ*lipM* strains in the absence of supplements. In contrast, growth of an *E. coli* Δ*lipB* strain can be complemented with *lipM*, but not *lipL*. These data demonstrate that LipM and LipL are both required for lipoyl domain octanoylation and hence lipoic acid biosynthesis. Unexpectedly, *B. subtilis* requires an additional gene for lipoic acid biosynthesis.

In Chapter Five I characterize the product of this extra gene and discover a novel enzyme used in lipoic acid biosynthesis. This new enzyme lacks an accessory domain and is the third *B. subtilis* lipoyl ligase homologue, LipL. I report that LipM specifically modifies the glycine cleavage system protein, GcvH, and therefore another mechanism must exist for modification of other lipoic acid requiring enzymes (*e.g.*, pyruvate dehydrogenase). I show that this function is provided by LipL which catalyzes the amidotransfer (transamidation) of the octanoyl moiety from octanoyl-GcvH to the E2 subunit of pyruvate dehydrogenase. LipL activity was demonstrated *in vitro* with purified components and proceeds via a thioester-linked acyl-enzyme intermediate. As predicted, *ΔgcvH* strains are lipoate auxotrophs. LipL represents a new enzyme activity. It is a GcvH:[lipoyl domain] amidotransferase that probably employs a Cys-Lys catalytic dyad. Although the active site cysteine residues of LipL and LipB are located in different positions within the polypeptide chains, alignment of their structures show these residues occupy similar positions. Thus, these two homologous enzymes have convergent architectures.

I Chapter Six I summarize my findings, provide a personal narrative to the work presented, and discuss the implications of the research presented. This Thesis not only expands on previous knowledge but also defines new paradigms in lipoic acid metabolism. This is especially true for the novel lipoylamidotransferase, which is an enzyme that has been overlooked through over fifty years of lipoic acid research. I also suggest further avenues for research resulting from examination of the phylogeny and distribution of lipoic acid metabolic enzymes. Further study of lipoylamidotransfer will define the role of LipL orthologues in pathogenic microorganisms. Also, I predict that a

lipoylamidotransferase isozyme is present in yeast and humans based on LplA family phylogeny and research from other labs. Overall, the work in this Thesis provides many avenues for further investigation and will improve our ability to understand and predict central metabolism in diverse organisms.

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"Hard work on interesting problems is enjoyable and preferable to aimless wasting of leisure time. It may also lead to unexpected findings that give insights into important related problems. Such unexpected findings–sometimes called "luck"–frequently happen to the active researcher, but only rarely to those who prefer talk to study and work. So one should study and work hard, on interesting problems of any nature, with the purpose of explaining nature and helping others."

- A message to students by Esmond E. Snell while at Osaka in 1971.

I am indebted to so many people for completing this thesis. Fortunately I think they will not collect. I hope that I one day I will repay them by helping others on their journey for greater understanding. I thank my PhD advisor, John Cronan, who has patience and generosity as deep as his knowledge of the physiology of *Escherichia coli*. I have also had the privilege to learn by his example the approach and attitude conducive to new discoveries. I thank my committee and other faculty for their words of wisdom. I have reflected on them on many occasions, which hopefully you find evident in this thesis. Thanks to my undergraduate advisor Michael Thomas and his wife Michelle Rondon for showing me the endlessly fascinating world of bacteriology and taking the time to teach a clueless undergrad the basics. I have benefited from quite a few caring and inspiring teachers throughout my life and I thank them. I want to thank my family for nurturing and guiding my quest for greater insight. Finally I thank my wife, Dana, for keeping me sane and sharing these lovely years with me.

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

THE DISCOVERY OF LIPOIC ACID

The discovery of lipoic acid was made through careful study of microbial metabolism. Lipoic acid was one of the last cofactors discovered at the end of the "vitamin era" (Carpenter, 2003). It was also covalently bound to protein and not very abundant. As a result, lipoic acid was independently discovered three times before its isolation. It was first discovered by Esmond E. Snell as a graduate student in W.H. Peterson's lab at the University of Wisconsin. He found that *Lactobacillus delbrueckii* grew poorly on a peptone-glucose-salts medium unless potato extract was also added. This factor could be partially extracted by ether. It could be replaced by acetate and so was called "acetate-replacing factor". Further studies using defined media with acetate were performed by various labs to study bacterial nutrition, and acetate-replacing factor fell by the wayside (Snell *et al.*, 1937; Snell, 1993). It was discovered a second time from the effort of multiple labs as a growth factor II for *Tetrahymena geleii* (Kidder *et al.*, 1945) and was later renamed "Protogen" (Stokstad *et al.*, 1949). The last discovery was by Dan O'Kane, a graduate student in the laboratory of Irwin C. Gunsalus. They discovered a factor for *Enterococcus (Streptococcus*) *faecalis* required for pyruvate oxidation; the factor was aptly named "pyruvate oxidation factor" (O'Kane *et al.*, 1947). This factor was found to be tightly bound but present in a variety of substances, and was found in acetate-replacing factor as well (Gunsalus, 1984). Shortly after this, acetate-replacing factor was investigated further by a graduate student in the Snell lab and the project was eventually given to Lester Reed, who was a friend and colleague of Snell at the University of Texas. Both the laboratory of Irwin Gunsalus and Lester Reed found purification of sufficient factor difficult, and both independently collaborated with Eli Lilly to obtain sufficient material. Both labs agreed to merge projects at the suggestion of Eli Lilly. Finally about 30 mg of lipoic acid was obtained from an estimated 10 tons of liver residue. In hindsight it is no wonder Lester Reed's lab could not isolate it from 6 L

bacterial cultures. The trivial name "α-lipoic acid" was proposed by Reed due to its lipophilicity and it being a carboxylic acid. Reed designated the compound "alpha" in order to differentiate it from a second isolated compound with a sulfur oxidized to the sulfoxide, which was designated "β-lipoic acid" (Reed *et al.*, 1951; Reed, 2001). Further characterization was made in a collaborative effort between Gunsalus, Reed and chemists at Eli Lilly who proved the natural isolate contained an aliphatic chain of eight carbons and two sulfur atoms (one of which was terminal), was acidic, and by polarimetry was of the dextro- (+)-configuration (Reed *et al.*, 1953). Different positions for sulfur attachment were possible candidates for the correct structure for RLA, depending on attachment of sulfur at C4, C5 or C6, which were designated 4-, 5-, or 6-thioctic acid. Synthesis of the racemic compound proved the tentative assignment of 6-thioctic acid had been correct (Bullock *et al.*, 1952). While the optical activity of biological lipoic acid was known as (+), the configuration was found to be the *R* enantiomer (Mislow *et al.*, 1956). This was later supported by synthesis of the *S* enantiomer (Brookes *et al.*, 1983) (Figure 1-1). The discovery and characterization of lipoic acid led to elucidation of the the mechanism of lipoic acid dependent complexes, and was followed by understanding of the critical role of lipoic acid in aerobic metabolism.

LIPOIC ACID DEPENDENT COMPLEXES

Lipoic acid was discovered as a factor necessary for pyruvate metabolism, and it is in this capacity that it has been most studied. The pyruvate dehydrogenase (PDH) complex is the only enzyme capable of energy-conserved decarboxylation of pyruvate in an aerobic environment (Figure 1-2). Generally lipoate biosynthesis is not required anaerobically, because oxygen-labile ferredoxin oxidoreductases, such as pyruvate-formate lyase, can be used instead (Buckel *et al.*, 2006). The PDH serves as a model for related complexes, which have similar structures. PDH is composed of three different enzymes which are designated E1-3 in order of decreasing molecular weight, which also corresponds to the reaction order (Figure 1-3A). The E1 is a thiamine diphosphate-

dependent decarboxylase (EC 1.2.4.1), that catalyzes the decarboxylation of pyruvate and the reductive acetylation of protein bound lipoic acid. Lipoic acid is covalently attached to the ε-amino group of a conserved lysine of the lipoyl domain (LD). The lipoyl domain is typically found at the N terminus of the E2 (EC 2.3.1.12) catalytic subunit, which is a dihydrolipoyl acetyltransferase that transfers the acyl group to CoA. The E3 dihydrolipoamide dehydrogenase (EC 1.8.1.4) regenerates the disulfide bond in the lipoyl group and transfers the electrons to NAD+ via a flavin cofactor. This prepares the lipoyl group for another round of catalysis. The energy from decarboxylation is conserved by thioester formation and through formation of NADH (Perham, 1991; Perham, 2000; Reed, 2001) (Figure 1-3A).

2-Oxoacid and acetoin dehydrogenase (ADH) complexes have a central E2 core and the other subunits surround this core to form the complex. In the space between these two subunits, the LD ferries intermediates between the active sites. The geometry of the PDH E2 core, which doesn't include lipoyl domains, can either be cubic or dodecahedral depending on the organism and complex. The LD itself is attached by a flexible linker typically to the E2 core and the number of LDs varies from one to three for a given organism and complex. The number of domains has been experimentally varied in *E. coli* PDH. This seemed to have little effect on growth until over nine are present, although the presence of more than three decreased activity of the complex (Perham, 2000). The cloned LD is based on the catalytically competent product of proteolyzed purified complexes, which was determined for *Bacillus stearothermophilus* and *E. coli* (Packman *et al.*, 1987; Packman *et al.*, 1988).

The 2-oxoglutarate dehydrogenase and branched-chain keto-acid dehydrogenase (BDH) complexes closely resemble the PDH complexes. They are comprised of three subunits, and the E3 or lipoamide dehydrogenase for all these complexes can orginate from the same gene. The PDH and ODH are critical components for a functioning citric acid cycle due to PDH supplying acetyl-CoA and ODH itself being an enzyme in the cycle (Figure 1-2). PDH-derived acetyl-CoA is also required as a substrate for fatty acid biosynthesis. As such, lipoic acid is required for aerobic

respiration in *E. coli* and other organisms (Cronan *et al.*, 2005). The BDH is widely used in catabolism of isoleucine, leucine, and valine. BDH performs this role in organisms from all three domains of life (Massey *et al.*, 1976; Brosnan *et al.*, 2006; Sisignano *et al.*, 2010). In bacteria that synthesize branched fatty acids, the BDH is required for synthesis of branched-CoA starting units derived from amino acids. Branched fatty acids are used to regulate membrane fluidity, disruption of which results in a defect in virulence and tolerance to environmental stress (Kaneda, 1991; Keeney *et al.*, 2009). BDH is also required for secondary metabolite biosynthesis in some bacteria (Chan *et al.*, 2009).

Lipoic acid is also required for catabolism of acetoin (3-hydroxy-2-butanone or acetylmethylcarbinol) by the acetoin dehydrogenase (ADH) complex (EC 2.3.1.190) (Figure 1-2). Acetoin is produced as a fermentation product by certain microbes and is produced by decarboxylation of alpha-acetolactate (Xiao *et al.*, 2007). Enzymatic condensation of acetaldehyde with pyruvate to form acetoin is possible, although little has been done to determine the physiological relevance of this pathway (Neuser *et al.*, 2000; Xiao *et al.*, 2007). Yeast pyruvate decarboxylase appears to be good at this reaction (Neuser *et al.*, 2000). Condensation products of acetoin can be oxidized to diacetyl or reduced to 2,3-butanediol, which is of particular interest to the brewing industry (Ostergaard *et al.*, 2000). The production of acetoin has been used as an identification criterion using the Voges-Proskauer test (Eddy, 1961). Acetoin and related C4 compounds are not acidic and are advantageous during stationary phase compared to acetic acid production. This has been well studied in *Vibrio cholerae*, where different fermentation strategies are proposed to be responsible for the dominance of El tor strain over the classical strain (Yoon *et al.*, 2006; Pradhan *et al.*, 2010). The acetoin dehydrogenase (ADH) is homologous to the PDH complex and catalyzes a similar reaction except ADH produces acetyl-CoA and acetaldehyde (Figure 1-2). Although other pathways for utilization of acetoin have been proposed, acetoin dehydrogenase is the only one proven so far (Xiao *et al.*, 2007).

The glycine cleavage system (also called glycine decarboxylase and glycine dehydrogenase) is significantly different from the other complexes, and accordingly has

a different nomenclature. The glycine cleavage system is technically not a complex, as all the component proteins dissociate easily and behave as independent proteins. The H protein, GcvH, is a free LD similar to E2 subunit lipoyl domains except the H protein contains additional helices. The GcvH lipoyl domain, unlike other lipoyl domains, exists as a free protein. The L protein, GcvL, is a dihydrolipoamide dehydrogenase (EC 1.8.1.4) which can also be replaced by the E3 lipoamide dehydrogenase, *lpd*, in some organisms such as *E. coli*. The P protein, GcvP, is the PLP dependent glycine decarboxylase (EC 1.4.4.2), which transfers methylamine from PLP to lipoyl-GcvH. The T protein is an aminomethyltransferase (EC 2.1.2.10) that transfers methylamine from lipoate to tetrahydrofolate (THF), yielding methylene-THF and ammonia. Methylene-THF can then be used by serine hydroxymethyltransferase in a reversible reaction to synthesize serine from glycine, although the glycine cleavage system itself is not reversible (Douce *et al.*, 2001) (Figure 1-2). This system is used in many organisms and plays a crucial role in the photosynthetic carbon cycle. In *Escherichia coli* the glycine cleavage system is not required for growth of wild type *E. coli* strains (Plamann *et al.*, 1983), but glycine cleavage is required for growth of *Arabidopsis* (Engel *et al.*, 2007) where the H protein can be present at millimolar concentrations in chloroplasts (Bauwe *et al.*, 2003).

From the large amount of research summarized above, it is apparent that lipoic acid is indeed a central cofactor for aerobic life. All of the complexes mentioned above play important roles in degredation and carbon source utilization. In particular, the pyruvate and 2-oxoglutarate dehydrogenase are essential components of the citric acid cycle. The pyruvate and branched chain dehydrogenase also provide essential substrates for biosynthesis of fatty acids and other molecules. The diverse roles of lipoic acid dependent complexes are due to their role in central metabolic processes; this is why lipoic acid is important for aerobic life.

LIPOIC ACID AS A DIETARY SUPPLEMENT

Considerable attention has been given to the effects of lipoic acid as a dietary supplement; the effects of which are currently speculated to occur by thiol oxidation

and reduction. Although it has been uncertain if free lipoate can affect thiol oxidation-reduction (redox) in a physiologically relevant way, recent studies have promising results. Lipoic acid has been considered as a remedy to a variety of ailments beginning shortly after its discovery. Due to its role in metabolism and abundance in mammalian liver, it was the subject of a variety of studies with questionable controls and relevance. An early study finds that some rats on a diet of B vitamins prefer lipoic acid to ethanol, although liver was preferred even more (Mardones *et al.*, 1954). It was first administered to humans by injection to 63 patients suffering from various diseases and 32 of them responded well to lipoic acid. These patients had a sense of "well-being" and one subject woke from a vegetative coma after being administered lipoic acid. In the same report it was suggested that some people suffer from a deficiency of lipoic acid (Rausch, 1955). Addition of lipoic acid to liver slices increased metabolism of glucose and pyruvate (without addition of another reducing agent) (Bornstein *et al.*, 1955). Later these physiological effects were considered to be due to various biochemical properties making it an "antioxidant" (when lipoic acid acts as reductant). Lipoic acid has been called this because of the ability of reduced dihydrolipoate to reduce thiols, to scavenge reactive oxygen species, and to chelate metals in biochemical assays. More recent studies have also demonstrated it can also act as a "prooxidant", so perhaps it is best to consider lipoic acid as neither an antioxidant nor prooxidant (Moini *et al.*, 2002).

Lipoic acid has a short lifetime when injested or injected in mammals; this casts doubt on the hypothesis that lipoic acid consumption directly affects physiology. Through the use of lipoic acid radiolabeled at carbons 7 and 8, it was found that over 70% of lipoic acid and its degradation products are excreted within 24 hours by various mammals (Schupke *et al.*, 2001). In humans, lipoic acid is rapidly metabolized; being present in plasma for less than an hour, while only 0.2% is excreted unmodified (Teichert *et al.*, 2003). With such a short lifetime, it is doubtful that biochemical studies of free lipoic acid reactions have physiological relevance. Studies determining if the metabolites of lipoic acid have any effect on cell physiology may yield insight.

Instead of a direct catalytic effect of lipoic acid, recent evidence supports lipoic acid affects cellular regulation. No studies have provided data supporting a mechanism for this effect, although there are changes in the levels of the regulators NRF2 and NF-κB due to lipoic acid supplementation. It is clear that the transcription of glutathione-S-transferase is increased after administration of lipoic acid, providing a possible mechanism for effects attributed to lipoic acid reduction. It has been proposed that regulatory changes are due to changes in the redox state of the cysteine residues of regulator proteins, but thus far no evidence supports this idea (Shay *et al.*, 2008; Shay *et al.*, 2009). It is interesting to note that peak NRF2 induction occurs 24 hours after injection of 40 mg/kg lipoic acid. The long response time suggests NRF2 induction is indirect and may be the result of a lipoyl degradation product (Suh *et al.*, 2004). A technical issue with the use of lipoic acid as a supplement is the large dose required: at least 600 mg daily (Moini *et al.*, 2002; Shay *et al.*, 2008; Shay *et al.*, 2009). Uncertainty about the mechanism of action is a significant barrier to further insight into the physiological effects and therapeutic benefits of lipoic acid.

THE EMERGING ROLE OF LIPOIC ACID IN HYDROPEROXIDE REDUCTION

Lipoic acid is typically a cofactor that participates in oxidative metabolism, but recent findings demonstrate its role in reductive metabolism as well (Figure 1-3B). Certain types of lipoamide dehydrogenases, or E3 subunit proteins, are present in microorganisms, without the other components of the multienzyme complexes, and are also membrane associated (Danson *et al.*, 1987). These less common E3 proteins were found to have an attached N-terminal lipoyl domain and were found to be a surface antigen in *Neisseria meningitides* (Li de la Sierra *et al.*, 1997). Lipoyl-domain-containing E3 proteins, or Lpd, were found to be a virulence factor first in *Streptococcus pneumoniae*, deletion mutants being deficient in carbohydrate metabolism (Smith *et al.*, 2002). An orthologous gene was shown to be important to virulence of *Mycoplasma gallisepticum* (Hudson *et al.*, 2006). The physiological role of these orphan E3 proteins

has yet to be established, although they would be ideal participants in reductive metabolism by lipoic acid.

The function of Lpd was uncertain until the recent discovery of a lipoic acid dependent peroxidase, Ohr, from *Xyllela fastidosa* (Cussiol *et al.*, 2010). This peroxidase was dependent on dithiol reduction by lipoic acid, which could not be done with thioredoxin, glutaredoxins, or glutathione. Catalytic production of reduced lipoic acid required a lipoamide dehydrogenase as well. Cussiol *et al.* extend these finding to a related protein from *E. coli*, OsmC, which is periplasmic. Both *ohr* and *osmC* mutants have been shown to be sensitive to hydroperoxides (Mongkolsuk *et al.*, 1998; Conter *et al.*, 2001), so lipoic acid appears to be indirectly involved in scavenging of hydroperoxide. An ongoing study in the laboratory of Jon Beckwith also has evidence that lipoic acid can serve as a cellular reductant in *E. coli* in the absence of thioredoxin and glutathione (Beckwith, 2009). Reductive metabolism by lipoic acid is a new discovery so much more work is required to understand how widespread these systems are and their physiological relevance.

DEGRADATION AND SCAVENGING OF LIPOIC ACID

The discovery of lipoic acid depended on the study of natural auxotrophs which could be grown without it. This facilitated further study into utilization of lipoic acid, or the relevant basic form lipoate. Reed *et al.* found that Lipoic acid was found to be utilized via lipoate-protein ligase (EC 2.7.7.63) (Reed *et al.*, 1958). Using partially purified protein from *E. faecalis* and *E. coli* they demonstrated ligation proceeded via a lipoyl-adenylate intermediate with production of pyrophosphate (Figure 1-4). Strangely, *E. faecalis* lipoyl ligase could be separated into two fractions, whereas the *E. coli* ligase could not. One fraction was responsible for adenylation while the other was required for transfer and could be destroyed by trypsin (Reed *et al.*, 1958). Two forms of lipoic acid ligase were later purified from *E. coli* (Brookfield *et al.*, 1991), but this appears to be an artifact of the purification procedure as only one form was observed in a later study (Green *et al.*, 1995). The existence of a lipoyl ligase findings were extended to

Azotobacter vinladii and *Phaseolus radiates* (mung bean), which also contained a lipoyl ligase capable of adenylation and transfer (Mitra *et al.*, 1965).

The gene encoding a lipoate:protein ligase, *lplA*, was first isolated from *E. coli* (Morris *et al.*, 1994). LplA was the first such ligase purified to homogeneity. Assays with a fully defined system have demonstrated that LplA with lipoate and Mg-ATP are sufficient to reconstitute lipoylation *in vitro* (Morris *et al.*, 1994; Green *et al.*, 1995). Strains with *lplA* disrupted showed that LplA does not play a role in lipoic acid biosynthesis but rather acts to scavenge lipoic acid from the environment (Morris *et al.*, 1994; Morris *et al.*, 1995). This conclusion was further validated by isolation of spontaneous mutants of *lplA* conferring resistance to the toxic analog selenolipoate (Reed *et al.*, 1994).

In addition to selenolipoate, *E. coli* lipoate:protein ligase is able to use various alternative acyl substrates with reduced effeciency. It is also able to use octanoate, which is equivalent to the acyl chain of lipoic acid (Brookfield *et al.*, 1991; Green *et al.*, 1995; Hermes *et al.*, 2009). Purified lipoic acid ligase from *E. coli* was shown to use 8-methyllipoate as a substrate, but to have reduced activity with (S)-lipoic acid (Brookfield *et al.*, 1991). *E. coli* lipoic acid auxotrophs are reported to have restored growth on analogs of different lengths as well as on the S isomer. Contamination by trace amounts of R-lipoate is a concern, as all analogs required higher concentrations. Incorporation of the shorter bisnorlipoic (Figure 1-1) acid was confirmed by selective antibody (Loeffelhardt *et al.*, 1996). Incorporation of (R)-[S^{35}]-lipoic acid is significant in *E. coli*, while no incorporation of (S)-[S^{35}]-lipoic acid was detected (Oehring *et al.*, 1992). Capitalizing on lipoyl ligase promiscuity, mutant lipoyl ligases have been developed to use fluorescent substrates for protein labeling studies (Uttamapinant *et al.*, 2010).

Due to the role of lipoic acid in central metabolism, it is not surprising that it is required for virulence. This was first proven by identifying a lipoyl ligase of *Listeria monocytogenes*, *lplA1*. As the name suggests another ligase homologue, *lplA2*, was also apparent. LplA1 was required for intracellular growth but not growth on rich media (O'Riordan *et al.*, 2003). Lipoic acid scavenging has also been shown to be required for

virulence in acomplexan parasites *Toxoplasma gondii* and *Plasmodium falciparum* (Crawford *et al.*, 2006; Allary *et al.*, 2007). The presence of small lipoyl peptides was detected in cytosol and *lplA1* is required for efficient utilization of these, although the mechanism for their utilization is unclear (Keeney *et al.*, 2007). The loss of BDH and the resulting change in membrane composition are responsible for the avirulence of *lplA1* mutants (Keeney *et al.*, 2009; Sun *et al.*, 2010). These studies establish scavenging of host lipoic acid as a general pathogen strategy. Also, they reveal scavenging of lipoic acid is more complex than previously thought.

Lipoic acid is synthesized while covalently attached to protein and yet lipoate:protein ligases are widely distributed. In the process of testing various lipoylamide compounds as inhibitors of lipoyl ligation, it was observed that *E. faecalis* could use these compounds as a source of lipoic acid, which was dependent on ATP. This led to the discovery and characterization of a lipoyl-X-hydrolase (Reed *et al.*, 1958), which was also capable of cleaving lipoic acid from lipoyl domains and so was renamed lipoamidase. It appeared in this study that lipoamidase activity was present in two separate fractions, suggesting there may be two hydrolases (Suzuki *et al.*, 1963). This was also seen in a later purification of lipoamidase (Liu *et al.*, 1995). No lipoamidase activity was found in *E. coli* (Reed *et al.*, 1958). Also, no exchange of lipoate was seen after five hours in pulse-chase experiments with *E. coli* suggesting lipoyl domains aren't degraded appreciably during exponential phase in this organism (Griffith *et al.*, 1974). Lipoamidase activity was also found in extracts from many different mammalian tissues. When measured, these enzymes were capable of hydrolyzing a variety of substrates making their physiological role of the enzyme uncertain (Oizumi *et al.*, 1997). Through a cosmid library approach, the gene encoding lipoamidase, *lpa*, was isolated and identified from *Enterococcus faecalis*. Pure Lpa also had some biotinidase activity with biotinyl-lysine, but less than lipoamidase activity (Jiang *et al.*, 2005). *L. monocytogenes* has been shown to be able to utilize lipoamide and smaller lipoyl-peptides, but not intact lipoyl-domains. This ability is dependent on one of two lipoyl ligases (LplA1), but the mechanistic details are unknown (Keeney *et al.*, 2009).

Little is known about the degradation and utilization of aliphatic sulfides such as lipoic acid, with the exception of cysteine. A strain of *Pseudomonas putida* was isolated that could use lipoic acid as a sole carbon, sulfur, and energy source. An abundant intermediate was the shorter bisnorlipoic acid (Shih *et al.*, 1975). Oxidative products lipoic sulfinate and bisnorlipoic sulfinate were also detected, which may be intermediates of sulfur utilization (Furr *et al.*, 1978). A selection for mutants of *Bacillus subtilis* which require lipoic acid led to the identification of 3'-phosphoadenosine 5'-phosphosulfate sulfotransferase, CysH, which was required for sulfate utilization. This study demonstrated that lipoic acid can serve as a sulfide or sulfite source in this organism (Mansilla *et al.*, 1997). Although fatty acid degradation enzymes are likely involved, gene products responsible for use of lipoic acid as a sulfur source are unknown. Lipoic acid is metabolized in a variety of ways when given as a dietary supplement in mammals (Schupke *et al.*, 2001). Lipoic acid is partially degraded by a variety of transformations, which can occur in various combinations. Degradation to tetranorlipoic acid, oxidation of one or both of the sulfur atoms to the sulfoxide, and S-methylation of the sulfide were observed. Conjugation of unmodified lipoic acid to glycine was detected especially in mice (Schupke *et al.*, 2001). This is interesting because lipoic acid has been found to competitively inhibit benzoic acid excretion in rats, which occurs via conjugation to glycine (Gregus *et al.*, 1996). Degradation of lipoic acid is similar in humans, although it is not clear if the sulfur atoms become significantly oxidized (Teichert *et al.*, 2003). Apparently mammals are not capable of utilizing the dithiolane moiety as a sulfur source.

The mechanism of transport of lipoic acid is uncertain. Studies of lipoic acid transport in *E. coli* find that transport is not saturable at a physiologically relevant concentration (Oh *et al.*, 1969; Griffith *et al.*, 1983). Data of transport rates at different concentrations of lipoic acid are lacking. In two separate selections for lipoic acid utilization, no mutants of *E. coli* have been found that are unable to take up lipoate from the media (Reed *et al.*, 1994; Morris *et al.*, 1995). This suggests that in bacteria lipoate is sufficiently membrane permeable, or that it uses multiple transporters. However,

lipoic acid is transported across an epithelial-cell-culture layer in a proton-dependent manner competitive with octanoic acid (Takaishi *et al.*, 2007). A sodium dependent transporter was identified that also transports biotin and pantothenate. As this transporter is similar to many other transporters, including *E. coli* PanF, it is not clear which are used for lipoic acid transport (Prasad *et al.*, 1998). It appears that transport may be important in Eukarya, but not in bacteria. Clearly more work remains to understand lipoic acid transport.

LIPOIC ACID BIOSYNTHESIS

Although scavenging of lipoic acid was explored after the discovery of lipoic acid, its biosynthesis remained a mystery. Octanoate was shown to serve as a precursor to lipoic acid in *E. coli*. The sulfur atoms were exchanged for protons at carbons 6 and 8 without loss of protons at carbons 5 and 7, suggesting a direct replacement without an unsaturated intermediate (Parry, 1977). Introduction of the sulfur at carbon 6 proceeded with inversion of configuration (Parry *et al.*, 1978; White, 1980a; White, 1980b). A multiple step insertion was proposed with hydroxylation as an intermediate, as direct sulfur insertion was unprecedented (Parry *et al.*, 1978). Precursor feeding studies suggested direct sulfur insertion, as 8- and 6-hydroxyoctanoate could not be used for biosynthesis but 8- and 6-thiooctanoate could. 6-thioocanoate yielded 10-20% less lipoic acid than 8-thiooctanoate (White, 1980a). Injection of various radiolabeled compounds into rats and determination of the amount of lipoic acid labeled demonstrated that cysteine and octanoate were the most direct carbon and sulfur precursors for lipoic acid biosynthesis (Dupre *et al.*, 1980).

Mutants of *E. coli* that required lysine and methionine for growth were also found to grow with supplementation of acetate and succinate or lipoic acid. Supplementation was found to not be required anaerobically (Vise *et al.*, 1967). Selection for mutants of *E. coli* requiring acetate and succinate identified mutants that also responded to lipoic acid. Multiple mutations mapped to a single locus designated *lip* (Herbert *et al.*, 1968). It is still not clear why supplementation with lysine and

methionine allows growth without active pyruvate dehydrogenase. It was suggested that pyruvate oxidase may supply sufficient acetate (Vise *et al.*, 1967). Large innocula are required for lysine and methionine bypass, suggesting nutrient carryover may contribute to bypass (Herbert *et al.*, 1968). Expression of a subgene encoding the PDH lipoyl domain in a *lip*$^-$ strain resulted in octanoylation at the same lysine as lipoylation, suggesting it may be a biosynthetic intermediate (Ali *et al.*, 1990). The *lip* locus was identified by transposon mutagenesis (Vanden Boom *et al.*, 1991) and cosmid library complementation (Hayden *et al.*, 1993). The locus was found to contain two genes required for lipoic acid biosynthesis: *lipA* and *lipB*. The previously described *lip* mutants were found to be in the *lipA* gene (Vanden Boom *et al.*, 1991). The *lip* locus was also identified in *Salmonella typhimurium* using the Mu *d*J transposon creating a *lacZ* fusion. The expression of this locus was unchanged under a variety of conditions, suggesting expression is constituitive (Smith *et al.*, 1991).

Initially both LipA and LipB were purified but found to be inactive. The translation start site for *lipA* was found to be 40 codons upstream from where it was originally annotated (Reed *et al.*, 1993). Point mutants of *lipA* were found to be partially or fully deficient in the use of 6 and 8-thiooctanoate, indicating that LipA was responsible for sulfur insertion (Hayden *et al.*, 1993). LipB and LplA appeared to have somewhat redundant roles in lipoic acid biosynthesis. Overexpression of *lplA* bypassed the requirement for *lipB,* while the converse did not restore growth (Morris *et al.*, 1995). This and the inexplicable purification of two forms of ligase (Brookfield *et al.*, 1991) caused confusion resulting in LipB being incorrectly called lipoate protein ligase B (Kim do *et al.*, 2005; Gunther *et al.*, 2007). Enzymatic activity of LipB was elusive until it was determined that translation started at a TTG codon upstream of the predicted ATG start codon. LipB was shown to be a lipoyl (octanoyl)-Acyl Carrier Protein:Protein Transferase (EC 2.3.1.181). LipB was shown to be inhibited by octanoyl-CoA, and had a similar reaction rate with octanoyl-ACP; but had tenfold less affinity than with lipoyl-ACP (Nesbitt *et al.*, 2005). LipB was shown to proceed via a thioester intermediate with a conserved cysteine (Zhao *et al.*, 2005). The structure of the *M. tuberculosis* LipB

suggests a conserved lysine interacts with the cysteine nucleophile. The crystal structure was only obtained with a covalent decanoyl thioether, presumably from *cis*-3-decanoate (Ma *et al.*, 2006). The structure of *Thermus thermophilus* LipB was obtained as a covalent dimer linked by the conserved cysteine active site residues (Kim do *et al.*, 2005; Kim do *et al.*, 2008). Attempts to crystalize *E. coli* LipB have failed, presumably due to the flexible nature of the enzyme which was seridipitously overcome in currently solved structures.

It was noticed that LplA was able to complement a LipB mutation when overexpressed, but the reason was not known (Morris *et al.*, 1995). Low transferase activity was also detected with LplA (Jordan *et al.*, 2003). This transferase activity was likely due to trace amounts of octanoate in the preparation of octanoyl-ACP, evident by the extremely low rate. It was determined *lplA* mutations conferring increased affinity were responsible for the most common class of *lipB* supressors. This demonstrated that LplA's ability to bypass LipB depends on ligation of trace amounts of intracellular free octanoic acid, not transferase activity (Hermes *et al.*, 2009). Because of this phenomenon, use of an *E. coli lipB (*or *lipB lplA*) strain to demonstrate complementation does not distinguish well between an octanoyltransferase or lipoyl ligase.

LipA was purified and shown to be an iron-sulfur protein similar to biotin synthase (Busby *et al.*, 1999; Ollagnier-de Choudens *et al.*, 1999; Ollagnier-De Choudens *et al.*, 2000). LipA was found have lipoyl synthase activity (EC 2.8.1.8). It inserts sulfur atoms into PDH-bound octanoate using S-adenosylmethionine as a substrate to generate a radical electron (Miller *et al.*, 2000). Coexpression of the *isc* system increased LipA yield suggesting it is involved in LipA iron sulfur cluster assembly (Kriek *et al.*, 2003). It was unclear whether LipA or LipB came first in the biosynthetic pathway until biochemical assays of LipA demonstrated it preferred octanoyl-lipoyl domain over octanoyl-ACP as a substrate (Zhao *et al.*, 2003). The thioester intermediate attached to LipB after purification is octanoate and no lipoic acid was detected (Hassan *et al.*, 2011). This further supports octanoyl-ACP as the physiologically relevant substrate for LipB, confirming that lipoic acid is synthesized on requiring enzymes (Figure 1-4). LipA was

also shown to be active using octanoyl-GcvH and uses two molecules of S-adenosylmethionine (Cicchillo *et al.*, 2004). The enzyme contains two 4Fe-4S clusters, one having a novel motif (Cicchillo *et al.*, 2004). Both sulfur atoms are derived from the same enzyme molecule (Cicchillo *et al.*, 2005). The enzyme proceeds in a stepwise manner, first inserting the sulfur at position 6, then 8 (Douglas *et al.*, 2006). The structure of LipA is unsolved, but efforts in at least two labs are underway.

LIPOIC ACID METABOLISM IN EUKARYA

The discovery and elucidation of lipoic acid metabolism was largely achieved in bacteria. This knowledge has been extended to understand lipoyl metabolism in eukarya. The compartementalization of cells into organelles and differentiation of cells into different tissues makes the study of lipoic acid metabolism technically and theoretically more complicated. Opportunites for further study exist in these areas, and emerging evidence suggests new enzymes are on the horizon.

Orthologues of lipoic acid ligase from mammals differ from the bacterial counterparts. The bovine orthologue was purified and shown to be unable to adenylate lipoic acid, but was able to use various adenylates as substrates for lipoyl domain modification, and so is called lipoyltransferase (Fujiwara *et al.*, 1994; Fujiwara *et al.*, 1997). As expected, the human lipoyltransferase orthologue had similar properties. Shortly after the discovery of the bacterial lipoate protein ligase, adenylation of lipoic acid was detected from beef liver homogenate, and lipoate-activating enzyme was partially purified. Interestingly, no activity was found from heart homogenate (Tsunoda *et al.*, 1967). Recently this enzyme was purified from mitrochondria, and although it had a higher affinity for ATP, it had a better rate with GTP as a substrate (Fujiwara *et al.*, 2001). Lipoyl-activating enzyme is the same enzyme as a xenobiotic acyl-CoA ligase (Vessey *et al.*, 1995), whose substrate preference can vary greatly depending on monovalent ion concentrations (Vessey *et al.*, 2000). Expression of lipoyl activating enzyme was shown to vary in different cell lines. Also, incorporation of lipoic acid and other analogs into PDH is possible in cell free assays (Walden *et al.*, 2008). Endogenous

lipoic acid biosynthesis is required for embryogenesis in mice. The *lipA* homologue, *LIAS*, was disrupted in mice, and homozygous progeny perished during embryogenesis. Injection of lipoic acid failed to allow growth of embryos (Yi *et al.*, 2005). This shows lipoic acid scavenging does not occur in the embryo, although it does not rule out lipoic acid scavenging by adults. Although much is known about lipoic acid metabolism in mammals, direct evidence for lipoic acid scavenging is lacking.

The lipoic acid metabolic enzymes in plants appear to be similar to those characterized in *E. coli*. A homolog of *E. coli lipB*, *LIP2*, was identified in *Arabidopsis thaliana*. This gene could complement *E. coli lipB* and was actively expressed in leaves. Another *lipB* homologue, which was presumably the lipoyl ligase, was found but was not actively expressed in leaves. LIP2p was also found to be located in the chloroplast, which provides a mechanism for lipoylation of the chloroplast PDH (Wada *et al.*, 2001a; Wada *et al.*, 2001b). The lipoyl synthase of *A. thaliana*, *LIP1*, was identified, shown to be actively expressed, and to complement an *E. coli lipA* mutation. LIP1p was also shown to be also localized to the chloroplast, consistent with LIP2p (Yasuno *et al.*, 1998; Yasuno *et al.*, 2002). Unlike mammalian systems the lipoyl ligase homologue from rice, OsLplA, is able to ligate lipoic acid. The lipoyl ligase from rice was found to be able to complement a *lipB lplA* strain of *E. coli* and careful measurement of growth showed a significant improvement upon addition of free lipoic acid, demonstrating lipoyl ligase activity. This gene was actively expressed in various tissues including leaves, which is in contrast to *A. thaliana* ligase (Kang *et al.*, 2007). Disruption of fatty acid biosynthesis in the chloroplast of *A. thaliana* reduced lipoylation in some tissues but did not eliminate it. This suggests either that ligation is widespread or that our knowledge of fatty acid biosynthesis is incomplete (Ewald *et al.*, 2007). Lipoylation in plants is similar to *E. coli* but futher study is required to understand lipoic acid transport and scavenging.

Apicomplexan parasites are unique in that lipoic acid biosynthesis, but not scavenging, are dispensible. Lipoic acid metabolism in these organisms is complicated by the PDH being localized in the apicoplast while other lipoate-requiring enzymes are localized in the mitochondrion. The LipA of *T. gondii* is also located in the apicoplast

(Thomsen-Zieger *et al.*, 2003). Lipoylation in the apicoplast is dependent on fatty acid biosynthesis and use of the inhibitor 8-bromooctanoate demonstrates that scavenging of lipoic acid is required for growth (Crawford *et al.*, 2006). The *P. falciparum* LipA homologue is also localized to an organelle outside the mitochondrion, likely the apicoplast. One ligase homologue LplA1 is localized to the mitochondrion (Wrenger *et al.*, 2004). *P. falciparum* also possesses a second lipoyl ligase homologue, LplA2, which weakly complements an *E. coli lipB lplA* strain. External lipoic acid is primarily incorporated into the mitochondrion, as in *T. gondii* the inhibitor 8-bromooctanoate arrests growth in *P. falciparum* (Allary *et al.*, 2007). Disruption of *lipB* is not lethal but results in reduced lipoylation of the PDH. LplA2 is localized to both the apicoplast and the mitochondrion, suggesting it is redundant with LipB; although it is not clear what reaction it is catalyzing (Gunther *et al.*, 2007). Disruption of *lplA1* is not possible without a complementing copy, demonstrating it is essential for growth and virulence (Gunther *et al.*, 2009). It appears these parasites always have access to host lipoic acid and so biosynthesis is dispensible in these organisms under the conditions tested.

Lipoic acid biosynthesis in yeasts initially appeared to by similar to *E. coli*. A *lipA* homologue in *Saccharomyces cerevisiae*, *LIP5*, was identified that resulted in elevated levels of $tRNA^{fMet}$ by an unknown mechanism. The mutation was slightly rescued by ethanol, but not by lipoic acid (Sulo *et al.*, 1993). A homologue of *E. coli lipB*, *LIPB* of *Kluveromyces lactis*, was found to be required for growth and glycine utilization. Growth could be restored with ethanol and succinate, but unexpectedly, lipoic acid had no effect (Chen,1997). An orthologue of *LIPB*, *LIP2*, was characterized in *S. cerevisiae*. Mutants of *LIP2* behaved like *LIPB* and lacked PDH activity (Marvin *et al.*, 2001). Even though there was an absence of ligase activity, a lipoyl ligase homologue is encoded in the genome. Disruption of this gene, *LIP3 (*also *LPLA* and *AIM22*), resulted in loss of lipoylation of the PDH and OGH which is not consistent with a lipoyl ligase. Unexpectedly, the glycine cleavage system H protein of yeast, GCV3, was still lipoylated in a *LIP3* strain. Disruption of *GCV3* also resulted in a lipoic acid deficient strain. Mutation of the lipoylation site of GCV3 and conserved catalytic residues of LIP3 also

resulted in loss of lipoylation. The reasons for these novel phenotypes are not known, although a model where a protein complex is required is proposed (Schonauer *et al.*, 2009). The uncertainty regarding lipoic acid metabolism in yeast suggests novel enzymes are employed. A novel mechanism for lipoic acid biosynthesis in yeast is provided by the work of this thesis.

AIMS AND SCOPE OF THIS THESIS

Previous work has established the role of lipoic acid dependent complexes in central metabolism of a variety of organisms (Figure 1-2 and 1-3). Although a complete model for lipoic acid metabolism is known for *E. coli* (Figure 1-4), the details of lipoic acid metabolism in other organsims is less clear. In Chapter 2 a careful analysis of the *Thermoplasma acidophilum* lipoyl ligase yields greater insight into the biochemical function of this protein. In light of this finding, analysis of the lipoate protein ligase family reveals the potential for novel enzymes. Chapters 3, 4, and 5 of this Thesis focus on *B. subtilis* as a subjet of study. The genome of *B. subtilis* encodes two proteins that from Chapter 2 are predicted to be novel enzymes. *B. subtilis* does not have an obvious orthologue to *E. coli* LipB, and I find a new octanoyltransferase isozyme called LipM. In collaboration with Natalia Martin, we also find another gene, *lipL*, required for lipoic acid biosynthesis; this is described in Chapter 3. In Chapter 4 I find that LipL is a novel amidotransferase enzyme, and that *B. subtilis* employs a novel biosynthetic pathway. Finally, in Chapter 5, I summarize our knowledge of the lipoate protein ligase family. I also describe how this new enzyme impacts our understanding of lipoic acid metabolism in diverse organisms.

FIGURES

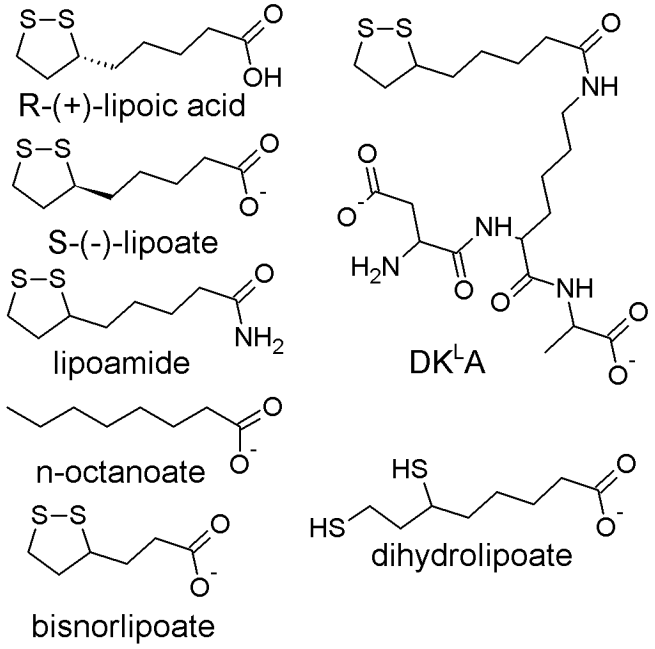


Figure 1-1. Lipoic acid and related compounds. Lipoic acid is the oxidized, uncharged, protonated, acid form, while lipoate is the basic salt form; the salt form is the physiologically relevant form. Lipoate is reduced to dihydrolipoate inside bacteria and mitochondria. The chiral center at carbon six means that a molecule of lipoic acid must be one of two enantiomers, the R configuration being the one biosynthesized. Octanoate can be a precursor to biosynthesis, while bisnorlipoate is a degradation product after a single round of β-oxidation. Asp-Lys-Ala (DKA) is the conserved amino acide residue sequence of mammalian lipoyl domains and the lipoylated DK^LA tripeptide is the primary source of lipoic acid utilized by *L. monocytogenes* during infection.

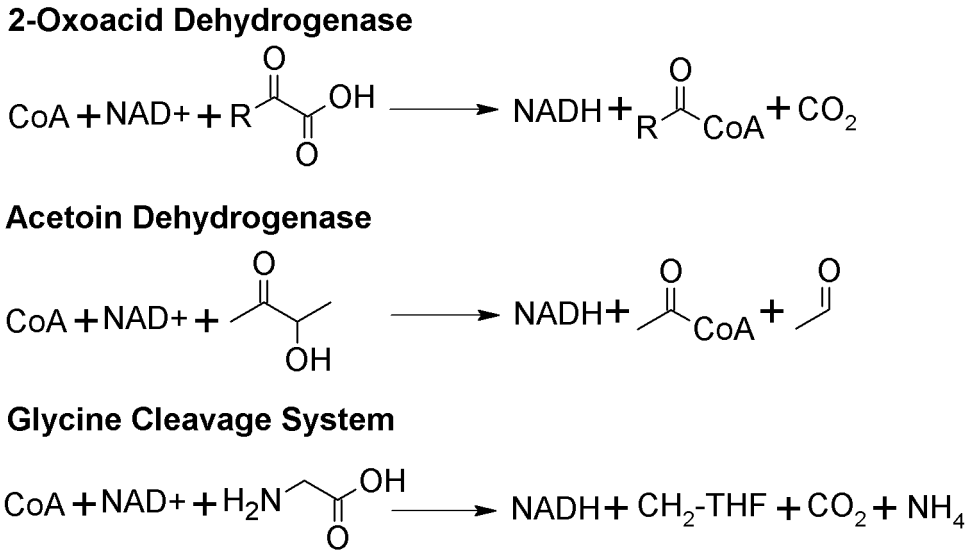


Figure 1-2. Net reactions of lipoic acid dependent systems. The net reaction for 2-oxoacid dehydrogenase complexes (pyruvate, 2-oxoglutarate, and branched-chain keto acid) is the same with different acyl groups. The acetoin dehydrogenase complex is homologous to 2-oxoacid dehydrogenases, but produces acetaldehyde instead of CO_2. The glycine cleavage system is different from the other systems at every step except for oxidation of the dihydrolipoyl group.

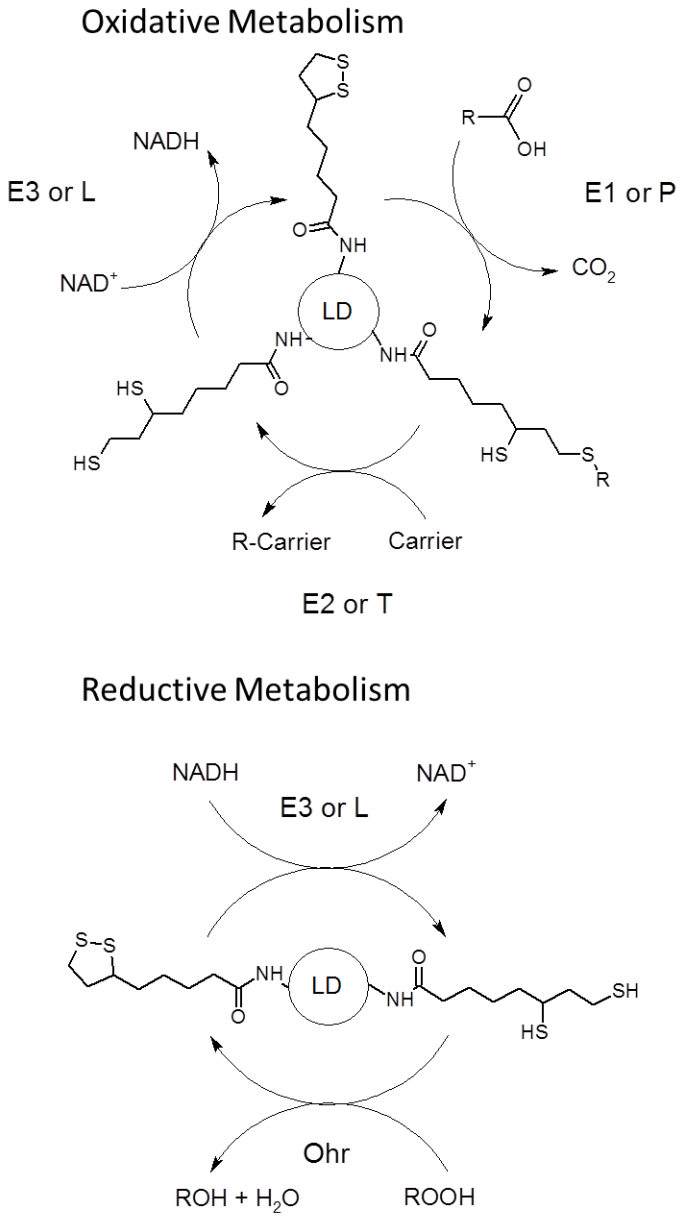


Figure 1-3. Cycle of the lipoyl group in oxidative and reductive catalysis. In well characterized lipoic acid dependent enzyme systems, lipoic acid is oxidized for another round of catalysis which results in the production of NADH. In the novel organic hydroperoxidase (Ohr) system, reduced lipoic acid is the substrate which can be regenerated by a dihydrolipoamide dehydrogenase.

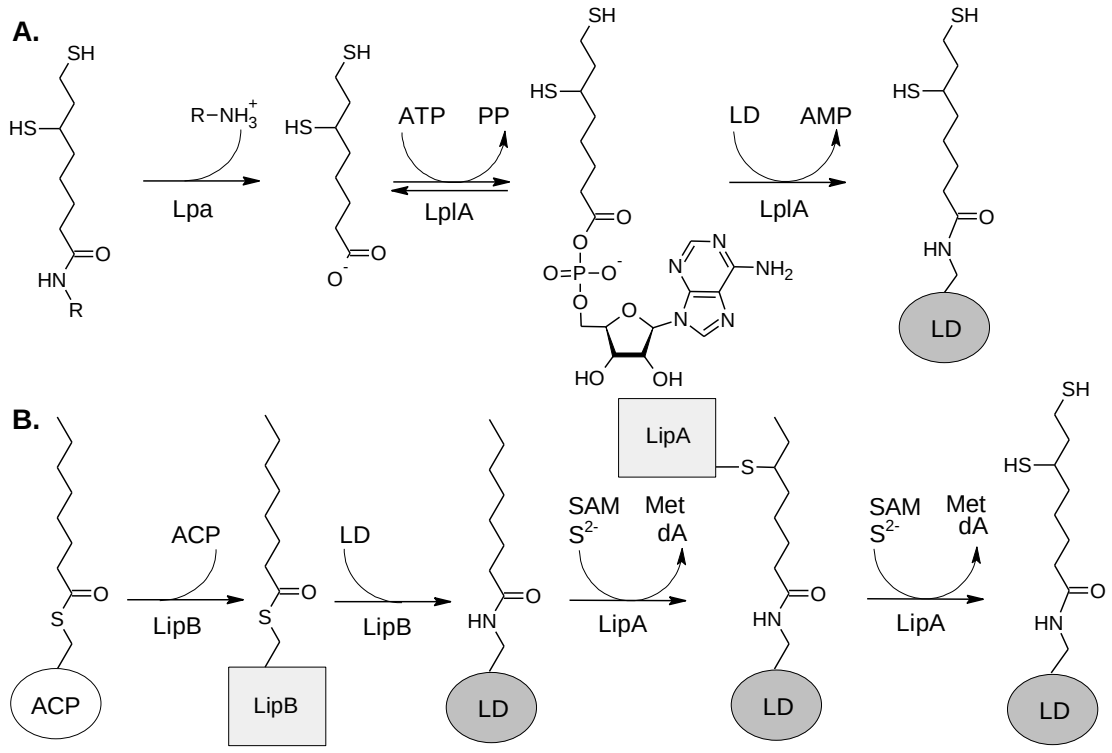


Figure 1-4. The current model for lipoic acid synthesis and scavenging in *E. coli* is shown. *E. coli* uses two independent pathways for lipoic acid attachment. Panel A: Lipoic acid is cleaved from peptides or proteins by lipoamidation. Exogenous lipoate (or octanoate) is transferred to unmodified acceptor lipoyl domains (LD) in an ATP-dependent two-step reaction catalyzed by LplA. Panel B: The endogenous synthesized octanoate is transferred from acyl carrier protein (ACP) to apoproteins by LipB, an octanoyl-ACP:protein-N-octanoyltransferase. The octanoylated domains then become substrates for sulfur insertion by LipA.

CHAPTER 2

THE *THERMOPLASMA ACIDOPHILUM* LPLA-LPLB COMPLEX DEFINES A NEW CLASS OF BIPARTITE LIPOATE-PROTEIN LIGASES

INTRODUCTION

Recently crystal structures of *E. coli* LplA and of LplA homologues have been reported including an *E. coli* LplA-lipoic acid complex (Fujiwara *et al.*, 2005; Kim do *et al.*, 2005; McManus *et al.*, 2006). The reported structures of the unliganded proteins agree well and show *E. coli* LplA to be a two-domain protein consisting of a large N-terminal domain and a small C-terminal domain (Figures 2-1 and 2-2). However, the *E. coli* LplA-lipoic acid complex is difficult to interpret because lipoic acid molecules were heterogeneously bound to LplA molecules within the crystals and were poorly resolved. In one case the lipoic acid carboxyl was hydrogen-bonded to Ser-72, whereas in another case Arg-140 was the hydrogen bond donor (Fujiwara *et al.*, 2005). Because 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 in the interdomain cavity was artifactual. Moreover prior work (Reed *et al.*, 1994) had isolated LplA mutants resistant to inhibition by an analogue of lipoic acid in which the sulfur atoms had been replaced with selenium. Because 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 Ser (Morris *et al.*, 1995)) was distal from the lipoate binding site reported. This dilemma was resolved by two lipoic acid-containing structures of an LplA homologue from the archaeon *T. acidophilum* (Kim do *et al.*, 2005; McManus *et al.*, 2006) 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 (Figure 2-2). 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 (McManus *et al.*, 2006). Moreover addition of lipoic acid to a complex of the *T. acidophilum* LplA with ATP gave lipoyl-AMP showing that the lipoic acid was bound in a physiologically meaningful manner (Kim do *et al.*, 2005). The lipoyl-AMP was bound in a U-shaped pocket and was well shielded from solvent. Thus, it seems that the locations of the lipoate moieties in the two *T. acidophilum* LplA structures indicate that these represent catalytically competent lipoate binding sites (rather than the sites of *E. coli* LplA where lipoate bound).

A caveat was that the *T. acidophilum* LplA was inactive in catalysis of the overall LplA reaction (McManus *et al.*, 2006). Because *T. acidophilum* LplA lacks the C-terminal domain (CTD) of *E. coli* LplA (Kim do *et al.*, 2005; McManus *et al.*, 2006), this suggested that the missing domain was required for activity, and a second protein was proposed to interact with *T. acidophilum* LplA to allow the complete reaction (McManus *et al.*, 2006). If this were the case, the *T. acidophilum* lipoyl ligase would provide an unusually facile system to investigate the role of the CTD in lipoate-protein ligases.

If the lipoate-protein ligase reaction can be catalyzed by a heteromeric protein this may allow better discrimination of lipoyl ligases from acyl carrier protein:protein octanoyltransferases. Although the two classes of LD-modifying enzymes, LplA and LipB, show very low amino acid sequence conservation and utilize different chemistries, the proteins surprisingly show structural conservation and have related active site architectures (Ma *et al.*, 2006; Kim do *et al.*, 2008) (Figure 2-2). The *Mycobacterium tuberculosis* LipB and *T. acidophilum* LplA can be superimposed by using all matching Cα positions. This yields a root mean square deviation of ~2.5 Å with good topological matching of most secondary structural elements (Ma *et al.*, 2006). Hence in length and structure, LipBs resemble LplAs that lack the C-terminal domain. Although the *E. coli* LipB and LplA sequences align very poorly, a large number of proteins in the data bases have similarities to both proteins, and therefore annotation of a given protein as a ligase or octanoyltransferase is not straightforward. If an LplA CTD can be a separate protein, an additional criterion to distinguish lipoate ligases and octanoyltransferases would be

available. It should be noted that biotin ligases also show structural (but not sequence) conservation with LipB and LplA, and this group of proteins comprises the Pfam family PF03099 (Finn *et al.*, 2010). However, all known biotin ligases have a C-terminal domain that greatly aids in their annotation. I report that, as predicted (McManus *et al.*, 2006), the CTD function essential for lipoate-protein ligase activity is encoded by a gene located immediately upstream of *T. acidophilum lplA* that I call *lplB*.

EXPERIMENTAL PROCEDURES

Chemicals, Bacterial Strains, and Growth Media

Strains and plasmids used in this study are listed in Table 2-1. LB rich and M9 minimal media were prepared as described previously (Miller, 1972). Bacterial cultures were grown at 37 °C. *T. acidophilum* DSM 128 genomic DNA was purchased from the ATCC. Oligonucleotides were synthesized by Integrated DNA Technologies and their sequences are shown in Table 2-2. PCR amplification was performed using *Pfu* polymerase (Stratagene), and A overhangs were added with *Taq* polymerase (New England Biolabs). The TOPO TA Cloning kit was used for cloning PCR products into the pCR2.1 vector (Invitrogen) using the Topoisomerase Cloning kit was used for pET101 (Invitrogen). DNA constructs were sequenced by the Roy J. Carver Biotechnology Center of the University of Illinois. All reagents and biochemicals were obtained from Sigma-Aldrich unless otherwise noted. Radiolabeled [α-^{32}P]ATP and [^{35}S]methionine were purchased from American Radiolabeled Chemicals.

All *E. coli* strains were derivatives of *E. coli* K-12. Strain QC146 was constructed by the method of Datsenko and Wanner (Datsenko *et al.*, 2000). The *lplA* gene was replaced with a kanamycin resistance cassette by transformation of the PCR product obtained using primers Q017 and Q018. The *lipB* gene was replaced with a chloramphenicol resistance cassette using the PCR product obtained using primers Q019 and Q020. The insertions were transduced with phage P1 into the wild type strain MG1655, the antibiotic cassettes were removed using the Flp protein encoded by the temperature-sensitive plasmid pCP20 (Datsenko *et al.*, 2000), and the *lipB lplA*

phenotype was verified on M9 0.2% glucose medium comparing growth with 5 µg/ml lipoic acid or 5 mM sodium acetate plus 5 mM sodium succinate. The strains are described in Table 2-1.

Plasmid Constructions

The TA0513 and TA0514 open reading frames were amplified as a single fragment by PCR from *T. acidophilum* genomic DNA using primers Q007 and Q008, which added terminal NcoI and HindIII sites. This product was directly cloned into the same sites of pBAD322G (Cronan,2006) under the control of an arabinose-inducible promoter to give pQC005. TA0514 was amplified with primers Q027 and Q008, which added NcoI and HindIII sites, and TA cloned into pCR2.1 to give pQC019 followed by insertion between the NdeI and HindIII site of pBAD322G to give pQC021. TA0513 was amplified with primers Q007 and Q025 and TA cloned into pCR2.1 to give pQC020 followed by insertion between the NdeI and XbaI sites of pBAD322G to give pQC022. A derivative of TA0514 having an N-terminal hexahistidine tag was amplified from genomic DNA using primers Q026 and Q008, which also added the N-terminal hexahistidine tag sequence. The product was TOPO cloned directly into pET101 to give pQC017. TA0513m was amplified using primers Q069 and Q068, which added an EcoRI site, a ribosome binding site, an N-terminal hexahistidine tag, and an XhoI site. This product was directly cloned between the EcoRI and XhoI sites of pET19b to give pQC043.

The lipoyl domain of the E2 subunit of the branched chain dehydrogenase was determined by alignment to the lipoyl domain derived by proteolytic digestion of *Bacillus stearothermophilus* pyruvate dehydrogenase E2 subunit (Packman *et al.*, 1984). The domain contains the first 86 amino acids of the branched chain dehydrogenase E2 from open reading frame TA1436. The domain was amplified from genomic DNA with primers Q032 and Q033 creating an NdeI restriction site within the initiation codon and a stop codon following codon 86. This was TA cloned into pCR2.1 to give pQC028. The gene was then ligated between the NdeI and SacI sites of pET30 (a)+ to give pQC024. A putative *T. acidophilum gcvH*, open reading frame TA1366m, was found by similarity searches and was amplified with primers Q050 and Q049, which introduced a C-terminal

hexahistidine tag and a BspHI site that overlapped the initiation codon. The product was TA cloned into pCR2.1 to give pQC034 and subsequently inserted into the NcoI and XbaI sites of pET28 (b)+ to give pQC035. TA0514 was cut from pQC021 with NcoI and HindIII and inserted between the same sites of pRSF-1b to give pQC055. TA0513 was digested from pQC022 and ligated into the NcoI and XbaI sites of pRSF-1b to give pQC056.

Bioinformatic Analyses

Identification and bioinformatics characterization of single domain homologues of LplB were done using the Berkeley Phylogenomics Website (Glanville *et al.*, 2007). Twenty-six homologues were identified using the TA0513m protein sequence from 10 iterations of SHMM (subfamily hidden Markov model) and PSI-BLAST (position-specific iterative basic local alignment search tool). Genome comparison, similarity searches, and lipoic acid subsystem analyses were performed using the National Microbial Pathogen Database Resource (McNeil *et al.*, 2007). Alignments were generated using ClustalW2 (Larkin *et al.*, 2007).

Complementation and 2-Oxoacid Dehydrogenase Assays

Strains JK1 and TM131 containing pBAD322G expressing the indicated ligase proteins were grown in LB medium supplemented with 0.2% glucose, 5 mM sodium acetate, 5 mM sodium succinate, and 5 mg/L lipoic acid. The cells were harvested at an A_{600} of 0.6 by centrifugation and resuspended in 10 mM Tris-HCl buffer (pH 7.5) at ~100 mg of wet weight/mL. Cells were lysed by two passages through a French pressure cell at ~20,000 p.s.i. Lysates were cleared by centrifugation at 27,000 × *g* for 15 min. Assays of the pyruvate dehydrogenase and oxoglutarate dehydrogenase complex activities were a modification of previous continuous spectrophotometric assays (Amarasingham *et al.*, 1965; Guest *et al.*, 1997). The reaction mixture contained 150 mM Tris-HCl buffer (pH 8.5), 3 mMl-cysteine, 0.1 mM CoA, 0.5 mM thiamin pyrophosphate, and 2 mM acetylated NAD analogue (2-acetylpyridine adenine dinucleotide). For assays of pyruvate dehydrogenase, 5 mM $MgCl_2$ was also included to give maximal activity. Cell extract protein contents were determined using the using the Bio-Rad Protein Assay kit. The amount of extract assayed varied from 100 to 1 µg/ml final concentration. Each

assay began by addition of 50 mM substrate (pyruvate or 2-oxoglutarate) at 25 °C. The reduction of 2-acetylpyridine adenine dinucleotide was measured at 366 nm, and the extinction coefficient of the reduced form in assay buffer was experimentally determined to be 7.0 mM^{-1} cm^{-1}.

Purification of LplA and LplB

Purification of hexahistidine-tagged versions of LplA and LplB was done using Ni^{2+} affinity chromatography followed by anion exchange chromatography. Plasmids QC048 and QC049 were transformed into strain Rosetta2 DE3, and the transformants were grown at 37 °C in 2 liters of LB medium with 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. At an A_{600} of 0.6 expression was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h before harvest and storage of the cell pellets at −80 °C. All chromatography steps were performed at 4 °C. The cell pellets were resuspended in lysis buffer (50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1 mM dithiothreitol, and 10 mM imidazole) and lysed using a French pressure cell. The lysate was cleared at 48,000 × *g* for 15 min, and 5 ml of a 50% slurry of Ni^{2+}-NTA-agarose resin (Qiagen) was added to the cleared lysate and incubated for 1 h at 4 °C with mixing. The resin was packed into a 0.75-inch column and washed with at least 10 column volumes of wash buffer (50 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 20 mM imidazole) by gravity flow. The protein was eluted with the same buffer (elution buffer) containing 250 mM imidazole. The eluate was dialyzed overnight in 50 mM Tris-HCl (pH 8.0) and subjected to anion exchange using a 1-ml POROS HQ 20 column with a gradient of 10–500 mM NaCl using an AKTA Purifier 10 FPLC run at 5 ml/min. Fractions containing pure protein, as judged by SDS gel electrophoresis followed by Coomassie Blue staining, were dialyzed overnight in storage buffer that contained 10 mM sodium phosphate buffer (pH 7.0), 100 mM NaCl, 1 mM dithiothreitol, and 10% glycerol. The proteins were concentrated using Vivaspin concentrators (Sartorius), flash frozen in dry ice and ethanol, and stored at −80 °C. The masses of purified proteins were determined after dialysis against 10 mM ammonium acetate followed by drying under vacuum. The samples were submitted to the

University of Illinois at Urbana-Champaign Mass Spectrometry Laboratory for matrix-assisted laser desorption ionization mass spectrometry analysis.

Purification of Lipoyl Domain Substrates

The *E. coli* E2p hybrid domain was a gift from Dr. Xin Zhao of this laboratory and had been purified by precipitation and ion exchange chromatography as described previously (Zhao *et al.*, 2003). Plasmid pQC024 in Acella λDE3 cells (Edge Biosystems) was used to express the E2 domain in LB medium with 50 µg/ml kanamycin. The culture was induced at an A_{600} of 0.5 for 4 h. The collected cell pellet was washed with 10 mM sodium phosphate buffer (pH 7.0) and stored at −80 °C. Purification of *T. acidophilum* E2 domain of the branched chain dehydrogenase was performed as described previously for *E. coli* E2p domain. This anion exchange protocol allows resolution of the apo and holo forms of the domain as shown by 20% native PAGE. Pure apo domain was dialyzed and stored as described for LplA and LplB. The masses of apo- and holo-LDs were verified as described for LplA and LplB except that electrospray mass spectrometry was performed. All E2 domain preparations were found to lack the N-terminal methionine residue.

Plasmids pQC035 and pTara were used to express the putative glycine cleavage H protein TA1366m in the LD modification-deficient strain QC146 grown in LB medium containing 0.1% glucose, 5 mM sodium acetate, 5 mM sodium succinate, 50 µg/ml kanamycin, and 25 µg/ml chloramphenicol. When the cells reached an A_{600} of 0.6, 0.2% arabinose was added as an inducer, and cells were harvested by centrifugation 4 h later. GcvH was purified by Ni^{2+} affinity and anion exchange as described above for LplA and LplB.

Assay of Lipoyl Domain Modification

Protein concentrations were determined using extinction coefficients calculated from the ProtParam program on the ExPASy Tools Website. The assays contained 50 mM sodium phosphate buffer, 1 mM sodium lipoate, 5 mM disodium ATP, 5 mM dithiothreitol, 1 mM $MgCl_2$, and 20 µM apo-LD. The 2 µM LplA and 20 µM LplB were added as indicated. Three different apo-LDs were used. Reactions (20 µl) were

incubated at 55 °C for 1 h in the case of the *T. acidophilum* proteins and 16 h for the *E. coli* LD domain. Lipoyl domain modification was determined by gel shift by native polyacrylamide gel electrophoresis using 20% Tris-glycine gels.

Lipoyl-AMP was synthesized by the method of L. J. Reed *et al.* (Reed *et al.*, 1958). Lipoyl-AMP was weighed and dissolved in 100 mM sodium phosphate buffer (pH 7.0) before use as a substrate for lipoyl domain modification by LplA and LplB. The reaction contained 50 mM sodium phosphate (pH 7.0), 1 mM tris(2-carboxyethyl)phosphine, 0.1 mM $MgCl_2$, 20 µM apo-LD, and 1 mM lipoyl-AMP. LplA (2 µM) and LplB (20 µM) were added as indicated. Reactions (20 µl) were incubated at 55 °C for 30 min. Lipoyl domain modification was determined as described above.

Assay of Enzymatic Lipoyl-adenylate Intermediate Formation

Formation of lipoyl-AMP was assayed using radiolabeled [α-^{32}P]ATP. The reactions contained 50 mM sodium phosphate (pH 7.0), 1 mM tris (2-carboxyethyl)phosphine, 10 nM [α-^{32}P]ATP, 10 µM $MgCl_2$, and 0.1 mM sodium lipoate. 15 µM LplB and 10 µM LplA were added as indicated. The reaction was incubated for 30 min at 55 °C. 1 µl of the reaction was subjected to cellulose thin layer chromatography on plates containing a fluorescent indicator developed in isobutyric acid:NH_4OH:water (66:1:33). Lipoyl-AMP had an R_f of 0.68, whereas the R_f of AMP was 0.41. The thin layer chromatograms were dried and exposed to a phosphorimaging plate for 10 min to 10 h and visualized using a Fujifilm FLA-3000. The same reaction as above was also run with 0.1 µM [α-^{32}P] ATP and 1 µM LplA. Additionally 3 µM apo-LD was added to this and preincubated for 15 min without ATP to remove any enzyme-bound ATP or adenylate.

Isolation of enzyme-bound lipoyl-adenylate was performed after the reaction above with LplA and LplB using a 10-kDa Microcon centrifugal filter device (Millipore). The reaction was diluted with 600 µl of 50 mM sodium phosphate buffer (pH 7.0), applied to the filter, and centrifuged at 14 × *g* for 15 min. This was followed by four 400-µl buffer washes of the filter after which the protein was recovered. The fractions were analyzed by TLC and phosphorimaging as described above.

Determination of the Size and Stoichiometry of the LplA-LplB Complex

Strains QC049, QC108, QC164, and QC165 were used to determine the size and stoichiometry of the lipoyl ligase complex. These strains have *T. acidophilum* lipoyl ligase proteins expressed from a T7 promoter. All proteins were hexahistidine-tagged except for TA0513 in QC164 and TA0514 in QC165. LplA and LplB were expressed together in Rosetta2 DE3 cells using plasmids with compatible origins of replication. Strain QC164 contained pQC017 and pQC056 that expressed a hexahistidine-tagged LplA and native LplB. Strain QC165 contained pQC043 and pQC055 that expressed a hexahistidine-tagged LplB and native LplA. The strains were grown in 50 ml of LB medium with 100 µM ampicillin and 50 µg/ml kanamycin where appropriate. Cultures were grown to an A_{600} of 0.6, and expression was induced with 1 mM isopropyl 1-thio-β-d-galactopyranoside for 4 h. The cells were harvested by centrifugation and stored at −80 °C. Cellytic Express (Sigma) was dissolved at 50 mg/ml in 10 mM Tris-HCl (pH 8.0) and used to resuspend cells at ∼400 mg/ml wet weight. Lysis occurred after incubation at room temperature for 20 min. The extract was cleared by centrifugation at 18,000 × *g* for 20 min. The extract was then applied twice to a pre-equilibrated Ni^{2+}-NTA spin column (Qiagen). The column was washed thrice with 600 µl of wash buffer. Finally the products were eluted with 100 µl of elution buffer and were analyzed by SDS-PAGE or gel filtration chromatography.

Gel filtration chromatography was performed in 50 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl using a Superdex 200 10/300 GL column on an AKTA purifier 10 FPLC run at 0.5 ml/min. The void volume was found to be 7.7 ml using blue dextran. Low range molecular weight standards (GE Healthcare) were also run to establish a standard curve. The partition coefficient, K_{av}, was calculated from $K_{av} = (V_e - V_0)/ (V_t - V_0)$ where V_0 is the void volume, V_e is the elution volume, and V_t is the total column volume.

The ratio of LplA to LplB was determined by protein labeling with [^{35}S]methionine (Tabor *et al.*, 1985). Strain QC164 was grown in M9 medium with 0.4% glucose, 50 µg/ml l-methionine, 50 µg/ml ampicillin, 25 µg/ml kanamycin, and 12 µg/ml

chloramphenicol. Cells were grown to an A_{600} of 0.6, 1 mM isopropyl 1-thio-β-D-galactopyranoside was added, and 10 min later cells were washed thrice with M9 medium. Cells were added to M9 medium with 0.4% glucose, 1 mM isopropyl 1-thio-β-d-galactopyranoside, and 0.2 μg/ml rifampicin. After 15 min 5 μCi/ml l-[^{35}S]methionine and 50 μg/ml l-cysteine were added to label methionine residues and prevent labeling of cysteine residues. Cultures were allowed to grow for 2 h until harvest by centrifugation. The cells were lysed using Cellytic Express (Sigma) as above. The complex was purified using a Ni^{2+}-NTA spin column (Qiagen), and the eluate was subjected to gel filtration as described above. Fractions were subjected to SDS-PAGE followed by Coomassie Blue staining. The gel was dried and analyzed using a Fujifilm FLA-3000 and phosphorimaging plate. The ratio of LplA to LplB was determined by comparing the ratio of the intensities of each band. The ratio was corrected for the number of methionine residues in each protein (14 for hexahistidine-tagged LplA and 5 for native LplB). The ratio was further corrected for differences in acrylamide concentration, which is not included in the puplication corresponding to This chapter. Previously published data (Harding *et al.*, 1983) was plotted and fit to a line of $y=3.83e^{-0.11x}$. The acrylamide percentage at the positions of LplA and LplB was estimated by measuring 3 independent gels run with the same conditions as used for autoradiography.

RESULTS

Complementation of *E. coli* lplA Mutants by *T. acidophilum* lplA and lplB

An *E. coli lipA lplA* mutant was used for complementation studies because the lack of lipoyl ligase has no phenotype when lipoic acid biosynthesis is intact (Morris *et al.*, 1994; Morris *et al.*, 1995). The *lplA* null mutation of *E. coli* the host was complemented when *lplA* and *lplB* of *T. acidophilum* were coexpressed but not when the genes were expressed separately. This was shown by restoration of growth on minimal medium supplemented with lipoic acid (Figure 2-3A). Complementation was due to activation of 2-oxoacid complexes, an activity expected of a lipoyl ligase (Figure 2-3B). A slight increase in 2-oxoglutarate dehydrogenase activity was seen with LplA

alone suggesting that the protein has some ability to modify lipoyl domains. This was also seen by the very weak complementation of growth by LplA alone (Figure 2-3A). This was unexpected because previous *in vitro* assays demonstrated no activity (McManus *et al.*, 2006) and suggested that LplA has low levels of activity in the absence of LplB. The greater modification of 2-oxoglutarate dehydrogenase suggests that *T. acidophilum* lipoyl ligase may have a greater relative affinity for this E2 domain than does *E. coli* LplA.

Requirement of LplB in Lipoyl Ligation

The LplA/LplB ligation reaction was reconstituted *in vitro*. Purified hexahistidine-tagged LplA and LplB proteins were tested for the ability to modify the *T. acidophilum* E2 and GcvH LDs in the presence of lipoate and ATP. Lipoylation was observed only when both LplA and LplB were present (Figure 2-4). Although as mentioned above a very weak activity was observed with LplA alone *in vivo*, I detected no activity *in vitro* in agreement with a prior report (McManus *et al.*, 2006). Octanoate was also a substrate for LplA-LplB as was demonstrated previously with *E. coli* lipoyl ligase (Morris *et al.*, 1994; Green *et al.*, 1995). The *E. coli* E2p LD was a substrate, but full modification required very long incubations.

The Role of LplB in Lipoyl-AMP Formation

LplB provides a test for the function of the CTD of the canonical single subunit lipoyl ligases. Based on the reported synthesis of adenylate within LplA crystals (Kim do *et al.*, 2005) I hypothesized that LplB would be required for transfer of lipoate from lipoyl-AMP but not for adenylate formation. To test this hypothesis the interactions of LplA and LplB with the lipoyl-adenylate intermediate were tested by two approaches: by direct measurement of adenylate formation (Figure 2-5A) and by use of chemically synthesized lipoyl-adenylate as a substrate (Figure 2-5C). I found that lipoyl-adenylate was formed with [α-^{32}P]ATP as substrate in the absence of LD acceptor. The adenylate intermediate was resolved from other products by thin layer chromatography using the solvent system used for chemical lipoyl-adenylate synthesis (Reed *et al.*, 1958) followed by autoradiography and phosphorimaging (Figure 2-5A). About 1 eq of lipoyl-adenylate/eq of the LplA-LplB ligase was formed after 30 min of incubation, a result

similar to that obtained for *E. coli* biotin ligase (Xu *et al.*, 1994). The lipoyl-AMP intermediate fractionated with the ligase complex indicating that the intermediate remained protein-bound (Figure 2-5B). I also attempted to demonstrate association of the synthesized lipoyl-adenylate using Ni^{2+} affinity column fractionation. However, elution resulted in significant lipoyl-AMP hydrolysis due to nucleophilic attack of lipoyl-AMP by the imidazole eluant (Jencks,1957). This result suggests that little or no lipoyl-AMP remained bound to the Ni^{2+} affinity-purified enzyme. To our surprise, LplB was required for adenylate formation (Figure 2-5A). No adenylate was formed with LplA alone even in the presence of 100-fold molar excesses of ATP and lipoic acid. To eliminate the possibility that the active site contained unlabeled adenylate or ATP, the reaction was pretreated with apo domain (equimolar with enzyme) before addition of [^{32}P]ATP (data not shown). The results obtained were similar to those shown, and no lipoyl-adenylate was detected unless both LplA and LplB were present.

I also tested transfer of lipoate from chemically synthesized lipoyl-AMP to an LD. The results (Figure 2-5C) demonstrated that only the LplA protein was required for transfer of the lipoyl moiety and that LplB is not required for interaction with the LD. This latter finding is not surprising because the related LipB octanoyltransferases interact with LDs although they lack a CTD (Figure 2-2). Lipoic acid and AMP were also seen among the reaction products because of the lability of the lipoyl-AMP mixed anhydride. Based on findings with biotinyl-AMP, lipoyl-AMP could chemically modify the domain (Streaker *et al.*, 2006). However, little such modification was seen under our reaction conditions (Figure 2-5C).

Properties of the LplA-LplB Complex

The LplA-LplB complex was recovered during Ni^{2+} affinity, anion exchange, and size exclusion chromatographic separations indicating reasonably stable interaction. Affinity-tagged LplA bound native LplB (Figure 2-6). In contrast tagged LplB coexpressed with native LplA resulted in little or no protein upon elution of the Ni^{2+} affinity columns. This is presumably due to the poor solubility of LplB and the low efficiency of the LplB affinity tag that may be further decreased when LplB is complexed with LplA. Size

exclusion chromatography indicated an LplA-LplB complex size that was considerably smaller than the molecular weight calculated for an equimolar A-B complex. However, LplA and LplB when chromatographed alone also appeared smaller than their calculated masses such that a 1:1 complex is a reasonable interpretation. I have no explanation for this aberrant behavior but note that similar atypical elution was reported for another member of this protein family, *E. coli* LipB (Nesbitt *et al.*, 2005). Specific biosynthetic labeling of the proteins with [^{35}S]methionine allowed the ratio of LplA to LplB to be determined. An LplA to LplB ratio of 1.6 ± 0.2:1 was obtained by size exclusion chromatography. However, the elution positions of LplA and the complex overlap such that excess LplA present in the chromatographed extract could have falsely increased the ratio. Excess LplA might be expected because of greater relative expression resulting from the higher copy number of pET101 relative to pRSF-1b. In a second approach I purified the [^{35}S]methionine-labeled complex by Ni^{2+} affinity chromatography and separated the subunits by denaturing gel electrophoresis (Figure 2-6B). Phosphorimaging analysis of the gel gave a ratio of LplA to LplB of 1.4:1. Adjusting for the absorbance of the acrylamide gradient based on previous observations (Harding *et al.*, 1983), this ratio becomes 1.2:1. This result suggests that the complex is somewhat unstable and together with the gel filtration data indicate that an A-B heterodimer was the best fit of the data.

DISCUSSION

This chapter confirms the hypothesis of McManus *et al.* (McManus *et al.*, 2006) that LplB is the missing CTD of the *T. acidophilum* LplA. Although LplB is the defining member of the "bacterial lipoate-protein ligase C terminus" family (Pfam PF10437), it is doubly misnamed because it is not of bacterial origin, nor is it always a C-terminal domain. Many actinomycete LplA homologues have a domain at their N termini homologous to a CTD suggesting that they are circularly permuted LplAs (this is currently under investigation). As a result of the misnaming of the PF10437 family, it contains only one single domain sequence: that of *T. acidophilum* LplB. I suggest this

family might better be called the lipoyl ligase accessory domain family. I detected other single domain LplB candidates using a Phylobuilder global-global homology search. LplB homologues are present in many Archaea and Bacteria but are not necessarily encoded near the *lplA* gene. Alignments of selected sequences are shown in Figure 2-7. Although the number of sequenced Archaea is few, the *lplB-lplA* gene arrangement appears to be the typical form of archaeal lipoyl ligases. Exceptions are the putative lipoyl ligases of *Sulfolobus* that encode putative LplAs that both have and lack an LplB-like CTD. There are few LplB homologues in Bacteria with the exception that all sequenced *Bordetella* strains contain an LplB homologue and a *T. acidophilum*-like LplA. Although cultured methanogens do not require lipoic acid, a putative two-subunit lipoyl ligase is found in the genome of the uncultured methanogen RC1.

The existence of two classes of LplAs, with and without accessory domains, was proposed previously (McManus *et al.*, 2006). The results of our study show that lipoyl-AMP formation requires the accessory domain. Because LipB octanoyltransferases have only a catalytic domain but bind and modify their protein substrate, I hypothesize that lipoyl ligase homologues lacking an accessory domain in the genome perform a different reaction. This is the case for the mammalian lipoyl ligase homologues, which have a CTD that has almost no sequence similarity with LplB (Figure 2-7). Like *T. acidophilum* LplA, these enzymes are unable to catalyze lipoyl-adenylation but transfer the lipoyl moiety when lipoyl-AMP is provided (Fujiwara *et al.*, 2007). This raises the question of the function of the mammalian CTD if it is not involved in adenylation. I found that both LplB and LplA are required for efficient complementation of an *lplA* mutant of *E. coli* and for efficient activation of the pyruvate and 2-oxoglutarate dehydrogenases. *In vitro* both I and McManus *et al.* (McManus *et al.*, 2006) found that LplA failed to modify the E2p lipoyl domain. However, it was previously reported that upon first soaking LplA crystals in ATP and then in lipoate the crystals contained lipoyl-AMP (Kim do *et al.*, 2005). I was unable to detect lipoyl-AMP formation by LplA despite use of a sensitive radiochemical assay. Moreover because LplA alone can catalyze lipoyl transfer from exogenously supplied lipoyl-AMP to LD (Figure 2-3C), McManus *et al.* (McManus *et al.*, 2006) and I

should have observed lipoylation activity if lipoyl-AMP had been formed. The difference between the crystal and solution studies of LplA cannot be due to lipoyl-AMP stability. If this were the case I should have observed increased AMP accumulation in the lipoyl-AMP synthesis reaction; this was not the case (Figure 2-5A). It therefore seems possible that the function of LplB is to modulate the structure of LplA such that lipoyl-AMP synthesis can occur and that perhaps crystal packing somehow mimicked this modulation. However, the weak modification of the 2-oxoglutarate dehydrogenase seen in the presence of only LplA (Figure 2-3) suggests that some lipoyl-AMP is formed *in vivo*. This could be due to trace ligase activity by LplA alone or by the presence of an *E. coli* factor that can mimic LplB. The *E. coli* pyruvate dehydrogenase domains seem poor substrates for the *T. acidophilum* ligase. Expression of LplA alone or LplA and LplB together in *E. coli* results in more 2-oxoglutarate dehydrogenase activity than pyruvate dehydrogenase activity, although the latter enzyme has three LDs per E2 subunit, and a single lipoylated LD is sufficient for activity (Perham, 2000). Indeed the *E. coli* E2p domain was a poor substrate *in vitro* (Figure 2-4).

The available structures of lipoyl ligases provide a possible mechanism for LplB action. The structure of *E. coli* LplA shows that the conserved CTD Asp (Asp-42 in LplB) residue faces the catalytic domain and is close to the loop formed by residues 72–82, highly conserved residues that line the lipoyl-AMP binding pocket (Fujiwara *et al.*, 2005). The G76S mutation conferring resistance to selenolipoic acid is also present in this loop, consistent with its role in substrate binding (Reed *et al.*, 1994). In an unpublished *Streptococcus pneumonia* LplA structure (Protein Data Bank accession code 1VQZ), the conserved Asp of the accessory domain is also in close proximity to conserved residue Gln-47, and both residues are close to the conserved loop that lines the active site. A structure of a catalytically active lipoyl ligase of known activity complexed with lipoyl-adenylate is not yet available, but an analogous biotin ligase structure has been solved (Wood *et al.*, 2006). Although some LplB residues are conserved in the biotin ligase accessory domain, it appears that the interaction of the CTD with the catalytic domain is different. The biotin ligase accessory domain is shorter

and lacks the two outward facing α helices that are present in the lipoyl ligase accessory domain.

Because of their unique ATP binding motif, it has been argued that members of the biotin ligase superfamily originally evolved to bind small molecules and that adenylation activity arose independently (Wood *et al.*, 2006). The role of the accessory domain in adenylation suggests that it may be important for binding of a small molecule substrate. Indeed the CTD of biotin ligase has also been shown to be important for binding ATP (Chapman-Smith *et al.*, 2001). Within the lipoyl ligase family the lipoyl ligase accessory domain can be found as an N-terminal domain, a CTD, or as a separate protein or is not found at all (Figure 2-8). The accessory domains found as separate proteins are closely related (Figure 2-7), so the variable domain architecture of these lipoate ligases could be a recent development. The advantage of a multimeric lipoyl ligase complex is not obvious. Perhaps the accessory domain was initially absent and was later added during evolution of ligase function. This is reasonable given that the LplA protein family is rooted in such sequences (Figure 2-8). If so, the *T. acidophilum* lipoyl ligase complex may be an evolutionary relic. The activity of the *T. acidophilum* LplA and LplB complex with octanoate in place of lipoate was first reported for the *E. coli* enzyme (Morris *et al.*, 1994). However in *E. coli* the octanoyl-LD can be converted to lipoyl-LD by lipoyl synthase (LipA). In contrast, *T. acidophilum* lacks a recognizable lipoyl synthase homologue, and thus octanoylation would render the LD inactive. Hence to obtain function of its lipoic acid-requiring enzymes *T. acidophilum* would need an environment rich in lipoic acid and deficient in octanoic acid.

TABLES

Table 2-1. Strains and plasmids used in this chapter.

Strain/plasmid	Relevant characteristics	Source or Ref.
MG1655		CGSC*
JK1	*rpsL* (Strep^R)	(Morris *et al.*, 1995)
TM131	*rpsL lipA* ::*Tn1000 lplA* ::*Tn10*	(Morris *et al.*, 1995)
Acella DE3	*ompT hsdSB* (rB$^-$ mB$^-$) *gal dcm* (DE3) Δ*endA* Δ*recA*	Edge Biosystems
Rosetta2 DE3	*ompT hsdSB* (rB$^-$ mB$^-$) *gal dcm* (DE3)/pRARE2	Novagen
ZX221	*rpsL* Δ*lipB* ::*FRT* ::*aac*	This chapter
QC144	Δ*lplA* ::*FRT* ::*kan*	This chapter
QC145	Δ*lplA* ::*FRT* ::*kan* Δ*lipB* ::*FRT* ::*cat*	This chapter
QC146	Δ*lplA* ::*FRT* Δ*lipB* ::*FRT*	This chapter
QC038	*rpsL lipA* ::*Tn1000 lplA* ::*Tn10*/pBAD322G	This chapter
QC090	*rpsL lipA* ::*Tn1000 lplA* ::*Tn10*/pQC005	This chapter
QC091	*rpsL lipA* ::*Tn1000 lplA* ::*Tn10*/pQC021	This chapter
QC092	*rpsL lipA* ::*Tn1000 lplA* ::*Tn10* pQC022	This chapter
QC064	*ompT hsdSB* (rB$^-$ mB$^-$) *gal dcm* (DE3) Δ*endA* Δ*recA* /pQC024	This chapter
QC096	*ompT hsdSB* (rB$^-$ mB$^-$) *gal dcm* (DE3) Δ*endA* Δ*recA*/pQC035	This chapter
QC049	*ompT hsdSB* (rB$^-$ mB$^-$) *gal dcm* (DE3)/pRARE2, pQC017	This chapter
QC108	*ompT hsdSB* (rB$^-$ mB$^-$) *gal dcm* (DE3)/pRARE2, pQC043	This chapter
QC164	*ompT hsdSB* (rB$^-$ mB$^-$) *gal dcm* (DE3)/pRARE2, pQC017, pQC056	This chapter
QC165	*ompT hsdSB* (rB$^-$ mB$^-$) *gal dcm* (DE3)/pRARE2, pQC043, pQC055	This chapter
QC166	Δ*lplA* ::*FRT* Δ*lipB* ::*FRT*/pTara/pQC035	This chapter
pBAD322G	Gm^R arabinose-inducible expression vector	(Cronan, 2006)
pTARA	Chl^R arabinose-inducible T7 polymerase	(Wycuff *et al.*, 2000)
pET19b	Amp^R T7 expression vector	Novagen
pET28b+	Kn^R T7 expression vector	Novagen
pET30b+	Kn^R T7 expression vector	Novagen
pET101/TOPO	Amp^R T7 expression vector	Invitrogen
pRSF-1b	Kn^R T7 expression vector with RSF origin	Novagen
pCR2.1	TOPO TA cloning vector	Invitrogen
pQC005	pBAD322G encoding a TA0513-4 operon	This chapter
pQC017	pET101 encoding N-terminal hexahistidine-tagged TA0514	This chapter
pQC019	pCR2.1 with TA0514 insert	This chapter
pQC020	pCR2.1 with TA051 insert	This chapter
pQC021	pBAD322G encoding TA0514	This chapter
pQC022	pBAD322G encoding TA0513	This chapter
pQC028	pCR2.1 encoding E2 LD gene	This chapter
pQC024	pET30 (a)+ encoding the E2 LD	This chapter
pQC034	pCR2.1 encoding C-terminal hexahistidine-tagged GcvH	This chapter
pQC035	pET28 (b)+ encoding C-terminal hexahistidine-tagged GcvH	This chapter
pQC043	pET19b encoding N-terminal hexahistidine-tagged TA0513m	This chapter
pQC055	pRSF1b encoding TA0514	This chapter
pQC056	pRSF1b encoding TA0513	This chapter

*CGSC, The Coli Genetic Stock Center.

Table 2-2. Oligonucleotides used in this chapter.

Oligonucleotides	**Sequence**
Q007	tagccatggttctcaattatactatgcat
Q008	tacaagcttacagggatatcgagacgtt
Q017	agcgagaaaaaagagtgacccattactacaagaaaggaaatcgttgtgtaggctggagctgcttc
Q018	aaaatccggcaaatcgaagagaaagttgcccgcatgggcgggtaacatatgaatatcctccttag
Q019	cccccactttttactcattctccacggagatgccgttttgtatcagtgtgtaggctggagctgcttcgaa
Q020	gtaatgacccagtgtaaattgggccattgatgtatggaattaagccatatgaatatcctcctt
Q025	tcagatcaccctcaaagc
Q026	caccatgcatcatcatcatcatcatatggaaggcaggcttctt
Q027	tagccatggaaggcaggcttctt
Q032	aatcatatgtacgaattcaaactgccagacataggtg
Q033	attgagctcttaaggctgctgtaccggagcc
Q049	tcatgacagaggtaccagagggttt
Q050	ttaatgatgatgatgatgttgtattaacttcctgtactccgatg
Q068	actcgagtcagatcaccctcaaagc
Q069	ttctagataaggaggagaccaatgcatcaccatcaccatcacatgcatatgatgtacagc

FIGURES

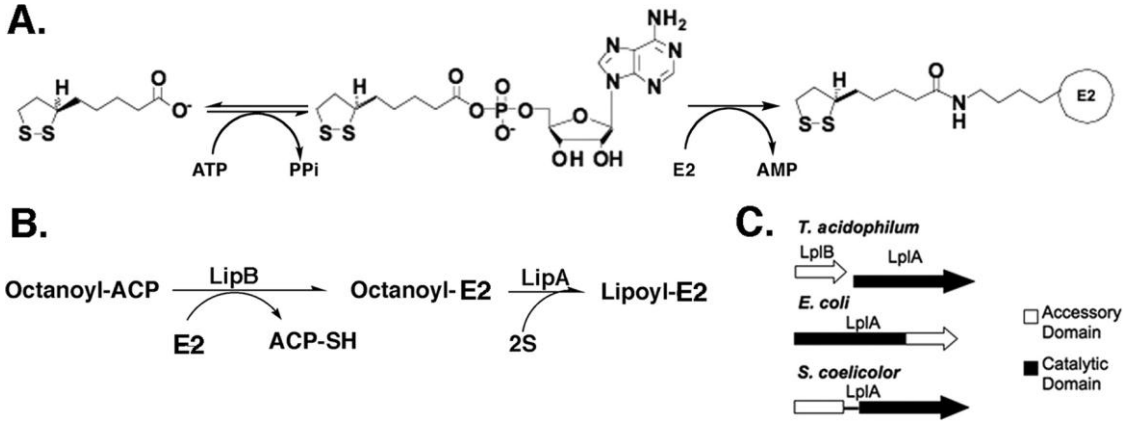


Figure 2-1. Lipoic acid metabolism in *E. coli*. Panel A: the lipoyl ligase (LplA) reaction that proceeds through the lipoyl-adenylate intermediate. In *E. coli* LplA acts to scavenge lipoic acid from the growth medium. Panel B: schematic of lipoic acid synthesis in *E. coli*. LipB transfers an octanoyl moiety from the fatty acid biosynthetic intermediate, octanoyl-acyl carrier protein, to the LD domain of a lipoate-accepting protein (in this case the E2 subunit of a 2-oxoacid dehydrogenase). The octanoylated LD 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. Panel C: the differing arrangements of genes and domains found in lipoate ligases in *T. acidophilum*, *E. coli*, and *Streptomyces coelicolor*. Only a single nucleotide lies between the *T. acidophilum* LplB and LplA coding sequences.

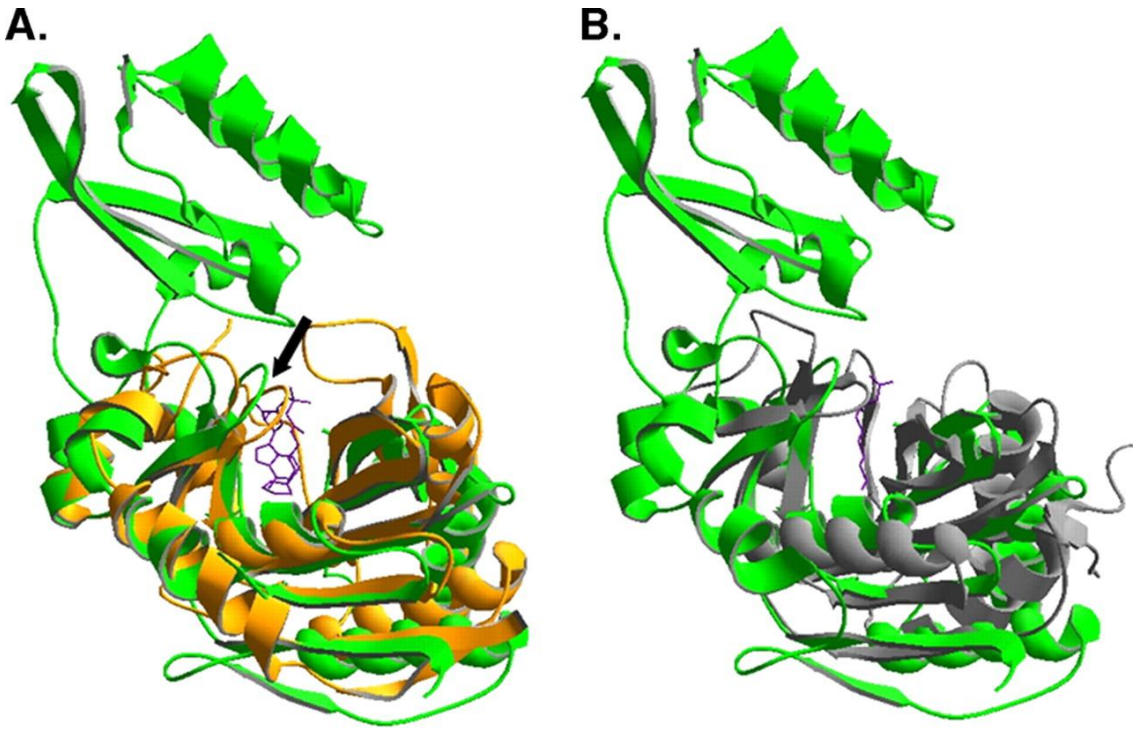


Figure 2-2. Alignments of LplA and LipB structures. Previously published crystal structures were aligned using DeepView (Guex *et al.*, 1997). Panel A: *E. coli* LplA (Protein Data Bank code 1X2H in *green*) aligned with *T. acidophilum* LplA (Protein Data Bank code 2ART in *orange*). The lipoyl-adenylate intermediate bound to *T. acidophilum* LplA is shown in *purple*. The adenylate binding loop is indicated with an *arrow.* Panel B: *M. tuberculosis* LipB (Protein Data Bank code 1W66 in *gray*) is aligned with the *E. coli* LplA structure of *panel A*. The *purple line* denotes the covalent decanoate adduct present in the *M. tuberculosis* LipB structure. The substrate binding pocket is conserved among members of the protein family. The accessory domain is not part of the binding pocket and appears to play an indirect role in catalysis.

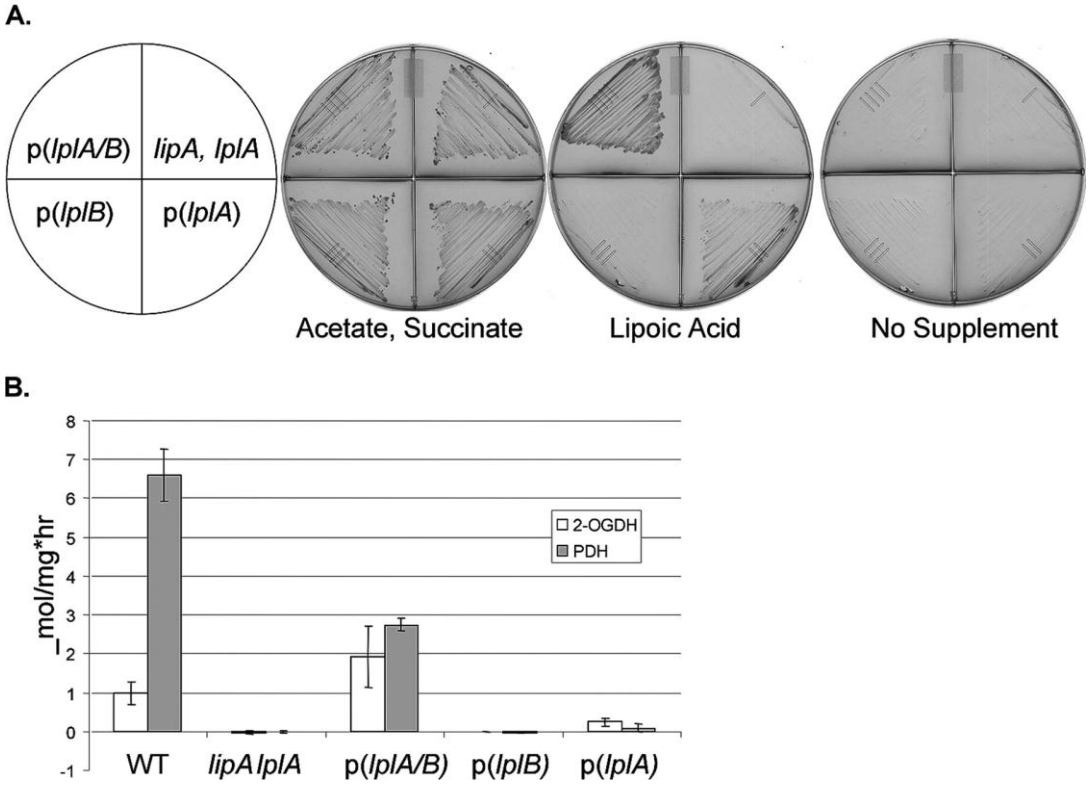


Figure 2-3. Complementation of an *E. coli* lipoyl ligase null mutant strain. The *lipA lplA* strain, TM131, was transformed with pBAD322G-derived plasmids, p (*lplA*), p (*lplB*), and p (*lplA plus B*), expressing the *T. acidophilum* genes (as indicated) from an arabinose-inducible promoter. The wild type (*WT*) and *lipA lplA* both with an empty vector are the control strains. The complementation assays were performed on M9 minimal agar containing 0.2% arabinose and 0.1% vitamin assay casamino acids. Where indicated, 5 mM sodium acetate plus 5 mM sodium succinate or 5 µg/ml lipoic acid was added. To prevent carryover of lipoic acid, before testing the strains were grown on the same medium containing 5 mM sodium acetate, 5 mM sodium succinate, and 24 mg/L gentamicin. Panel A: ability of expression of LplA and LplB alone or together to restore growth of *E. coli* strain TM131 (*lplA lipA*) when supplemented with lipoic acid. Representative plates of three replicate experiments are shown. Panel B: activation of the pyruvate dehydrogenase (*PDH*) and 2-oxoglutarate dehydrogenase (*OGDH*) complexes upon expression of LplA and LplB alone or together was assayed using an acetylated NAD analogue as a substrate for the overall reaction of the dehydrogenase complex. Results are reported as µMol of NAD analogue reduced/mg of cell extract h^{-1}. The error bars denote twice the S.D. from at least four assays.

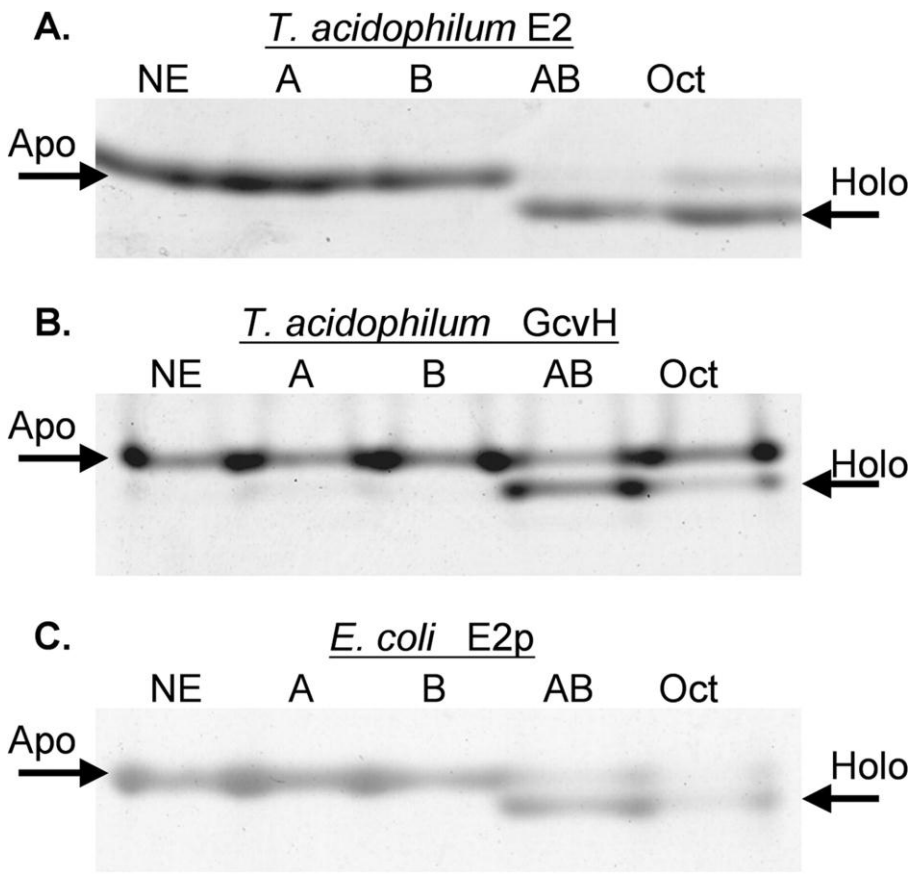


Figure 2-4. Gel shift assay for lipoyl domain modification. The modified lipoyl domain loses a charge upon modification of the target lysine residue resulting in more rapid migration on native gels than the unmodified domain. Representative gels of three independent experiments are shown. Lane designations given in parentheses below are the same in all three panels. Lane 1, no enzyme (NE); lane 2, LplA (A); lane 3, LplB (B); lane 4, LplA plus LplB (AB); lane 5, LplA plus LplB with octanoate in place of lipoate (Oct). Panel A: modification of the *T. acidophilum* branched chain dehydrogenase E2 lipoyl domain by lipoate or octanoate attachment. Panel B: modification of the putative *T. acidophilum* GcvH. *Panel C*, modification of the *E. coli* hybrid E2p domain. Note that efficient lipoylation of the *E. coli* domain required a 16-fold longer incubation time than the *T. acidophilum* domains.

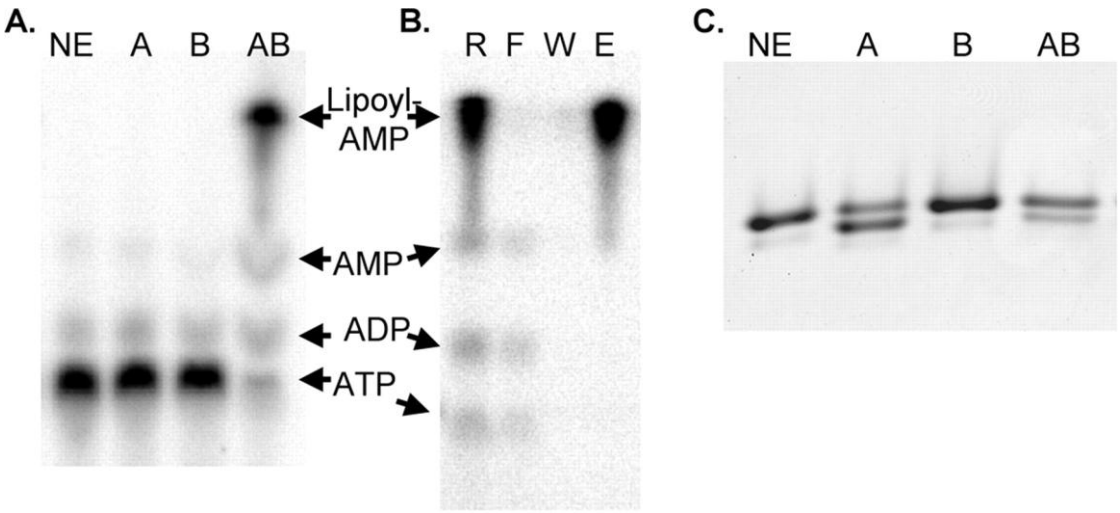


Figure 2-5. The role of LplB in lipoyl ligation. Panel A: synthesis of ^{32}P-labeled lipoyl-adenylate from [α-^{32}P]ATP was analyzed by cellulose thin layer chromatography and visualized by autoradiography. Lane 1, no enzyme (NE); lane 2, LplA (A); lane 3, LplB (B); lane 4, LplA plus LplB (AB). Panel B: demonstration of enzyme-bound lipoyl-AMP with a centrifugal filter device. Lane 1, reaction (R); lane 2, flow-through (F); lane 3, final wash (W); lane 4, retained enzyme fraction (E). Panel C, transfer of lipoate from synthetic lipoyl-adenylate to LD assayed by gel shift. Lanes 1–4 are labeled as in panel A.

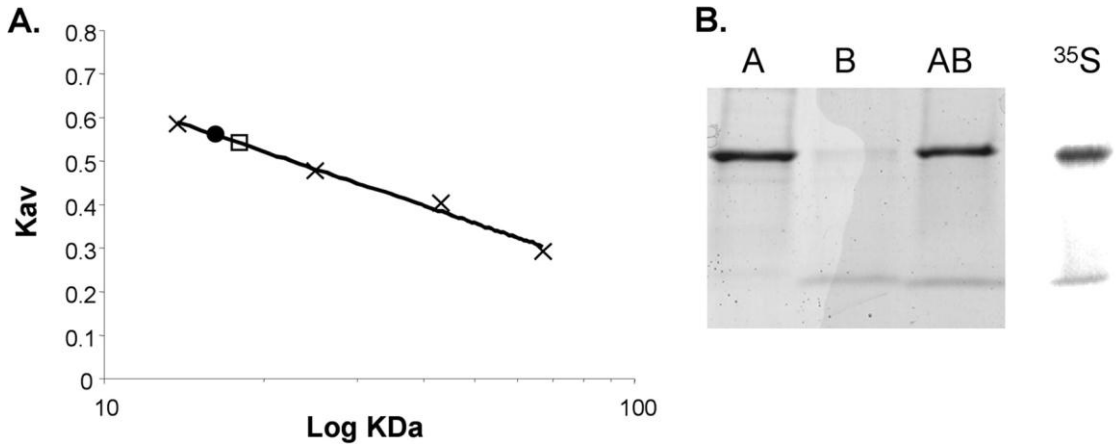


Figure 2-6. Characterization of the LplA-LplB complex. Panel A: size exclusion chromatography of the purified proteins. The calibration curve was prepared using (in order of ascending molecular mass) ribonuclease A, chymotrypsinogen, ovalbumin, and bovine serum albumin as standards, shown as "×" symbols. Hexahistidine-tagged LplA is shown as a *circle* and was estimated to have a mass of 16.6 ± 0.5 kDa, whereas mass spectrometry gave a mass of 30.8 kDa. Hexahistidine-tagged LplB eluted after the linear range of the column (<10 kDa), although it had a mass of 11.2 kDa as determined by mass spectrometry. The lipoyl ligase (LplA-LplB) complex is shown as a *square* and elutes soon after LplA and has an estimated mass of 18.2 ± 0.4 kDa and a mass calculated from the individual mass spectra of 41.1 kDa. The estimated sizes are the average of four runs with independent protein samples. The S.D. is also reported. Panel B: demonstration of the lipoyl ligase complex by Ni^{2+} affinity chromatography. The elution products were subjected to SDS-PAGE on a 4–20% gradient Tris-glycine gel and visualized with Coomassie Blue R-250. LplB copurified with hexahistidine-tagged LplA (*lane AB*). Hexahistidine-tagged LplA and LplB were also purified individually as references (*lanes A* and *B*, respectively). The *rightmost lane* is a phosphorimaging scan of L-[^{35}S]methionine-labeled lipoyl ligase complex after SDS-PAGE on a 4–20% gradient Tris-glycine gel.

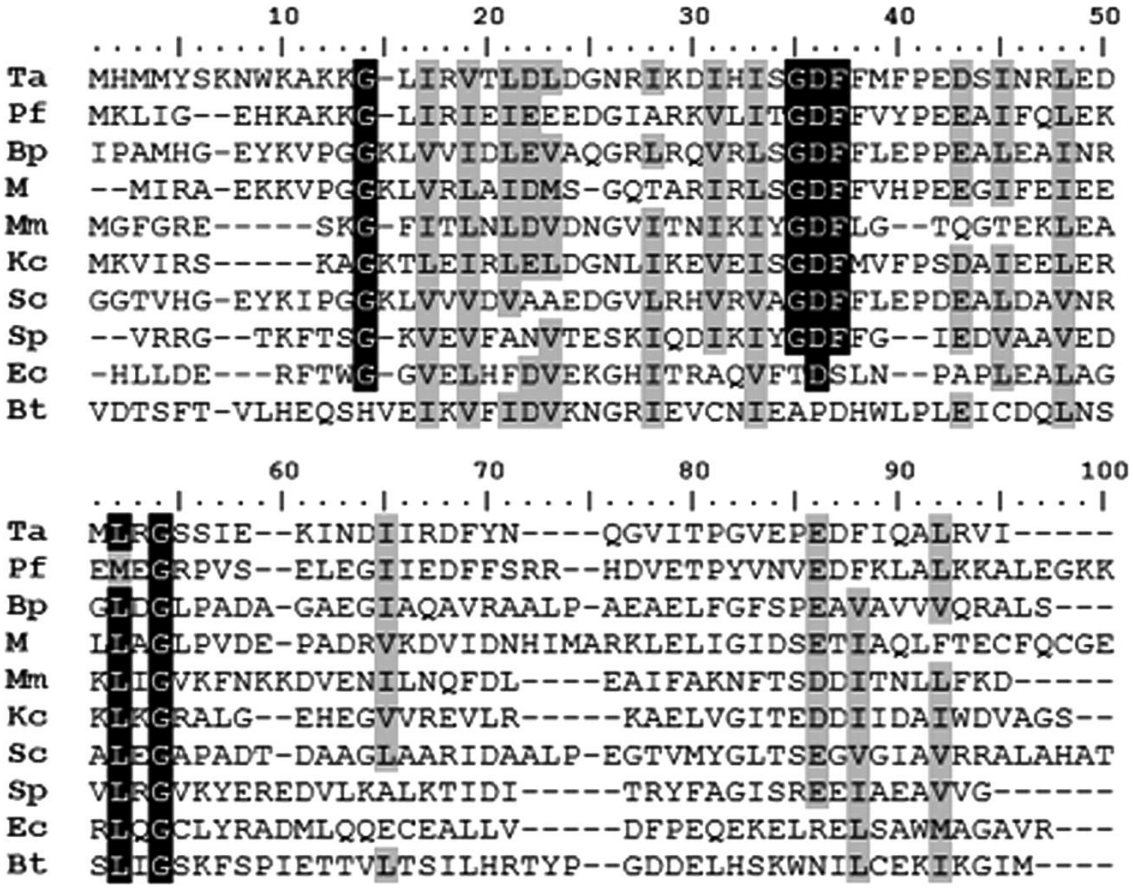


Figure 2-7. ClustalW alignment of representative LplB homologues**.** The first six sequences are representative single domain LplB homologues. Black shading denotes identical residues whereas gray shading denotes residues of similar properties. The putative *S. coelicolor* (*Sc*) ligase has an N-terminal LplB domain. *E. coli* LplA (*Ec*) is the canonical C-terminal domain ligase. The putative *S. pneumoniae* (*Sp*) LplA also has a C-terminal domain. The *Bos taurus* (*Bt*) bLT has a C-terminal domain of unknown function. Conserved Gly residues correspond to flexible loops in the *E. coli* LplA structure (Fujiwara *et al.*, 2005). Most conserved residues are predicted to be in the interior of the protein and probably serve structural roles. Close relatives to LplB, including single domain proteins, contain a GDFF motif. The aspartate residue of this motif (Asp-41 in LplB) is well conserved among LplB homologues. The Genbank accession numbers of the aligned sequences (all previously published) are (from top to bottom) NP_393989, NP_579363.1, NP_880065, YP_687213.1, ZP_02512737, ACB07333.1, CAA18910, NP_345629, AAC77339, and BAA24354. *Ta*, *T. acidophilum*; *Pf*, *Pyrococcus furiosus*; *Bp*, *Bordetella pertussis*; *M*, *methanogen*, *uncultured*; *MM*, *Mycoplasma mycoides*; *Kc*, *Korarchaeum cryptofilum*.

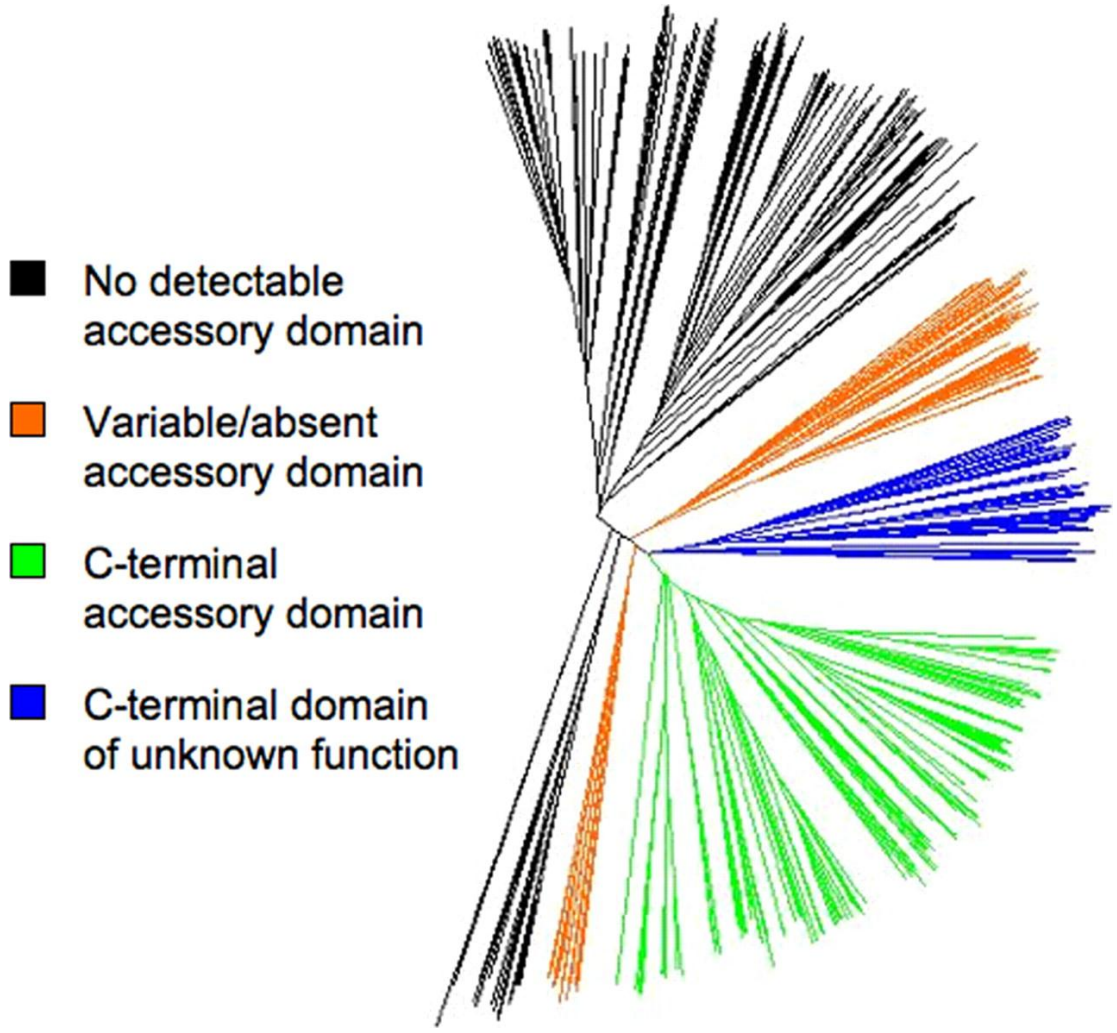


Figure 2-8. Phlylogeny of lipoyl ligase homologues. The lipoyl ligase subtree of the BPL_LplA_LipB Pfam family (PF03099) are displayed using Dendroscope (Huson *et al.*, 2007). Domain architecture is annotated by color. *Black* clade proteins contain a catalytic domain but no detectable LplB homologue in the genome. The *green* clade proteins have a C-terminal accessory domain. *Blue* clade proteins are found only in mammalian genomes and have a C-terminal domain of unknown function that has only background sequence similarity with the *green* clade C-terminal accessory domains (Figure 2-7). The *orange* clade proteins have a variable domain architecture including N-terminal accessory domains and independently coded accessory domains. The deeply branching and multiclade presence of LplAs with independent LplBs suggests that this architecture is an evolutionary relic. The tree root, determined with biotin ligases and octanoyltransferases as outgroups, is within the *black* clades. This suggests that the catalytic domain was originally independent of any other domains in the common ancestral protein.

CHAPTER 3

LIPOIC ACID SYNTHESIS: A NEW FAMILY OF OCTANOYLTRANSFERASES GENERALLY ANNOTATED AS LIPOATE PROTEIN LIGASES

INTRODUCTION

Although Bacilli have an unusually large number of lipoic acid-dependent enzymes, the annotation of their genomes argues that these bacteria have an incomplete lipoic acid synthesis pathway. Although *Bacillus subtilis* encodes a readily recognized and functional LipA homologue (Martin *et al.*, 2009), 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 firmicutes including important organisms such as the Staphylococci and Clostridia. I 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 (Sambrook *et al.*, 2001). 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

Strains used in this chapter are listed in Table 3-1, plasmids in Table 3-2, and primers in Table 3-3. *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 optical density at 600 nm using a Beckman DU600 spectrophotometer unless otherwise indicated. Standard techniques (Sambrook *et al.*, 2001) 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 (Henry *et al.*, 1992) 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 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 sodium acetate, 5 mM sodium 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 (Cronan,2006). 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 Q044 and Q046 and inserted into the NcoI (using a PciI end) and HindIII sites of pBAD322G to give pQC007. Note that the construction of pQC007 is incorrect in the publication corresponding to this chapter. The *E. coli* MG1655 *lipB* coding sequence was obtained by NcoI and HindIII digestion of pSJ112 (Jordan *et al.*, 2003). 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). 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 (Studier *et al.*, 1986).

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 (1.25 V/cm) 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 sodium acetate, 5 mM sodium 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 optical density 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 sodium acetate, 5 mM sodium 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 (Overbeek *et al.*, 2005). 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 (Finn *et al.*, 2010). An unweighted alignment of protein sequences was performed using T-Coffee (Notredame *et al.*, 2000) 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 (Waterhouse *et al.*, 2009) so only the catalytic domain remained. A minimum evolution tree was constructed with bootstrap analysis of 1000 replicates using Mega4 (Tamura *et al.*, 2007) 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 (Martinez *et al.*, 2010). 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 (Chapter 2). The modification state of AcpP was verified using conformationally sensitive native PAGE using 20% acrylamide, 2.5 mM urea gels buffered with Tris-glycine (Cronan *et al.*, 2009). 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 (Quadri *et al.*, 1998). 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 described (Chapter 2). Pooled fractions containing Sfp were precipitated with ammonium sulfate (35−55% of saturation) as described previously (Quadri *et al.*, 1998) 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 (Cronan *et al.*, 2009). 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 (Jiang *et al.*, 2006). 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 (Cronan *et al.*, 2009). Proteins were quantified by use of extinction coefficients calculated using PROTPARAM on the EXPASY website (Gasteiger *et al.*, 2003). 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, I used the lipoic acid auxotrophic strain QC146. The strain was grown in LB with 0.1% glucose, 5 mM sodium acetate, and 5 mM sodium succinate (pH 7.0) to an optical density 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 (Chapter 2) except that the reducing agent was 5 mM TCEP instead of DTT. Unmodified LipM was obtained using the *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. I 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 (Chapter 2) 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 (Hallmann *et al.*, 2008; Hermes *et al.*, 2009). 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 (Green *et al.*, 1995).

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, I 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) (Laskey *et al.*, 1975). 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 (Chapter 2). 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.

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 (Hermes *et al.*, 2009). 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, I 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 (Hermes *et al.*, 2009). 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 (Clarke *et al.*, 1976). The eight cosmid clones that allowed growth of the *lipB lplA* strain carried overlapping fragments of the *B. subtilis* genome (Table 3-3) 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 3-1). Because the growth observed could be caused by either octanoyltransferase activity or high-level lipoyl ligase activity, I 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 3-8 and 3-9), YqhM lacks lipoate ligase activity and instead has octanoyltransferase activity (Figure 3-1). Therefore, I have renamed *yqhM* as *lipM.*

Purification of GcvH

To provide a substrate for *in vitro* studies of LipM, I 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 3-2A), but electrospray mass spectrometry gave a major peak at 14944.2 amu (Figure 3-2B). This is 1.2 amu lower than that theoretically predicted, which I attribute to deamidation of the protein (which results in a negligible mass difference) perhaps at N73 which should be unusually labile because it is followed by a glycine residue (Jordan *et al.*, 1997; Robinson *et al.*, 2001). Deamidation of other LDs has been observed and does not affect modification (Jordan *et al.*, 1997). Note that GcvH migrates aberrantly during SDS−PAGE analysis (Figure 3-2A). 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, I purified the protein obtained after high-level expression in *E. coli*, and it appeared homogeneous by SDS−PAGE (Figure 3-2A) 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 3-2D). 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-translationally modified [the initiator methionine was retained as expected from the specificity of *E. coli* methionine aminopeptidase (Frottin *et al.*, 2006; Xiao *et al.*, 2010)]. 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 3-2C). To determine if the protein was post-translationally modified, I digested LipM with trypsin and subjected the resulting peptides to liquid chromatography−tandem mass spectrometry analysis. I 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 (Figure 3-3). 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 (Zhao *et al.*, 2005).

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 (Ma *et al.*, 2006). 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, I 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 (Henry *et al.*, 1992).

To reduce the level of the octanoyl-enzyme intermediate, I added triclosan, an inhibitor of fatty acid biosynthesis (McMurry *et al.*, 1998), to the medium during LipM expression. I 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 (Hallmann *et al.*, 2008). This method showed that the addition of triclosan resulted in a significant reduction in the

level of the octanoyl-enzyme intermediate (Figure 3-4), and thus by addition of triclosan and use of the *fabA* strain, I obtained LipM in a largely unmodified and active form.

In vitro Demonstration of LipM Activity

I 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 3-5). 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, I expect that the relative levels of transfer in the forward and reverse directions reflect the equilibria of the transfer reactions. I 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 3-6). This is not unexpected as CoA is a substrate mimic of ACP. To determine which of the substrates LipM preferred, I 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 modification of GcvH, although some modification caused by transfer from octanoyl-CoA was observed (Figure 3-6).

C150 and K165 Are Key LipM Catalytic Residues

I constructed point mutations in *lipM* to test the importance of the predicted active site residues. The residues mutated were the Cys found to be modified by LC-MS/MS (LipM C150), and the conserved lysine found in all members of PF03099 (LipM K165) (Reche,2000). 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 3-5C).

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 3-7 and 3-8). 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 (Hillis *et al.*, 1993). 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 3-8). 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 necessary to determine if there are physiologically relevant differences between the LipB and LipM types of octanoyltransferase.

Using the SEED subsystem database (Overbeek *et al.*, 2005), I 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, I 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 (Hermes *et al.*, 2009). I and others have found that overproduction of *E. coli lplA* complements *lipB* null mutants (Figure 3-1) (Morris *et al.*, 1994; Hermes *et al.*, 2009), and thus, I 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 3-1) (Hermes *et al.*, 2009). Other possibilities are that our library is biased and lacked *yhfJ* clones and that *yhfJ* does not encode a lipoate ligase. I 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 (Ferrari *et al.*, 1981). Although I used a vector with a single-copy F factor replication origin vector, basal expression from the second *trfA*-dependent replication origin (Wild *et al.*, 2002) 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 (Wild *et al.*, 2002).

Upon purification of LipM, I 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 (Ma *et al.*, 2006), 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, I 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 (Hermes *et al.*, 2009). 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 (Zhao *et al.*, 2005), whereas LipM C150A does not. Moreover, the LipB C169S protein forms a dead-end octanoyl ester (Zhao *et al.*, 2005), 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 (Reche,2000). Loss of the charge of the conserved lysine side chain inactivates *M. tuberculosis* LipB (Ma *et al.*, 2006), whereas the LipM mutant protein retained some activity (Figure 3-6). 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 (Schulz,1992). 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 (Hermes *et al.*, 2009), 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 (Chapter 2)(Nesbitt *et al.*, 2005). 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 (Nesbitt *et al.*, 2005). 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 (Chapter 2), 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 (Chapter 2) (Posner *et al.*, 2009). Although the absence of an accessory domain indicates that the short proteins annotated as lipoate ligases lack lipoyl ligase activity (Chapter 2), 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 (Chapter 2). 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* (Chapter 2).

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 3-7). 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 (Doolittle, 1994; Omelchenko *et al.*, 2010). 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 (Doolittle ,1994).

TABLES

Table 3-1. Strains used in this chapter.

strain	relevant characteristics	source
MG1655	*rph-1*	CGSC*
W3110	*rph-1* IN (*rrnD-rrnE*)	CGSC*
JK1	*rpsL8*	(Morris *et al.*, 1995)
168	*trpC2*	BGSC*
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	(Morris *et al.*, 1995)
TM131	*rpsL lipA*::Tn1000 *lplA*::Tn10	(Morris *et al.*, 1995)
Acella DE3	*ompT hsdS*B (rB- mB-) *gal dcm* (DE3) *ΔendA ΔrecA*	EdgeBio
MFH120	JC7623 *lacZ*ΔM15 Δ (*fabA-lacZ*)1 (Hyb)*cat fadAB poxB*::pMFH23 (*fabA*$^{+}$)	(Henry *et al.*, 1992)
ZX221	*rpsL8 ΔlipB*::FRT::*cat*	(Chapter 2)
QC144	*rph-1 ΔlplA*::FRT::*aph*	(Chapter 2)
QC146	*rph-1 ΔlplA*::FRT *ΔlipB*::FRT	(Chapter 2)
QC038	*rpsL lipA*::Tn1000 *lplA*::Tn10/pBAD322G	(Chapter 2)
QC035	*rpsL*/pBAD322G	(Chapter 2)
EMM99	*E. coli* BL21 (DE3)/pEM88	(Martinez *et al.*, 2010)
QC032	*rpsL lipB::Tn1000 lplA::Tn10*/pQC004	This chapter
QC034	*rpsL lipB::Tn1000 lplA::Tn10*/pQC006	This chapter
QC036	*rpsL lipB::Tn1000 lplA::Tn10*/pQC007	This chapter
QC057	*rpsL lipB::Tn1000 lplA::Tn10*/pBAD322G	This chapter
QC067	EPI300 *ΔlplA*::FRT::*aph*/pEAK2	This chapter
QC068	EPI300 *ΔlplA*::FRT::*aphΔlipB*::FRT::*cat*	This chapter
QC069	EPI300 *ΔlplA*::FRT *ΔlipB*::FRT	This chapter
QC087	*rpsL lipA::Tn1000 lplA::Tn10*/pQC004	This chapter
QC088	*rpsL lipA::Tn1000 lplA::Tn10*/pQC006	This chapter
QC089	*rpsL lipA::Tn1000 lplA::Tn10*/pQC007	This chapter
QC097	Acella DE3/pQC015	This chapter
QC101	EPI300 *ΔlplA*::FRT *ΔlipB*::FRT/pQC039	This chapter
QC111	EPI300/pCC1FOS	This chapter
QC112	EPI300 *ΔlplA*::FRT *ΔlipB*::FRT/pCC1FOS	This chapter
QC134	*rph-1* IN (*rrnD-rrnE*)1 DE (*fabA-lacZ*)1 (Hyb)*cat*	This chapter
QC142	*rph-1* IN (*rrnD-rrnE*)1 DE (*fabA-lacZ*)1 (Hyb)*cat*/pCY598, pQC015	This chapter
QC161	*rph-1 ΔlplA*::FRT *ΔlipB*::FRT/pQC057, pTARA	This chapter

*CGSC, The Coli Genetic Stock Center. BGSC, The Bacillus Genetic Stock Center.

Table 3-2. Plasmids Used in this chapter.

plasmid	relevant characteristics	source
pBAD322G	arabinose inducible expression vector	(Cronan, 2006)
pTARA	pACYC origin, arabinose inducible T7 polymerase	(Wycuff *et al.*, 2000)
pCC1Fos	cosmid cloning vector	Epicenter
pCY598	RSF origin, arabinose inducible T7 polymerase	(Cronan,2003)
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	(Kouzminova *et al.*, 2004)
pSJ112	*E. coli lipB* expression plasmid	(Jordan *et al.*, 2003)
pNRD136	*sfp* expression plasmid	(De Lay *et al.*, 2007)
pMM88	*B. subtilis acpP* expression plasmid	(Martinez *et al.*, 2010)
pQC004	*B. subtilis* 168 *yqhM*	This chapter
pQC006	*E. coli lipB*	This chapter
pQC007	*E. coli lplA*	This chapter
pQC015	*B. subtilis* 168 N-terminally hexahistidine-tagged *yqhM*	This chapter
pQC036	*B. subtilis gcvH* C-terminally hexahistidine tagged	This chapter
pQC039	cosmid carrying *B. subtilis* 168 (2538341..2570341) containing *yqhM*	This chapter
pQC057	*B. subtilis* 168 *gcvH* C-terminally hexahistidine-tagged	This chapter

Table 3-3. Oligonucleotides used in this chapter.

primer	sequence
Q005	TAAGAATTCACCATGCAAAAAGAAACTTGGCG
Q006	TACGTCTAGAATCTTCCATACTTGGTGTTGTC
Q022	CACCATGCATCATCATCATCATCATATGCAAAAAGAAACTTGGCG
Q023	CCATTACAAGTTTACACTAATGAACTTG
Q044	ACATGTCCACATTACGCCTGCT
Q046	CGGCAAATCGAAGAGAAAGTT
Q047	CATATGAGCATACCAAAAGATTTGCG
Q048	TTAATGATGATGATGATGATGGTCTTCTTGTGTCATCTCTTCGTATTG
Q051	CACCTAAATAGCTTGGCG
Q053	TGCAGGTCGACTCTAGAG
Q133	GGTTGAGGGGCGCGCGGTGGCGGGAAGC
Q134	GCTTCCCGCCACCGCGCGCCCCTCAACC
Q135	GTGGTTGAGGGGCGCCGTGTGGCGGGAAGCGCG
Q136	CGCGCTTCCCGCCACACGGCGCCCCTCAACCAC
Q137	TCCGCGGTCATCTGTTGCTTTTGACGCGCCTTCG
Q138	CGAAGGCGCGTCAAAAGCAACAGATGACCGCGGA
Q139	CGCGGTCATCTGTTTCTTTTGACGCGCCTTC
Q140	GAAGGCGCGTCAAAAGAAACAGATGACCGCG

FIGURES

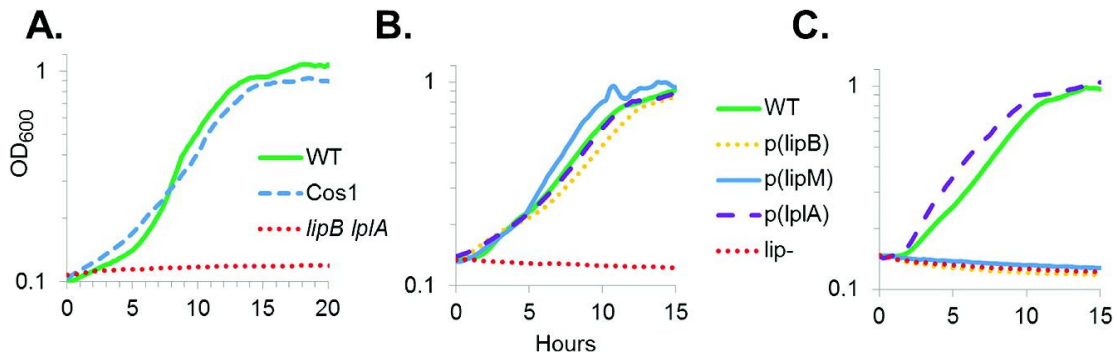


Figure 3-1. Complementation of *E. coli* lipoic acid auxotrophs. Growth curves of lipoic acid auxotrophs carrying various plasmids are shown. Panel A: Growth tests for *lipB* complementation in minimal glycerol medium. Growth curves of the wild type (WT) and *lipB lplA* strains with the empty pCC1FOS vector are shown. Cosmid isolate 1 (Cos1) or pQC039 was also tested in the *lipB lplA* strain. Panel 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. Panel 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.

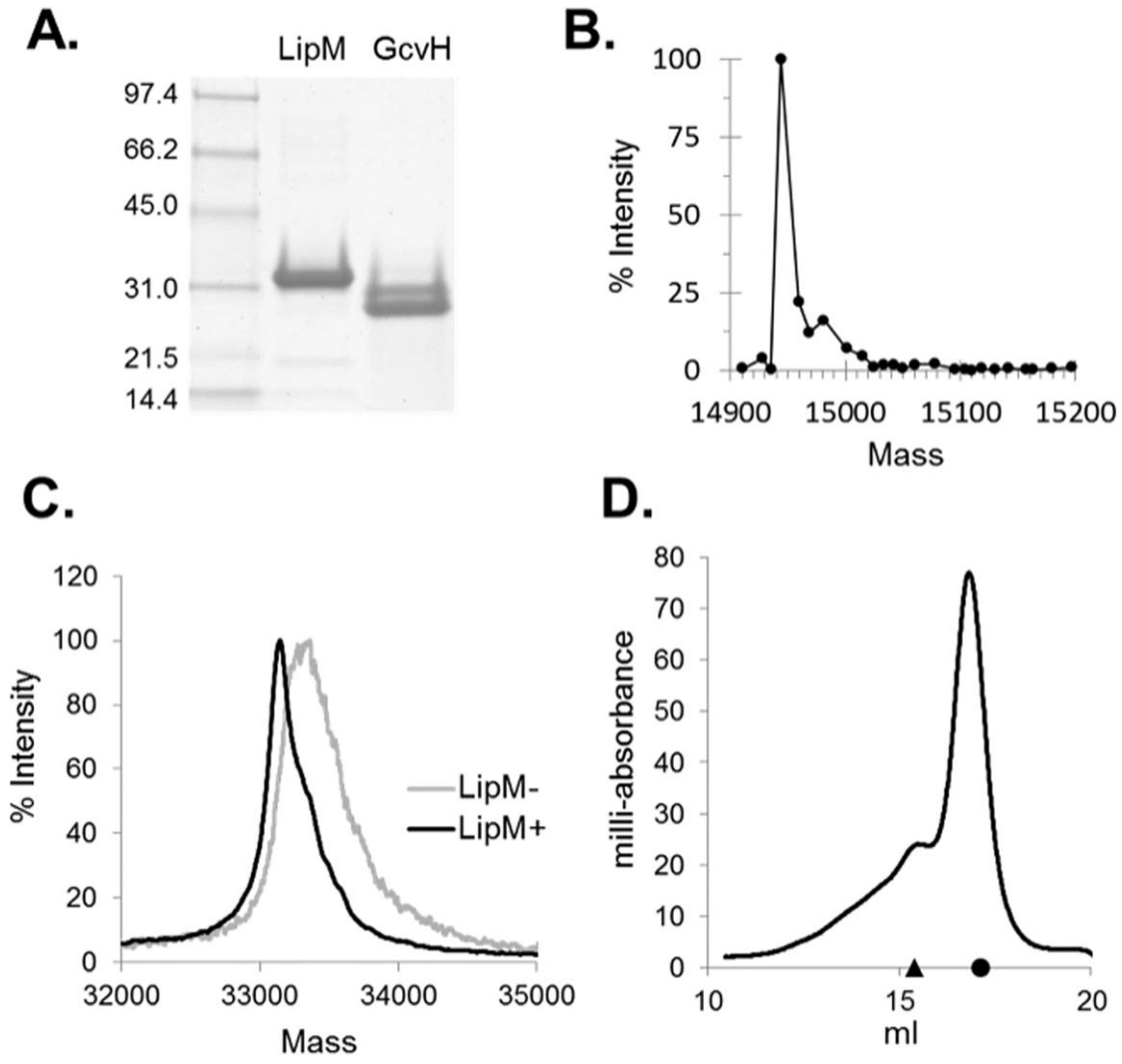


Figure 3-2. Analysis of purified proteins. Panel A: SDS−PAGE of 0.2 µMol of purified LipM and 0.2 µMol of purified GcvH. Molecular mass standards are indicated in kilodaltons. Panel B: Electrospray ionization mass spectrum of GcvH. Calculated masses are represented by black circles. Panel C: MALDI mass 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 *ΔfabA* strain grown with triclosan and oleate. The peak mass values for the LipM− and LipM+ proteins were 33300 and 33179, respectively. Panel 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.

A.

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

B.

```
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
```

Figure 3-3. Liquid chromatography−tandem mass spectrometric analysis of LipM tryptic peptides. Panel 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. Panel B: Sequence coverage of LipM. The LipM peptide sequences detected are shown in bold.

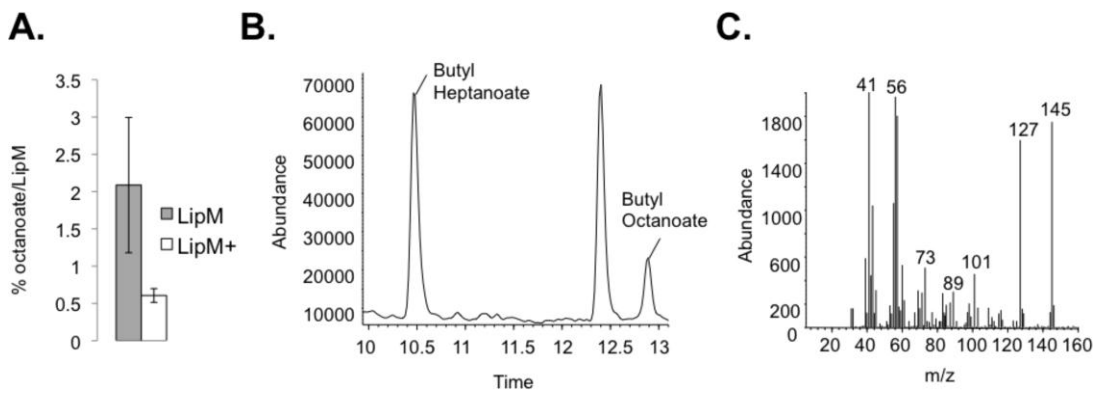


Figure 3-4. Gas chromatography−mass spectrometric analysis of LipM-bound octanoate. LipM-bound octanoyl moieties were assayed by transesterification to the butyl esters followed by GC−MS analysis. Panel A: The values are the molar percentages of octanoic acid per LipM preparation. The gray bar is for 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 *ΔfabA* strain. Panel B: Representative gas chromatogram of a LipM preparation. The butyl heptanoate internal standard and the analyte, butyl octanoate, are indicated. Panel C: Mass spectrum of the butyl octanoate from a LipM preparation.

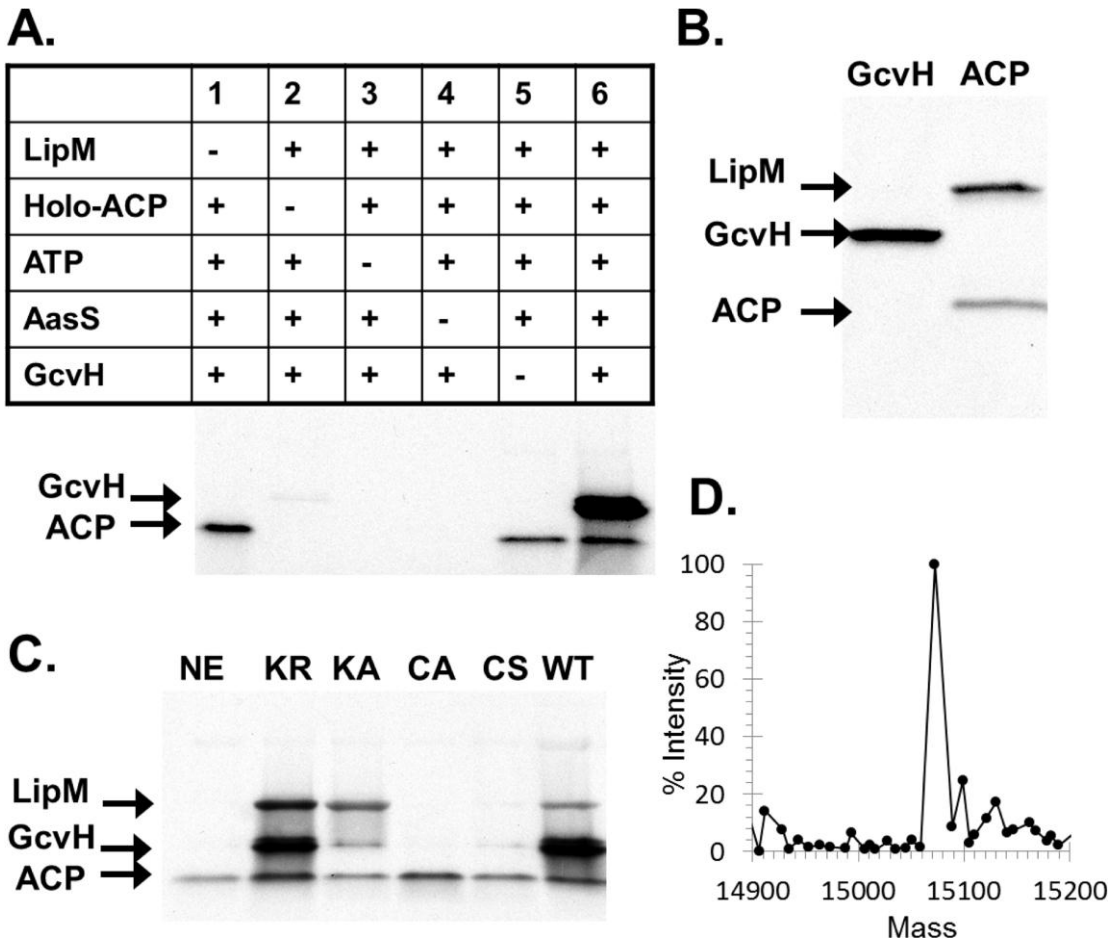


Figure 3-5. Octanoyltransferase activities of wild type and mutant LipM proteins. Assay of the transfer of the octanoyl group from octanoyl-ACP to *B. subtilis* GcvH octanoyl-ACP as the substrate. Autoradiograms of dried SDS−PAGE gels are shown. Panel A: The [1-^{14}C]octanoyl-ACP was synthesized via AasS added to the reaction mixture. Synthesis of octanoyl-ACP required AasS, ATP, and holo-ACP, whereas formation of octanoyl-GcvH required apo-GcvH, LipM, and octanoyl-ACP. Panel B: Transfer of octanoate from purified [1-^{14}C]octanoyl-LipM to an equimolar amount of either GcvH or holo-ACP as indicated. Panel 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. Panel 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 3-2) and in good agreement with the octanoyl moiety mass (126.2 amu).

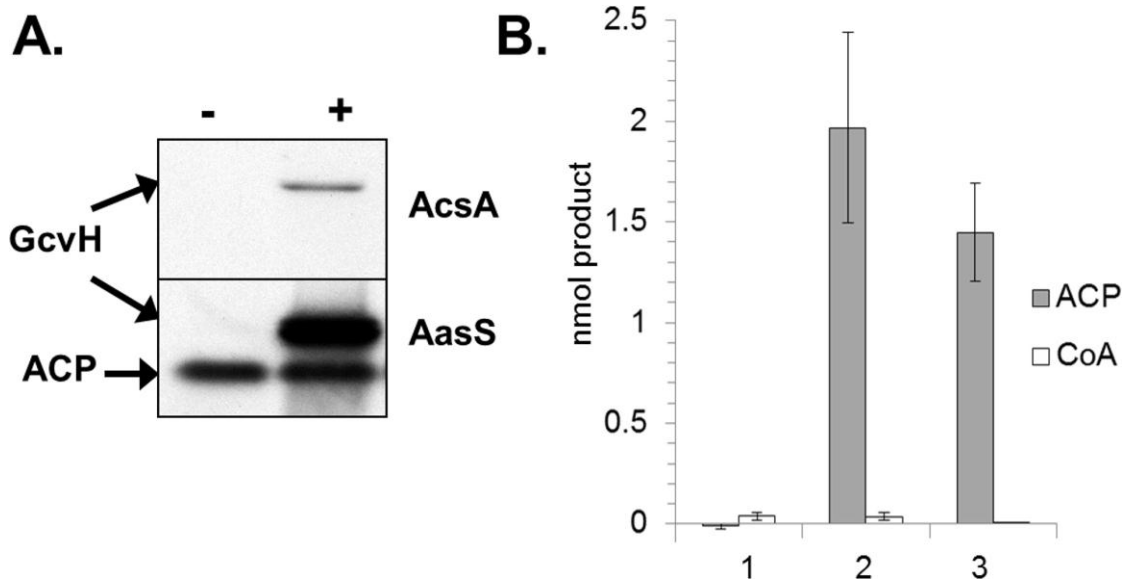


Figure 3-6. Comparison of the octanoyl thioesters of ACP and CoA as LipM substrates. Assays of the transfer of the octanoyl group to *B. subtilis* GcvH using either octanoyl-CoA or octanoyl-ACP. Page A: Autoradiograms of dried SDS−PAGE gels are shown. [1-^{14}C]octanoate was converted to [1-^{14}C]octanoyl-ACP or [1-^{14}C]octanoyl-CoA by AasS or acyl-CoA synthetase (AcsA), respectively. The presence (+) or absence (−) of LipM is indicated. Page 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.

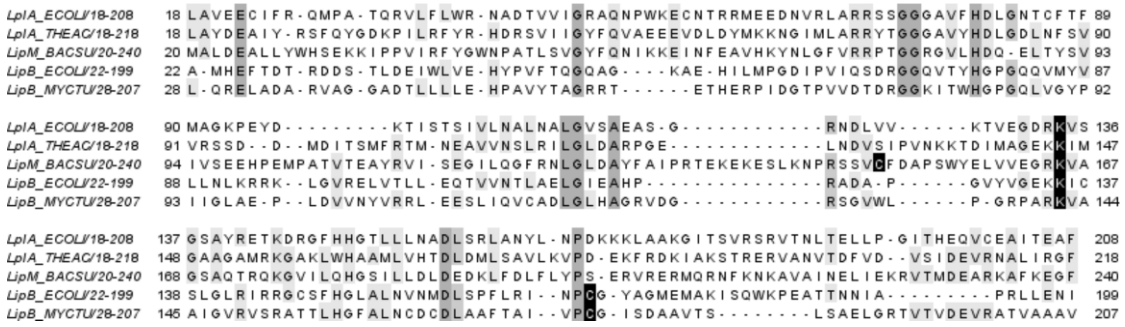


Figure 3-7. Alignment of LipM with enzymatically characterized LipB and LplA proteins. The alignment was performed as described in materials and methods and displayed using Jalview (Waterhouse *et al.*, 2009). The sequence name indicates the enzyme type; the Uniprot code indicates the organism of origin, 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.

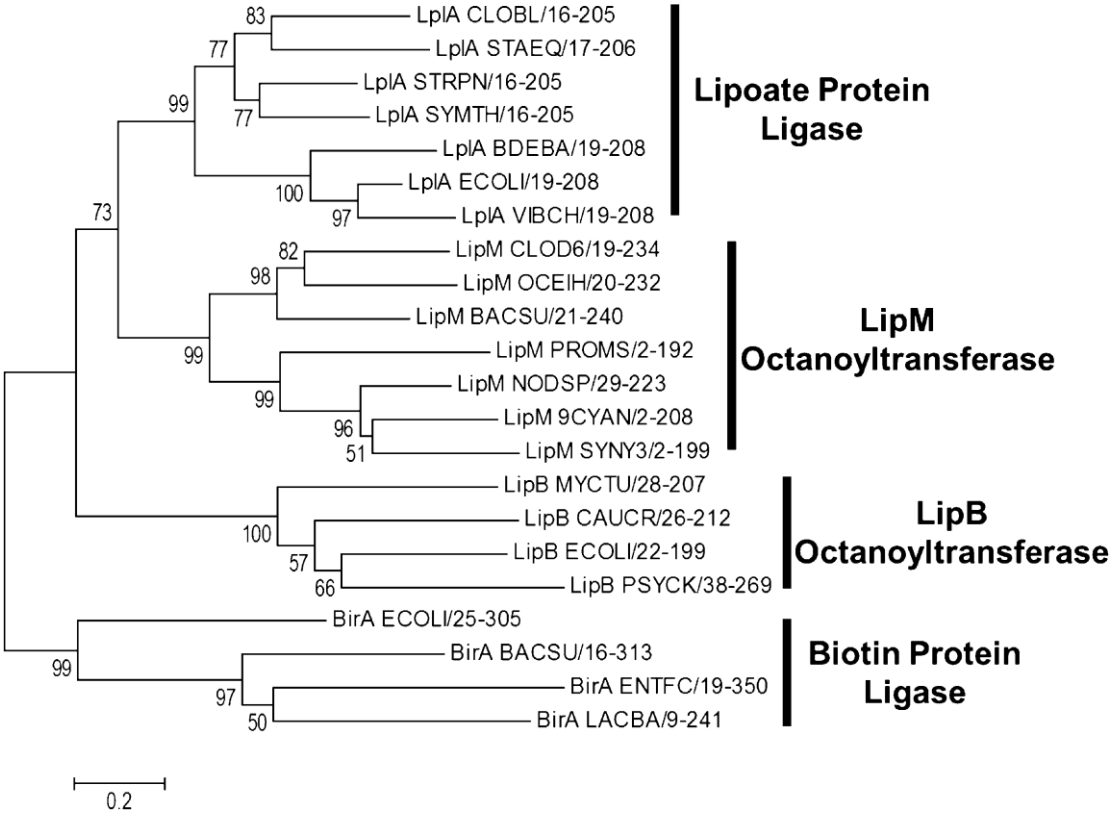


Figure 3-8. Phylogeny of LipM. The minimum evolution tree of selected PF03099 protein sequences with bootstrap percentage confidence values shown for each branch is given. Phylogenetic analyses were conducted as described in Materials and Methods. The scale bar corresponds to a 20% difference in compared 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.

CHAPTER 4
A NOVEL TWO-GENE REQUIREMENT FOR THE OCTANOYLTRANSFER REACTION OF *BACILLUS SUBTILIS* LIPOIC ACID BIOSYNTHESIS

INTRODUCTION

Homologues of the *E. coli* lipoic acid metabolism proteins are found in all domains of life, and thus unraveling the pathways by which this cofactor is synthesized and transferred to lipoate-dependent proteins is of broad biological significance. In contrast to the wealth of knowledge available on lipoic acid synthesis and utilization in *E. coli*, the existing information about these pathways in Gram-positive bacteria is fragmentary. It has been found that *Listeria monocytogenes* defective in proteins homologous to the *E. coli* LplA enzymes are unable to scavenge lipoic acid for modification of lipoyl domains (Keeney *et al.*, 2007). However, *L. monocytogenes* is a natural lipoate auxotroph and does not encode the enzymes necessary for lipoic acid biosynthesis. Lipoic acid synthesis and attachment to target proteins is less understood in other organisms. Despite the presence of homologues to the *E. coli* enzymes in fungi, plants, protists and mammals, the mechanistic details of lipoic acid synthesis still remain unclear.

Since *B. subtilis* cells grown on minimal media were known to contain essential lipoate-modified proteins, this bacterium must synthesize lipoic acid, and this was confirmed by demonstration of a functional lipoyl synthase (Martin *et al.*, 2009). Moreover, the lipoate requirement of *B. subtilis* Δ*lipA* strains for growth in minimal medium was bypassed by addition of acetate and a mixture of three short-branched-chain carboxylic acids: 2-methyl butyrate, isobutyrate, and isovalerate, metabolites that yield the products of two lipoylated enzymes, PDH and BKDH (acetate utilization is mediated by acetyl-CoA synthetase, the product of the *acs* gene, while an unknown enzyme converts the carboxylic acids to their CoA esters *in vivo*). Succinate supplementation to bypass the OGDH deficiency engendered by lipoic acid starvation was not required (Martin *et al.*, 2009).

However, despite the presence of a functional LipA, the *B. subtilis* genome contained no open reading frame (ORF) that resembled the *E. coli* LipB octanoyltransferase, an activity required for production of the substrate for LipA. Recently, an LplA homologue named LipM was identified and shown to have octanoyltransferase activity *in vitro* (Chapter 3). We began the present study due to paucity of information on lipoic acid biosynthesis in Gram-positive bacteria. Although LipM together with LipA should be sufficient for lipoic acid biosynthesis, the only *in vivo* analyses reported were performed in *E. coli* (Martin *et al.*, 2009) (Chapter 3). Moreover, the additional uncharacterized LplA homologues encoded in the genome suggested that *B. subtilis* lipoic acid metabolism may be more complex than in *E. coli,* and we have found that this is the case. Natalia Martin, a collaborating graduate student in the laboratory of Diego de Mendoza, has now tested the role of LipM in *B. subtilis* and confirmed its proposed role as octanoyltransferase *in vivo*. Martin shows in this chapter that a *lipM* mutant is a lipoic acid auxotroph. In addition to *lipM,* the *B. subtilis* genome contains two ORFs, *yhfJ* and *ywfL*, which encode proteins having significant sequence similarity to characterized lipoyl ligases. In this chapter Martin and I show that these genes are involved in lipoic acid metabolism (see below), so we have renamed *yhfJ* as *lplJ* and *ywfL* as *lipL.* These genes have been characterized by genetic, physiological and biochemical analyses

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

Bacterial strains used in the present study are listed in Table 4-1. *E. coli* and *B. subtilis* strains were routinely grown in Luria Bertani (LB) broth (Sambrook *et al.*, 2001). Spizizen salts (Spizizen,1958), supplemented with 0.5% glucose, trace elements and 0.01% each of tryptophan and phenylalanine were used as the minimal medium for *B. subtilis*. Different supplements added as needed were 0.5 mM DL-α-lipoic acid, 10 mM sodium acetate and 0.1 mM each BCFA precursor (isobutyric acid, isovaleric acid and 2-methylbutiric acid). For the experiments involving gene expression under the control of

the xylose-inducible promoter (P*xylA*), 0.5% glycerol was used as a carbon source instead of glucose. Xylose was added to 0.1% as required. Antibiotics were added to media at the following concentrations (in μg ml^{-1}) sodium ampicillin (Amp), 100; chloramphenicol (Cm), 5; kanamycin sulfate (Km), 5 and spectinomycin sulfate (Sp), 50.

Genetic Techniques

E. coli competent cells were transformed with supercoiled plasmid DNA using the calcium chloride procedure (Sambrook *et al.*, 2001). Transformation of *B. subtilis* was carried out by the method of Dubnau and Davidoff-Abelson (Dubnau *et al.*, 1971). The *amy* phenotype was assayed with colonies grown for 48 h in LB starch plates by flooding the plates with 1% I_2-KI solution (Sekiguchi *et al.*, 1975). Under these conditions *amy* positive colonies produced a clear halo, whereas Δ*amy* colonies gave no halo.

Plasmids and Strains Construction

Plamids used in this chapter are listed in Table 4-2. In all cases DNA fragments were obtained by PCR using the oligonucleotides described in Table 4-3. Chromosomal DNA from wild type *B. subtilis* was used as a template. Sequencing corroborated the identity and correct sequence of all the cloned fragments. *Bacillus* mutants were constructed by Natalia Martin.

A strain with a deletion of the *yqhM* (*lipM*) gene was constructed by gene replacement with a kanamycin resistance determinant, through a double crossover event. For this purpose a 571 bp fragment, corresponding to the 5' end of *lipM* and the upstream gene was PCR amplified with primers I and II and cloned into SacI and XbaI sites of plasmid pJM114 (Perego,1993). A 525 bp fragment containing the 3' end of *lipM* and part of the downstream gene was PCR amplified with primers VII and VIII and cloned into SacI and XbaI sites of the previously generated plasmid. The plasmid obtained pNM07, was linearized with ScaI and used to transform strain JH642. Transformants were selected for kanamycin resistance. The resulting strain was named NM57.

An *yhfJ (lplJ*) knock out mutant was constructed as follows. A 520 bp fragment of the 5' end of *lplJ* plus upstream sequences was PCR amplified using oligonucleotides V and VI and cloned into the XbaI site of vector pJM134 (M. Perego, unpublished) orientation of this insert was checked by restriction enzyme digestion with EcoRI and PstI. Afterwards, a 516 bp fragment of the 3' end of *lplJ* and downstream region was PCR amplified using oligonucleotides XI and XII and inserted into the SalI and XhoI sites of that plasmid to give pNM48. Strain JH642 was transformed with pNM48 linearized with PvuII, yielding strain NM60.

To construct a strain containing a deletion mutation in *ywfL* (*lipL*) gene a 552 bp fragment of the 5'end of *lipL* plus upstream gene sequences was PCR amplified with oligonucleotides III and IV and inserted between the SacI and SmaI sites of plasmid pJM134 (M. Perego, personal communication). Then, a 581 bp fragment containing the 3' end of *lipL* and part of the downstream gene was PCR amplified using oligonucleotides IX and X and inserted between the HindIII and KpnI sites of the previously generated plasmid to render plasmid pNM03. Plasmid pNM03 was linearized with ScaI and used to transform strain JH642. The resulting strain was named NM51. It should be noted that essentially the entire coding sequences of the *lipM, lipL* and *lplJ* genes were removed in construction of the deletion strains.

Strain CM28 was constructed by transformation of strain NM57 with plasmid pNM03 previously linearized with ScaI. Plasmid pNM07 was linearized with *Sca*I and was used to transform strain NM60, rendering strain NM65. To construct strain NM67 the spectinomycin resistant cassette from pNM03 was replaced by a kanamycin resistance cassette from vector pJM114 (Perego, 1993), yielding plasmid pNM47. This plasmid was linearized by digestion with ScaI and use to transform strain NM60, to give strain NM67. Strain NM68 was constructed as follows. A replacement of the spectinomycin resistant cassette from pNM03 with a chloramphenicol resistant cassette from vector pJM105A (Perego,1993) was performed. The resulting plasmid, pNM53, was linearized by digestion with ScaI and used to transform strain NM65. For strains NM65, NM67 and

NM68 selection of transformants was carried out in LB supplemented with acetate and BCFA precursors.

To test complementation of strain NM57 with a wild type copy of the *lipM* gene, a 882 bp fragment containing *lipM* with its ribosome binding site was PCR amplified with oligonucleotides XVII and XVIII and the product inserted between the SalI and KpnI sites of pGES40 (Schujman, unpublished). This plasmid was digested with BamHI to obtain a fragment containing *xylR* P*xylA*-*lipM*, which was cloned into pJM116 previously digested with BamHI, yielding plasmid pNM57. Strain NM57 was transformed with this plasmid; transformants were screened for kanamycin and chloramphenicol resistance and *amyE* phenotype. The resulting strain was named NM08.

Strain NM51 was complemented with a wild type copy of *lipL* gene as follows. A 952 bp fragment containing *lipL* with its ribosome-binding site was PCR amplified with oligonucleotides XIII and XIV and cloned into SalI and KpnI sites of pGES40. This plasmid was digested with BamHI to obtain a fragment containing *xylR* P*xylA*-*lipL*, which was cloned into pJM116 previously digested with BamHI, yielding plasmid pNM58. Strain JH642 was transform with plasmid pNM58 to yield strain NM10 which was further transformed with plasmid pNM03 to give strain NM13.

To express *E. coli lipB* in *B. subtilis* plasmid pNM59 was constructed as follows. A 682 bp fragment containing the *lipB* gene was PCR amplified from plasmid pYFJ29 (Zhao *et al.*, 2005) with oligonucleotides XL and XLI and inserted between the SalI and KpnI sites of pGES40. This plasmid was digested with BamHI to obtain a fragment containing *xylR* P*xylA*-*lipB*, which was cloned into pJM116 previously digested with BamHI, yielding plasmid pNM59. Strain JH642 and NM57 were transformed with plasmid pNM59 to yield strains NM09 and NM11. Strains NM09 and NM11 were then transformed with pNM03 to yield strain NM12 and NM14, respectively.

For *E. coli* complementation analyses, coding sequences were amplified from genomic DNA by PCR and inserted into pBAD322G (Cronan, 2006). The *lplJ* coding sequence was amplified with primers Q003 and Q004 and the product was ligated to the vector NcoI and HindIII sites to give pQC003. The *lipL* coding sequence was amplified

with primers Q039 and Q040 and inserted into pCR2.1 using the TA cloning kit (Invitrogen) and then inserted into the NcoI and HindIII sites of pBAD322G to give pQC032. These manipulations placed these genes under the control of an arabinose inducible promoter.

For purification of the protein products, their respective genes were amplified with primers that added a sequence encoding an N-terminal hexahistidine tag. These PCR products were first inserted into pET101 using the TOPO Cloning Kit (Invitrogen). The *lplJ* coding sequence was amplified using primers Q021 and Q004 to give pQC014, whereas the *lipL* coding sequence was amplified using primers Q0043 and Q0040 to give pQC033. These manipulations placed these genes under control of a T7 polymerase-dependent promoter (Studier *et al.*, 1986).

Growth Curves of B. subtilis Cultures

Growth of Bacillus was measured by Natalia Martin. Strains were grown overnight on liquid minimal medium supplemented with acetate and BCFA precursors. Cells were washed once with minimal medium and used to inoculate fresh media at an OD_{600} of 0.1-0.15. Cells were grown using a Bioscreen C with 300 µL per well with continuous and strong shaking. Growth (OD_{600}) was measured every h.

Complementation of E. coli Lipoate Auxotrophs

B. subtilis genes were expressed in *E. coli* from plasmids with an arabinose inducible promoter as described (Chapter 3). Complementation of the *lipB lplA* strain TM136 was tested in M9 minimal medium containing 0.2% arabinose, 0.1% Vitamin-Assay Casamino Acids, and gentamycin. Complementation of the *lipA lplA* strain TM131 was tested using the same medium containing 5 µg/ml sodium lipoate. Growth was measured by optical density (OD_{600}) in a Beckman DU600 spectrophotometer.

The activities of the lipoate dependent dehydrogenases were assayed using the continuous spectrophotometric assay previously described (Chapter 2). Briefly, derivatives of TM136 carrying various plasmids were subcultured to an OD_{600} of 0.1 in LB with 0.2% arabinose, 5 mM sodium acetate and 5 mM sodium succinate pH 7.0. Cells were harvested in late exponential phase at an OD_{600} of 0.7. The cells were lysed by two

passages through a French pressure cell and protein was quantified using the Bradford assay reagent (Bio-Rad) with bovine gamma globulin (Pierce) as the standard. PDH and OGDH activities were measured spectrophotometrically at 366 nm following the reduction of acetyl-pyridine adenine dinucleotide.

Strain QC168 was used to test the complementation properties of *B. subtilis* genes in a strain deficient in unsaturated fatty acid biosynthesis. Cultures were grown on M9 minimal agar with 0.4% glycerol, 0.1% Vitamin-Assay Casamino Acids, 5 mM sodium acetate, 5 mM sodium succinate, 0.5 mM sodium oleate, and 0.1% Tergitol NP-40 and then restreaked on the same medium lacking acetate and succinate to test for complementation.

Purification of protein substrates

Purification of GcvH: Hexahistidine-tagged *B. subtilis* GcvH was heterologously expressed in *E. coli* and purified by nickel affinity and anion exchange chromatographic steps as previously described (Chapter 3). GcvH was verified to be in the unmodified form lacking the N-terminal methionine residue by electrospray mass spectrometry as previously described (Chapter 3) and was quantified by absorbance at 280 nm using a calculated extinction coefficient of 16,960 M^{-1} cm^{-1}.

Purification and Modification of *B. subtilis* AcpP: The native acyl carrier protein of *B. subtilis* was purified by nickel affinity and ion exchange chromatographic steps, as previously described. Holo and octanoyl forms of ACP were also prepared as previously described (Chapter 3).

Purification of Lipoate Metabolism Enzymes

LipB was purified by nickel affinity and anion exchange as described (Jordan *et al.*, 2003). LipB was quantified by absorbance at 280 nm using an extinction coefficient of 22,920 M^{-1} cm^{-1}. LplJ was purified from strain QC103 by nickel affinity and anion exchange as described for *T. acidophilum* LplA (Chapter 2). LplJ was quantified by absorbance at 280 nm using an extinction coefficient of 34,380 M^{-1} cm^{-1}. LipM and LipL were also purified from the *E. coli fabA* strains QC142 and QC143, respectively, as described for LipM and analyzed by MALDI MS as previously described (Chapter 3). LipL

was also purified from strain QC083 grown in the absence of triclosan. LipM and LipL were quantified by absorbance at 280 nm using extinction coefficients of 45,380 and 25,900 M^{-1} cm^{-1}, respectively. The purified proteins were concentrated with Vivaspin centrifuge concentrators (GE Healthcare) and flash frozen for storage as above except that the buffer contained 100 mM sodium chloride.

Lipoate ligation Assay

Lipoate ligase activity was assayed by observing a mobility shift upon modification by native gel electrophoresis as originally described by Miles and Guest (Miles & Guest, 1987). Assays contained 100 mM sodium phosphate (pH 7.0), 5 mM DTT, 1 mM sodium lipoate, 1 mM magnesium chloride, 1 mM ATP, 20 µM lipoyl domain and 1 µM LplJ. For assay of octanoylation by various enzymes, sodium octanoate was substituted for sodium lipoate and 10 µM enzyme was used. The assays were incubated at 37°C for 1 h and 10 µl of the assays were subjected to native Tris-glycine gel electrophoresis using a 20% acrylamide gel for four h at 100 V. The proteins were visualized by staining with Coomassie Blue R-250 (Sambrook *et al.*, 2001) or by western blotting with anti-lipoic acid antibody (Calbiochem) and anti-rabbit IgG HRP conjugate (Roche). Western blotting was carried out using standard methods with 5% dehydrated milk (Carnation) for blocking and antibody incubation steps (Ausubel, 1987).

Acyl-adenylate intermediate formation was assayed by thin layer chromatography and autoradiography as described for BirA (Xu *et al.*, 1997). The reactions contained 100 mM sodium phosphate (pH 7.0), 5 mM TCEP, 0.1 µM [α-^{32}P] ATP (6000 Ci/mMol), 1 µM $MgCl_2$, 0.1 mM sodium lipoate or octanoate, and 10 µM LplJ. GcvH was also added to 50 µM where indicated. Reactions were incubated at 37°C for 30 min before spotting onto a cellulose TLC plate.

Assay of Octanoyl-[Acyl Carrier Protein]:Protein N-Octanoyltransfer

For assays using *B. subtilis* extracts, 100 µg of extract protein was added instead of enzyme. Cultures were grown to an OD_{600} of 0.6, pelleted by centrifugation, resuspended 1:100 of the culture volume in 100 mM sodium phosphate buffer (pH 7.0), and sonicated for 10 min using a Misonix cup-horn sonicator cooled with circulating 50%

polyethylene glycol. Extracts were cleared by centrifugation and quantified using the Bradford assay (Bio-Rad) with bovine gamma globulin as a standard. For assay of transfer from octanoyl-ACP to both LipM and LipL, 50 µM sodium [1-^{14}C]octanoate and 10 µM enzyme were used in the absence of lipoyl domain. The reaction was analyzed using a modification of the method of Laskey and Mills (Laskey *et al.*, 1975) in which 10 µl of the reactions were subjected to SDS-PAGE on a 4-20% gradient gel which was soaked in Amplify fluorographic reagent (GE Healthcare), dried, and exposed to preflashed Biomax XAR film (Kodak) at -70°C for 24 h.

Immunoblotting Analyses

Immunoblotting analysis of *Bacillus* crude extracts was performed by Natalia Martin. Preliminary blotting experiments were also performed by me, but are not shown. *B. subtilis* wild type and mutants strains were grown overnight in minimal medium supplemented with acetate and BCFA precursors at 37°C. Cells were resuspended in fresh media of the same composition and cultured at 37°C. A 1-ml aliquot of each culture was harvested after 22 hours of growth. The samples were centrifuged and the pellets were washed with buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl). They were resuspended in 180 µl of lysis buffer (50 mM Tris-HCl [pH 8.0], PMSF 10 µM) per OD_{600} unit. Resuspended cells were disrupted by incubation with lysozyme (100 µg/ml) for 15 min at 37°C followed by 5 min of boiling in the presence of loading buffer. Each sample was fractionated by sodium dodecyl sulfate-gel electrophoresis in a 12% acrylamide gel. Proteins were electroeluted to a nitrocellulose membrane and detected using anti-lipoate rabbit antibody and a secondary anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Bio-Rad). The bands were visualized by use of the ECL Plus Western Blotting Detection System (GE). The blots were scanned and the intensity of the bands was quantified by ImageQuant 5.2.

Purification of the B. subtilis PDH Complex

Purification of the PDH complex was performed by Natalia Martin with guidance from me. Cultures were grown at 37°C in LB supplemented with 0.5% glucose, 10 mM sodium acetate, 0.1 mM of each BCFA precursor and the appropriate antibiotic until an

OD_{600} of 1-1.5 when cells were harvested. Purification was carried out similarly as previously described by (Hodgson *et al.*, 1983). Briefly, cell pastes were suspended in 50 mM sodium phosphate buffer, (pH 7.0) containing 5 mM EDTA, Complete EDTA-free protease inhibitor cocktail (Roche), lysozyme (6 mg/ml) (Sigma) and DNAse I (5 µg/ml) (Sigma) and stirred for 2 h. Cells were lysed by three passages through a French pressure cell at ~20,000 psi. Lysates were cleared by centrifugation at 44,000 x *g* for 30 min. The cleared lysate was treated with 32 µg/ml ribonuclease A and incubated at 15°C for 70 min to degrade ribosomes. The samples were centrifuged for 30 min. at 44,000 x *g*. The solution of approximately 50 mg of protein/ml was layered on top of a sucrose step gradient (12.5% w/v and 70% w/w). Centrifugation was performed for 3 h at 180,00 x *g.* The brown protein band at the interface of the 12.5% and 70% sucrose layers was drawn off. Sucrose was removed by overnight dialysis against 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM EDTA and 0.15 mM phenylmethanesulphonylfluoride. The samples were concentrated using Vivaspin concentrators (Sartorius), loaded onto a column of Sephacryl HR-500 26/16 (GE) and eluted with 50 mM sodium phosphate buffer containing 5 mM EDTA (pH 7.0). Fractions containing pure complex as judged by SDS PAGE were pooled and dialyzed overnight against 50 mM sodium phosphate buffer, 15% glycerol and 1 mM *tris* (2-carboxyethyl)phosphine (Sigma). Samples were then concentrated using Vivaspin concentrators (Sartorius), flash frozen in dry ice and ethanol, and stored at -80°C. PDH activity was assayed in the preparation purified from strain NM60 extracts to confirm that the purified complex was indeed the PDH complex.

Protease Digestion of the B. subtilis Pyruvate Dehydrogenase Complex

Digestion of the PDH complex was performed by Natalia Martin with guidance from me. *B. subtilis* pyruvate dehydrogenase complex (1 ml; 5-20 mg/ml) in 50 mM sodium phosphate buffer (pH 7.0) was digested at 30°C with *Staphylococcus aureus* V8 protease (Wako) (1% w/w) for 100 min at which time a second addition of protease (1% w/w) was made. Following a total digestion time of 180 min phenylmethanesulphonylfluoride was added to a final concentration of 1 mM to block further digestion. After digestion the samples were centrifuged for 30 min at 14,000

rpm in a bench top centrifuge and supernatants analyzed by 20% native polyacrylamide-gel electrophoresis. The peptides were sliced from the gel and submitted to the UIUC Mass Spectrometry Lab for LC-MS/MS analysis.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Analysis

Interpretation and search of LC-MS/MS data was done by both Martin and me. Trypsin digestion and LC-MS/MS analysis was performed by the University of Illinois Biotechnology Center Protein Sciences Facility as follows. Gel slices were crushed and rinsed with water. 25 µl of trypsin (12.5 µg/ml) in 25 mM ammonium bicarbonate was added and the samples were digested in a CEM Discover microwave reactor for 15 min at 55^{o}C and 50 watts. The gel pieces were extracted with 100 µl of 50% acetonitrile and 5% formic acid for 10 minutes with sonication. The extracts were dried down in a vacuum centrifuge and resuspended in 13 µl of 5% acetonitrile and 0.1% formic acid. Analysis by LC-MS was carried out on a Waters Q-Tof API-US Quad-ToF mass spectrometer with a nanoAcquity UPLC system. The columns used were Waters nanoAcquity UPLC (75 m x 150 mM 3 µm Atlantis dC18) and Atlantis dC18 5 µm Nanoease trap columns. A 60 min linear gradient of 1% to 60% acetonitrile in 0.1% formic acid was used to elute the peptides from the columns. MS/MS 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 by Mascot (Matrix Science). Database searches were performed against the NCBI non-redundant database with taxonomy restrictions

RESULTS

LipM is Responsible for Octanoyl Transfer In Vivo

B. subtilis was recently demonstrated to encode a functional lipoate synthase called LipA (Martin *et al.*, 2009). However, BLAST searches against *B. subtilis* genome showed no open reading frame (ORF) that resembled the *E. coli* LipB octanoyltransferase, an activity required for production of the substrate of LipA. Instead, two ORFs, YhfJ and YqhM, annotated as encoding putative lipoyl ligases were

present that, respectively, shared 33% and 23% identity with *E. coli* LplA. A third ORF, YwfL that encoded a protein of unknown function having 22% identity with YhfJ was also found. Since all three genes are involved in lipoic acid metabolism (see below), they have been renamed. In this paper we have renamed *yhfJ* and *ywfL* as *lplJ* and *lipL*, respectively, whereas *yqhM* was previously renamed *lipM* (Chapter 3).

Recently, cosmids containing *B. subtilis* genomic fragments were isolated that complemented growth of an *E. coli lipB* strain and failed to complement an *lplA lipA* strain (Chapter 3). All complementing cosmids contained the *lipM* gene, and this gene was shown to be responsible for restoration of lipoic acid synthesis to the *E. coli lipB* strain. The pattern of complementation indicated that *lipM* encoded an octanoyltransferase, and the LipM protein was shown to catalyze octanoyl transfer *in vitro* by the same general acyl-enzyme intermediate mechanism used by LipB (Chapter 3). Based on the frequency that complementing cosmid clones were found, it was suggested that LipM might be the sole *B. subtilis* octanoyltransferase. However, no lipoate ligase encoding cosmids were isolated, and since growth of the *B. subtilis lipA* strain on lipoic acid would require ligase activity, the question of cosmid bank bias was raised (Chapter 3). To definitively test whether or not LipM was the sole *B. subtilis* octanoyltransferase, we constructed strain NM57 in which *lipM* was replaced with a kanamycin-resistance determinant.

Strain NM57 (*ΔlipM*) was auxotrophic for lipoic acid when grown in minimal medium but grew as well as the wild type strain, JH642, in the presence of lipoic acid (Figures 4-2 and 4-3A). As previously observed for a *ΔlipA* mutant strain (Martin *et al.*, 2009) the requirement for lipoic acid could be bypassed by addition of both acetate and branched chain fatty acid (BCFA) precursors (Figures 4-2 and 4-3A), which upon conversion to their CoA esters are the products of the two lipoylated *B. subtilis* enzymes, PDH and BKDH, that are required for growth in minimal medium. Cells grown in this medium were devoid of lipoylated proteins detectable by western blotting (Figure 4-3). Mass spectrometry also demonstrated that *lipM* was required for modification of lipoyl domains by the biosynthetic pathway (see below).

To confirm that the growth phenotype observed in this mutant strain was due to the absence of *lipM*, complementation analyses were carried out. A construct in which *lipM* was placed under control of a xylose-inducible promoter (P*xylA*) was introduced into the Δ*lipM* strain NM57 giving the *lipM amyE*::P*xylA*-*lipM* strain, NM08. Induction of *lipM* expression in strain NM08 allowed growth in minimal medium thereby indicating that the absence of a functional copy of *lipM* (rather than a polar effect on the downstream genes) was the cause of the growth phenotype of strain NM57 (Figure 4-2A). Moreover, LipM could be functionally replaced by expression of *E. coli lipB*. The *lipB* gene was placed under P*xylA* and the construct was introduced into the Δ*lipM* strain NM57 to give the *lipM amyE*::P*xylA*-*lipB* strain, NM11. Upon induction of LipB expression, strain NM11 grew in minimal medium, indicating that the LipB octanoyl transferase activity functionally replaced LipM. Similar complementation experiments were performed with a *ΔlipL* strain (see below). These results, together with those of Chapter 3, demonstrate that LipM functions as the sole *B. subtilis* octanoyltransferase.

Bacillus subtilis LplJ is a lipoate:protein ligase

The ability of exogenous lipoic acid to allow growth of both a Δ*lipM* strain and a *lipA* conditional mutant indicated that *B. subtilis* must encode a lipoate scavenging activity (Martin *et al.*, 2009). The most likely candidate for this role was YhfJ (LplJ), which was annotated as a putative lipoyl ligase and has 33% identity to *E. coli* LplA. We first found that expression of *lplJ* restored *E. coli lipB lplA* strains to prototrophy (Figure 4-4A). Since prototrophy could result from either octanoyltransfer or ligation of traces of endogenous octanoate (Hermes *et al.*, 2009), we also tested complementation of a *E. coli lplA lipA* strain in the presence of lipoate. Growth was also restored in this strain (Figure 4-4B) and it was accompanied by activation (hence lipoate modification) of the *E. coli* 2-oxoacid dehydrogenases (Figure 4-4C). Therefore, in complementation tests LplJ behaved like LplA. We then constructed strain NM60, a *B. subtilis* Δ*lplJ* strain. The Δ*lplJ* strain grew normally in minimal medium in the absence of supplements (Figure 4-1) and had a wild type pattern of lipoylated proteins (Figure 4-3). This behavior was expected because *ΔlplA* strains of *E. coli* lacking lipoate scavenging activity show growth defects

only when the strains are additionally blocked in the lipoic acid synthetic pathway by a *lipA* or *lipB* mutation (Morris *et al.*, 1994; Morris *et al.*, 1995) (Figure 4-1). Based on this precedent the Δ*lipM ΔlplJ* double mutant strain, NM65, was constructed. As expected, this strain was found to be unable to grow in minimal medium either in the presence or absence of lipoic acid (Figure 4-1), indicating that LplJ is the sole *B. subtilis* lipoic acid salvage enzyme.

Given that proteins with significant sequence identity to *E. coli* LplA have been shown to catalyze octanoyl transfer, it could not be assumed that the *B. subtilis* lipoate salvage enzyme catalyzed a ligase reaction. Therefore, we purified the hexahistidine-tagged protein to homogeneity (Figure 4-5A) and assayed it for both the overall and first partial reaction of the ligase reaction. In the absence of an acceptor domain, synthesis of both octanoyl-adenylate and lipoyl-adenylate intermediates was readily demonstrated by use of ATP labeled in the α-phosphate. Moreover, upon addition of an acceptor lipoyl domain the adenylate intermediates were hydrolyzed to AMP (Figure 4-5B). Gel mobility shift assays showed that LplJ modified the *E. coli* lipoyl domain from PDH ($E2_{AceF}$) and GcvH with either lipoate or octanoate. Western blotting with anti-lipoic acid antibody confirmed lipoylation of the acceptor proteins and demonstrated that the antibody recognized lipoylated proteins and not octanoylated proteins (Figure 4-5C). Finally, modification of GcvH was confirmed by electrospray mass spectrometry of the reaction products (Figure 4-5D). From these data we conclude that *B. subtilis* LplJ catalyzes a classical lipoate ligase reaction analogous to that of *E. coli* LplA.

The Unexpected Requirement for LipL

A third ORF, *lipL (*formerly *ywfL*), encodes a protein annotated as of unknown function. LipL seems more divergent from authentic lipoyl ligases than LipM and has only 22% sequence identity with LplJ. Our results, together with those previously reported (Martin *et al.*, 2009) (Chapter 3), indicated that *B. subtilis* like *E. coli* has an octanoyltransferase (LipM), a sulfur insertion enzyme (LipA) and a lipoate ligase (LplJ). However, unlike *E. coli,* these enzymes were not sufficient for either synthesis or efficient scavenging of lipoic acid, as demonstrated by the phenotype of *B. subtilis ΔlipL*

strains. Strains devoid of LipL were unable to grow in minimal medium as observed for strains lacking either *lipM* (Figure 4-1) or *lipA* (Martin *et al.*, 2009). However, in contrast to the growth phenotypes of *lipM* and *lipA* strains, addition of lipoic acid only partially restored growth of the *ΔlipL* strain NM51. The growth behaviour of the *ΔlipL* strain denoted a block in the endogenous lipoylation pathway because, like the *lipA* conditionally mutant strain and Δ*lipM* strains, the *ΔlipL* strain grew as well as its wild type parent in minimal medium supplemented with acetate and BCFA precursors. The residual growth of the *ΔlipL* strain in minimal medium containing lipoic acid was due to LplJ activity because the Δ*lipL ΔlplJ* double mutant strain, NM67, failed to grow in this medium. Hence the *ΔlipL* strain retained some ability to transfer exogenously provided lipoate to the unmodified acceptor proteins. Expression of a functional copy of the *lipL* gene under the control of a xylose-dependent promoter in the non-essential *amyE* locus restored growth of the Δ*lipL* strain in minimal medium (Figure 4-1B) indicating that the absence of a functional copy of *lipL* was the cause of the growth phenotype of strain NM51.

The growth phenotype of the *ΔlipL* strain indicated that it should have decreased levels of protein lipoylation and any residual modification should be abolished upon introduction of a *ΔlplJ* lesion. This was the case (Figure 4-3). Immunoblot analysis of crude extracts of strain NM51 (Δ*lipL*) with anti-lipoate antibodies showed that GcvH was lipoylated to a level similar to that seen in the wild type strain. However, only one of the two high molecular weight bands observed in the wild type strain was detected in strain NM51 and thus the total amount of protein lipoylation of this strain was more than 3-fold less than those seen in extracts of the wild type strain (Figure 4-3). Strain NM51 required both acetate and BCFA precursors for growth indicating that the BKDH and PDH E2 subunits of were nonfunctional in absence of LipL. Thus, the band observed seems likely to be the nonessential OGDH E2 subunit that may migrate together with low levels of lipoylated PdhC insufficient to overcome the acetate requirement. The putative OGDH E2 band was not present in extracts of the Δ*lipL ΔlplJ* strain, NM67, (Figure 4-3) indicating that LplJ was responsible for the residual lipoylation seen in the

absence of lipoic acid supplementation. Interestingly, in this strain the immunoblot signal of GcvH was about two-fold stronger than that of extracts from the wild type strain (Figure 4-3). This observation shows that lipoyl-GcvH is more abundant in this strain. This suggests that when GcvH is lipoylated by the concerted action of LipM and LipA, lipoyl-GcvH accumulated because in the absence of LipL and LplJ the GcvH lipoyl moiety could not be transferred to other lipoyl acceptors. Another possible explanation would be that LplJ scavenges and attaches octanoate to the acceptor proteins to serve as a LipA substrate as occurs in *E. coli* (Hermes & Cronan, 2009). However, this does not seem to be the case in *B. subtilis* since no lipoylated proteins were detected in a *ΔlipM* strain (Figure 4-3).

To provide further evidence for the role of LipL in protein lipoylation, complementation experiments with *E. coli lipB* were performed. As described above, *lipB* was placed under a xylose-inducible promoter (P*xylA*) and this construct was introduced into the chromosome of *B. subtilis* strain NM51 to give strain NM12 (Δ*lipL amyE::Pxyl-lipB*). Induction of LipB expression allowed growth not only of the *ΔlipL* strain NM12, but also of the Δ*lipM ΔlipL* double mutant strain CM28 (Figure 4-6). However, when expressed in *E. coli* mutant strains, LipL had no detectable ability to replace either *E. coli* LplA or LipB (Figure 4-4). A plausible explanation for this observation was that LipL might become inactive when expressed in *E. coli*. When *B. subtilis* LipM is expressed in *E. coli*, some molecules carry a covalently attached decanoate moiety derived from *cis*-3-decenoyl-ACP, an essential intermediate of unsaturated acid biosynthesis in *E. coli* (Chapter 3) that is not present in *B. subtilis*. A similar inability to exclude *cis*-3-decenoyl-ACP was reported for *Mycobacterium tuberculosis* LipB (Ma *et al.*, 2006). In the case of LipM an unmodified protein was obtained by expression of the protein in an *E. coli fabA* mutant which lacks the ability to make *cis*-3-decenoyl-ACP. The inability of LipL to relieve the lipoate requirement cannot be due to formation of a covalently attached decanoate because a *fabA lipB lplA* strain also failed to grow (Figure 4-4D).

Although LipL expression failed to restore the growth of an *E. coli ΔlipB* strain, *lipB* expression allowed growth of a *B. subtilis ΔlipL* strain on minimal medium. These results indicated that LipM and LipL are both required for octanoylation of E2 lipoyl domains and that the two proteins either catalyze sequential reactions or participate in octanoyl transfer as a complex. The latter explanation seems unlikely since LipM expressed in *E. coli* is active both *in vivo* and *in vitro* (Chapter 3). Moreover, crude extracts of *B. subtilis ΔlipL* mutants readily catalyzed the LipM reaction, transfer of octanoate from octanoyl-ACP to GcvH (Figure 4-7).

Given that lipoic acid synthesis proceeds through several sequential steps, it seemed possible that intermediates attached to the lipoyl domains of the E2 subunits might accumulate in some mutant strains. Therefore we purified the PDH complexes of wild type and various mutant strains and released their lipoyl domains by limited proteolysis with the *Staphylococcus aureus* V8 glutamyl protease as previously described (Packman *et al.*, 1988). The isolated domains were resolved from the other digestion products by polyacrylamide gel electrophoresis run under native conditions. The gel slices containing the domain bands were excised, crushed and subjected to in-gel trypsin digestion. The resulting peptides were analyzed by liquid chromatography-tandem mass spectrometry as described in Experimental Procedures. The PDH complexes were purified from extracts of strains NM60 (Δ*lplJ*); NM65 (Δ*lipM ΔlplJ*); NM67 (Δ*lipL ΔlplJ*) and NM68 (Δ*lipM ΔlipL ΔlplJ*) as described in Experimental Procedures (the strains all lacked LplJ in order to preclude ligation of any traces of octanoic acid or lipoic acid present in the culture medium). PDH peptides were detected with significant scores in all samples (Figure 4-8) and modification was determined using an error tolerant search as described in Experimental Procedures to determine the presence or absence of posttranslational modifications of the target lysine residue (K43). K43 was found in three forms-unmodified, octanoylated and lipoylated-in the peptides derived from the Δ*lplJ* strain NM60, whereas the peptides of the other strains were unmodified (Figure 4-8). These results indicate that PDH was not

modified in *ΔlplJ* strains when either LipM or LipL was nonfunctional and that LipL is required for E2 lipoyl domain modification rather than for sulfur insertion.

Although no novel PDH E2p-bound intermediates were present, this does not rule out a sequential mechanism in which another protein functions as an octanoyl/lipoyl carrier. Indeed the observed accumulation of lipoylated GcvH in a *ΔlipL ΔlplJ* strain (Figure 4-3) suggested that this small protein was a good candidate for an octanoyl/lipoyl carrier.

DISCUSSION

Lipoic acid synthesis in *B. subtilis* is clearly more complex than in *E. coli. E. coli* requires only two proteins to make this cofactor, whereas in this paper we demonstrate that *B. subtilis* requires three proteins: LipA, LipM (an isozyme of LipB), and an unexpected protein LipL. Inactivation of any of the three genes that encode these proteins results in lipoic acid auxotrophy. The two LipA proteins are interchangeable between *B. subtilis* and *E. coli,* as are LipM and LipB. In contrast, LipL has a curious behavior. Its expression in *E. coli* fails to complement a Δ*lipB* mutation whereas expression of LipB complements a *B. subtilis ΔlipL* strain. Therefore, LipL and LipM have distinct roles in lipoate biosynthesis. A plausible hypothesis is that LipM and LipL might form a complex to transfer an octanoyl group from octanoyl-ACP to the acceptor proteins. However this explanation seems unlikely since LipM alone complements the function of LipB *in vivo* (Chapter 3) and the data in this and the companion paper indicates that LipM and LipL catalyze two sequential steps in octanoyl transfer with GcvH as an intermediate required for the lipoylation of most (if not all) of the other *B. subtilis* apoproteins.

Strains lacking LipL (Δ*lipL*), unlike Δ*lipM* strains, grow poorly in the presence of exogenously added lipoic acid (Fig. 4-2) indicating that LipL has a role in lipoic acid scavenging. Indeed, Δ*lipL* Δ*lplJ* strains fail to grow in the presence of exogenously supplied lipoic acid, confirming that LplJ is required for the low level of lipoylation of the complexes seen in the Δ*lipL* strain. These data suggest that its lipoyl ligase activity may

be less efficient in the absence of LipL. These results could be explained if the PDH and BKDH E2 subunits are much poorer LplJ substrates than GcvH. That is, LipL would be both a facilitator of LplJ action as well as a necessary intermediate in LipM action. Our results clearly indicate the presence of unexpected diversity in lipoic acid metabolism among bacteria. It should be pointed out that LipL homologues can be found in pathogenic bacteria closely related to *B. subtilis* such as *Staphyloccocus aureus* and *Bacillus anthracis*. Thus, utilization of this pathway for protein lipoylation may be more widespread than previously appreciated. Finally, due to the involvement of lipoic acid metabolic proteins in pathogenesis, multidrug resistance and intracellular growth of pathogens, the discovery of new enzymes should provide potential new targets for antimicrobial agents. In the next chapter we report biochemical and genetic data that support the above model in which GcvH is an octanoyl/lipoyl carrier and demonstrate that LipL is a novel amidotransferase. Further work is needed to determine the importance of this new pathway in pathogenesis. The strong phenotype of a Δ*lipL* strains suggests LipL might be an excellent target for antimicrobials.

TABLES

Table 4-1. Strains used in this chapter.

Strain	Relevant characteristics	Source or reference
B. subtilis		
JH642	*trpC2 pheA1*	Laboratory stock
NM57	JH642 *lipM*:: Km^r	This chapter
NM08	NM57 *amyE*::P*xyl-lipM*	This chapter
NM11	NM57 *amyE*::P*xyl-lipB*	This chapter
NM51	JH642 *lipL*:: Sp^r	This chapter
NM09	JH642 *amyE*::P*xyl-lipB*	This chapter
NM10	JH642 *amyE*::P*xyl-lipL*	This chapter
NM12	NM51 *amyE*::P*xyl-lipB*	This chapter
NM13	NM51 *amyE*::P*xyl-lipL*	This chapter
CM28	NM57 *lipL*:: Sp^r	This chapter
NM14	CM28 *amyE*::P*xyl-lipB*	This chapter
NM60	JH642 *lplJ*:: Sp^r	This chapter
NM65	NM60 *lipM*:: Km^r	This chapter
NM67	NM60 *lipL*:: Sp^r	This chapter
NM68	NM65 *lipL*:: Cm^r	This chapter
E. coli		
DH5α	*supE44 thi-1 ΔlacU169 (f80lacZΔM15) endA1 recA1 hsdR17* gyrA96 *relA1 trp6 cysT329*	Laboratory stock
MG1655	*rph-1*	CGSC*
EMM99	*E. coli* BL21 (DE3) / pEM88	(Martinez *et al.*, 2010)
TM131	*rpsL8 lipA::*Tn*1000 lplA*::Tn*10*	(Morris *et al.*, 1994)
TM136	*rpsL8 lipB::*Tn*1000 lplA*::Tn*10*	(Morris *et al.*, 1994)
JK1	*rpsL8*	(Morris *et al.*, 1994)
MFH120	JC7623 *lacZ*ΔM15 ϕ (*fabA-lacZ*)1 (Hyb)*cat fadBA poxB*::pMFH23	(Henry *et al.*, 1992)
QC031	*rpsL8 lipB::*Tn*1000 lplA*::Tn*10*/ pQC003	This chapter
QC032	*rpsL8 lipB::*Tn*1000 lplA*::Tn*10*/ pQC004	(Chapter 3)
QC035	*rpsL8* / pBAD322G	(Chapter 2)
QC038	*rpsL8 lipA::*Tn*1000 lplA*::Tn*10*/ pBAD322G	(Chapter 2)
QC057	*rpsL8 lipB::*Tn*1000 lplA*::Tn*10*/ pBAD322G	(Chapter 3)
QC080	*rpsL8 lipB::*Tn*1000 lplA*::Tn*10*/ pQC032	This chapter
QC081	*rpsL8 lipA::*Tn*1000 lplA*::Tn*10* / pQC032	This chapter
QC086	*rpsL8 lipB::*Tn*1000 lplA*::Tn*10*/ pQC003	This chapter
QC087	*rpsL8 lipB::*Tn*1000 lplA*::Tn*10*/ pQC004	(Chapter 3)
QC134	*rph-1* IN (*rrnD-rrnE*)1 ϕ (*fabA-lacZ*)1 (Hyb)*cat*	(Chapter 3)
QC142	*rph-1* IN (*rrnD-rrnE*)1 ϕ (*fabA-lacZ*)1 (Hyb)*cat* / pCY598, pQC015	(Chapter 3)
QC143	*rph-1 IN (rrnD-rrnE)1 φ (fabA-lacZ)*1 (Hyb)*cat* / pCY598,pQC033	This chapter
QC161	*rph-1* Δ*lplA*::FRT Δ*lipB*::FRT / pQC057, pTARA	(Chapter 3)
QC168	*rpsL lipB*::Kn *lplA*::Tc ϕ (*fabA- lacZ*)1 (Hyb)*cat*	This chapter
QC175	*rpsL lipB*::Kn *lplA*::Tc ϕ (*fabA- lacZ*)1 (Hyb)*cat* / pBAD322G	This chapter
QC176	*rpsL lipB*::Kn *lplA*::Tc ϕ (*fabA- lacZ*)1 (Hyb)*cat* / pQC003	This chapter
QC177	*rpsL lipB*::Kn *lplA*::Tc ϕ (*fabA- lacZ*)1 (Hyb)*cat* / pQC004	This chapter
QC178	*rpsL* (SmR) *lipB*::Kn *lplA*::Tc ϕ (*fabA- lacZ*)1 (Hyb)*cat* / pQC032	This chapter

*CGSC, *E. coli* genetic stock center.

Table 4-2. Plasmids used in this chapter.

Plasmids	Relevant Characteristics*	Source or Reference
pGES40	pBluescript (Stratagene) containing the *xylR* gene and P*xylA* promoter	(Schujman, unpublished)
pJM116	Integrative vector to construct transcriptional fusions to *lacZ*; integrates at the *amyE* locus of *B. subtilis*; Cm^r	(Dartois *et al.*, 1996)
pJM105A	Integrational vector; Cm^r	(Perego,1993)
pJM114	Integrational vector; Km^r	(Perego,1993)
pJM134	Integrational vector; Sp^r	(Perego,1993)
pBAD322G	arabinose inducible expression vector	(Cronan,2006)
pTARA	pACYC origin, arabinose inducible T7 polymerase	(Wycuff *et al.*, 2000)
pCY598	RSF origin, arabinose inducible T7 polymerase	(Cronan,2003)
pET101/D-TOPO	T7 promoter expression vector	Invitrogen
pCR2.1	TOPO TA cloning vector	Invitrogen
pNM03	pJM134 containing *lipL* interrupted with a Sp^r cassette	This chapter
pNM07	pJM114 containing *lipM* interrupted with a Km^r cassette	This chapter
pNM48	pJM134 containing *lplJ* interrupted with an Sp^r cassette	This chapter
pNM47	pJM114 containing *lipL* interrupted with a Km^r cassette	This chapter
pNM53	pJM105A containing *lipL* interrupted with a Cm^r cassette	This chapter
pNM57	Contains *xylR* P*xylA*-*lipM* into BamHI site of pJM116	This chapter
pNM58	Contains *xylR* P*xylA*-*lipL* into BamHI site of pJM116	This chapter
pNM59	Contains *xylR* P*xylA*-*lipB* into BamHI site of pJM116	This chapter
pSJ120	*E. coli* LipB expression vector	(Jordan *et al.*, 2003)
pQC003	LplJ expression vector	This chapter
pQC004	LipM expression vector	(Chapter 3)
pQC015	N-terminal hexahistidine LipM expression vector	(Chapter 3)
pQC032	LipL expression vector	This chapter
pQC033	N-terminal hexahistidine lipL expression vector	This chapter

*Cm^r, Gm^r Km^r, Sp^r, Amp^r denote resistance to chloramphenicol, gentamicin, kanamycin, spectinomycin and ampicillin, respectively.

Table 4-3. Primers used in this chapter.

Primer name	Sequence (5'-3')
I	ggagctcGTTGTAAATCTCAGTGCAGCC
II	CTTctAgaGCCTGAGTCTATAAACCGCCA
III	AgaGctcGACATACAAACGAGAATGAGC
IV	TTTCccggGCATCAGTAAATCAATC
V	gGagCtcGATGCTGATATAGAACAGTTTCA
VI	ATTctagaGCGGATCATTGATATTTTGATTG
VII	AAACgtcGacCGCTTCAGATGAATG
VIII	CTTcTcgAgGGGCATCATCTTCTTC
IX	CCGaAgcttTGAGCGGAATGCAAAG
X	GTgGtAccCCACCAGGAGTTTCGCG
XI	TGGGgtCgacACGAAAGAGGATTTC
XII	CAGCcTcgaGCTTGGCCACATAATA
XIII	AGTTgTCGacCAATAAGCCTAACATGAAAGGG
XIV	AGGgtAccgGatcCTTGAGATAAAAAATGCATG
XVII	CTATgtcgACGGTAAGGAAGGTCTTAAAATGCA
XVIII	CGGGTaccggatCCTGTTTACCGCTTATAATTC
XL	GTGTcgacGAATggaggCTTCATATGTATCAGG
XLI	AGgtacCGgatcCAAGCTTAAGCGGTAATATATTCG
XLVI	TGAAAgcTtTGGTATCTTCTTATTGTAGTGCTG
XLVII	CTTggtaccggATCCTGTTTTAGTCTTCTTGTG
L	AGTGGaTccAAGAGCATGGGAAAG
LI	TTgtCgACTCGTGTTCTCCTGAGTAA
LII	GAGtcGACACAAGAAGACTAAAACAG
LIII	AAAGActGCAGTGAAATTCACCGCCA
Q003	GAGACATGTTATTTATAGACAATCAAAATATCAATG
Q004	TATAAGCTTCTCCTGCCTCCATTATTT
Q021	CACCATGCATCATCATCATCATCATATGTTATTTATAGACAATCAAAATATCAATG
Q036	GAGCAATTGACCATGCATCATCATCATCATCATATGTTATTTATAGACAATCAAAATATCAATG
Q039	CACCATGGCAAACCAACCGATTGA
Q040	CGTTCACCCAAATACCTTTGCA
Q043	CACCATGCATCATCATCATCATCATATGGCAAACCAACCGATTGA

Lowercase letters indicate variations with respect to the wild-type *B. subtilis* sequence. Restrictions sites are underlined.

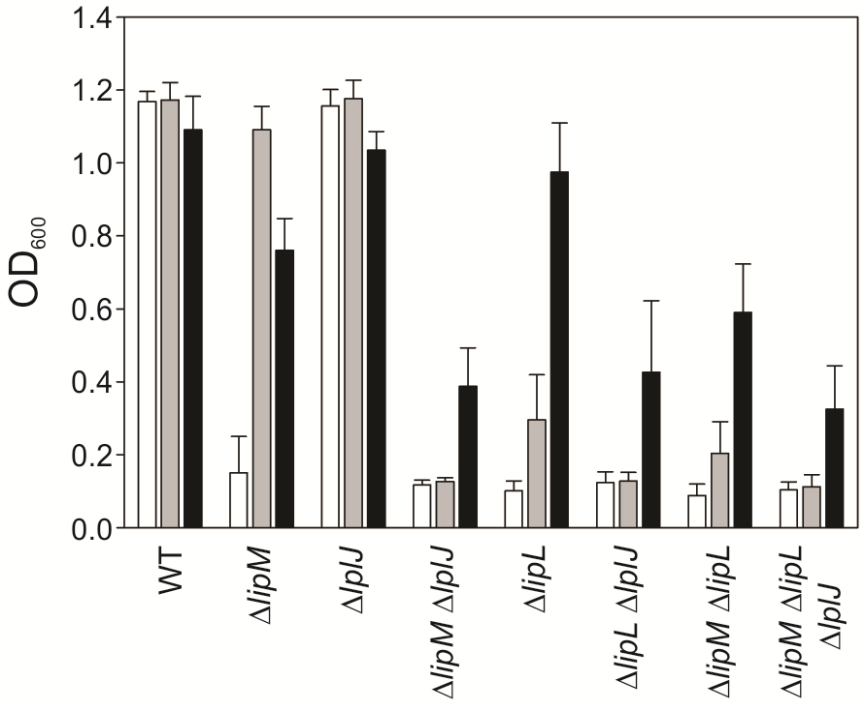


Figure 4-1. Growth phenotypes of *B. subtilis* mutant strains. The strains were grown overnight in minimal medium supplemented with acetate and branched chain fatty acid (BCFA) precursors. The cultures were centrifuged and the cells resuspended in minimal medium without supplements (white bars); minimal medium supplemented with lipoic acid (grey bars) or with acetate and BCFA precursors (black bars). The OD_{600} values of the cultures were measured after 17 h of growth at 37°C. The results shown are the averages of results from three independent experiments. These data were produced by Natalia Martin.

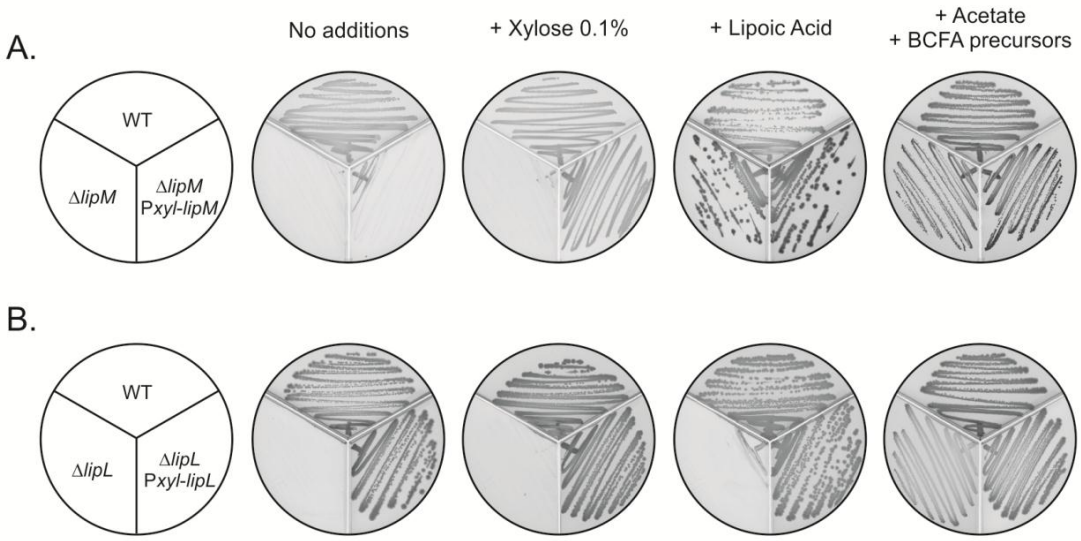


Figure 4-2. Characterization of *B. subtilis* strains blocked in lipoic acid (LA) synthesis. Panel A. Growth of bacterial strains JH642 (wild type), NM57 (Δ*lipM)* and NM08 (Δ*lipM amyE*::P*xyl*-*lipM*). Panel B. Growth of wild type (JH642), NM51 (Δ*lipL)* and NM13 (Δ*lipL amyE*::P*xyl*-*lipL*). The strains were streaked onto minimal medium-glycerol plates containing the supplements indicated above and incubated for 24 h at 37°C. These data were produced by Natalia Martin.

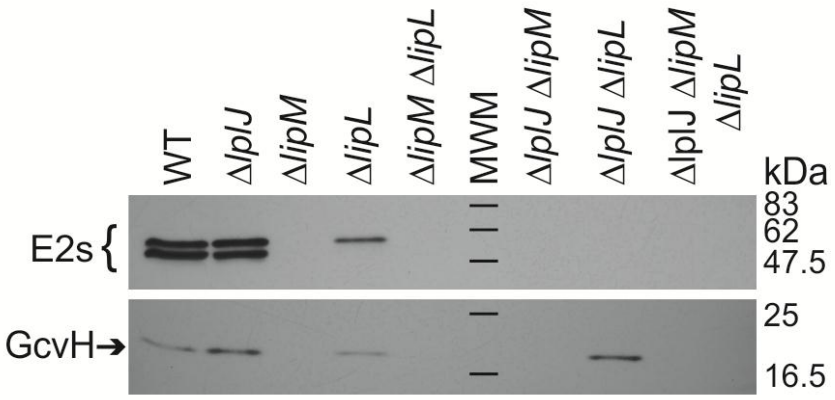


Figure 4-3. Immunoblotting analyses of mutant strains with an anti-lipoic acid antibody. The strains were grown overnight in minimal medium supplemented with acetate and BCFA precursors. The cells were diluted in fresh medium of the same composition and grown for 22 h before analysis. The black bars denote the molecular weight standards (MWM). These data were produced by Natalia Martin.

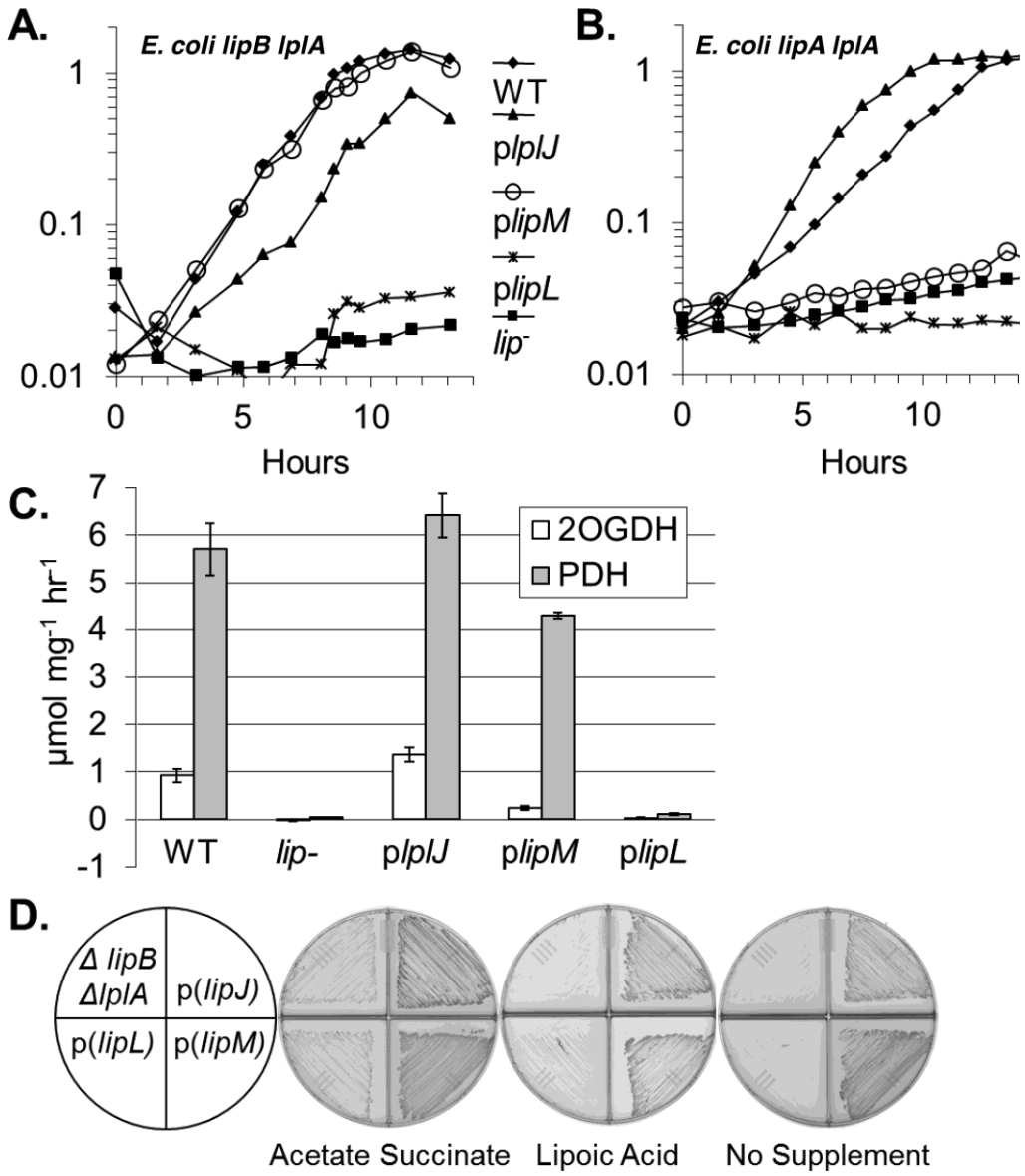


Figure 4-4. Complementation of *E. coli* lipoic acid auxotrophs with *B. subtilis* genes as described in Experimental Procedures. Panel A: Growth of the *lipB lplA* strain TM136 containing plasmids expressing the genes indicated on the figure. Culture optical density at 600 nm is reported. Panel B: Growth of *lipA lplA* strain TM131 containing plasmids expressing genes indicated as in panel A. Lipoic acid is also added to the medium as a substrate for ligation. Panel C: The 2-oxoacid dehydrogenase activities of the *lipB lplA* strain TM136 carrying plasmids encoding the genes indicated. The wild type strain was strain JK1. Panel D: Growth of *lipB lplA fabA* strain QC168 containing plasmids expressing the genes indicated. Note that the host strain was *fabA* to prevent possible decanoyl adduct modification of the *B. subtilis* proteins. Growth of the strain carrying the p (*lplJ*) plasmid on the unsupplemented plate is due to scavenging of the endogenous octanoic acid present in *E. coli* by high levels of ligase (Hermes *et al.*, 2009).

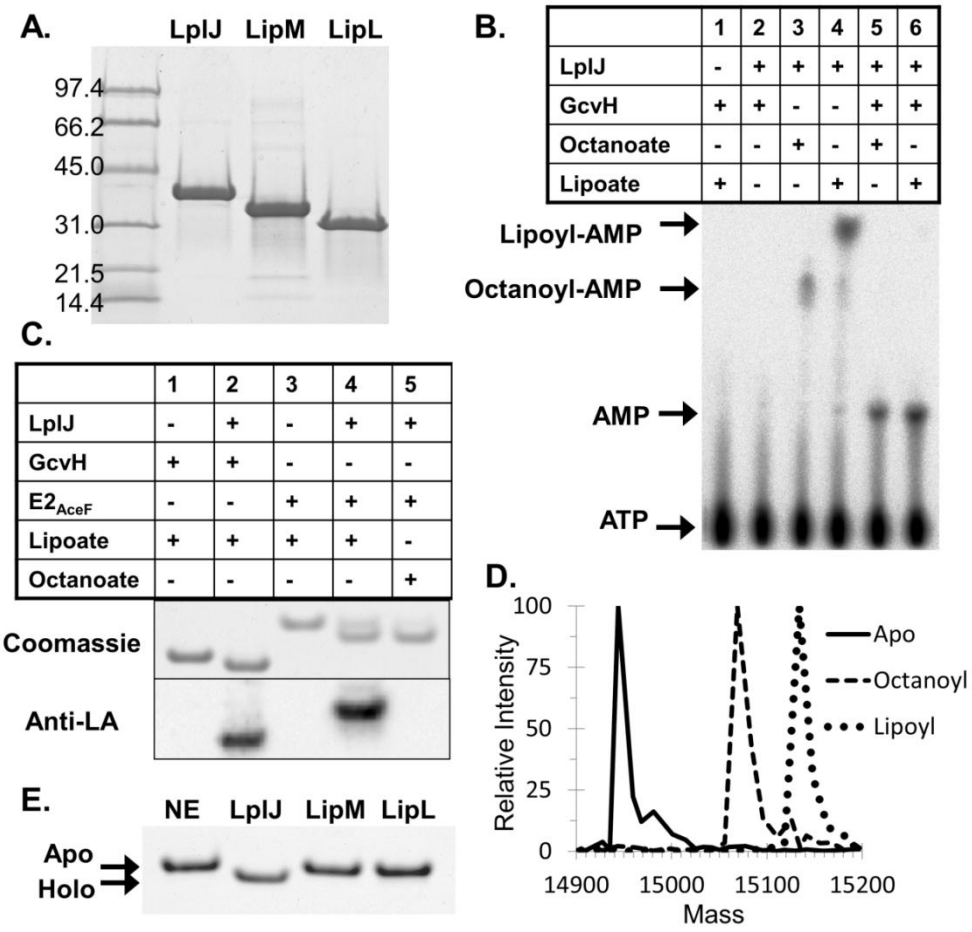


Figure 4-5. The *B. subtilis* lipoate salvage enzyme is a lipoate-protein ligase. Panel A: An SDS-PAGE gel (4-20% acrylamide) containing ~2 μg of each of the indicated purified proteins is shown. The molecular weight marker standards in kilodaltons are given. Panel B: Formation of the acyl adenylate intermediates by LplJ in the first partial reaction of the ligase reaction using [α-^{32}P]ATP incorporation assayed by thin layer chromatography. Addition of an acyl acceptor domain results in consumption of the intermediate with production of AMP. Panel C: Gel shift assay of LplJ using either the $E2_{AceF}$ *E. coli* hybrid lipoyl domain or *B. subtilis* GcvH as acyl acceptors. The proteins were visualized by either Coomassie R250 staining or by western blotting with anti-lipoic acid antibody (Anti-LA) as indicated. Panel D: Electrospray mass spectra of GcvH after LplJ treatment with either octanoate or lipoate as acyl donors. Panel E: Assay of purified proteins for octanoyl ligation using GcvH as the substrate acceptor domain. NE denotes the control reaction that lacked enzyme.

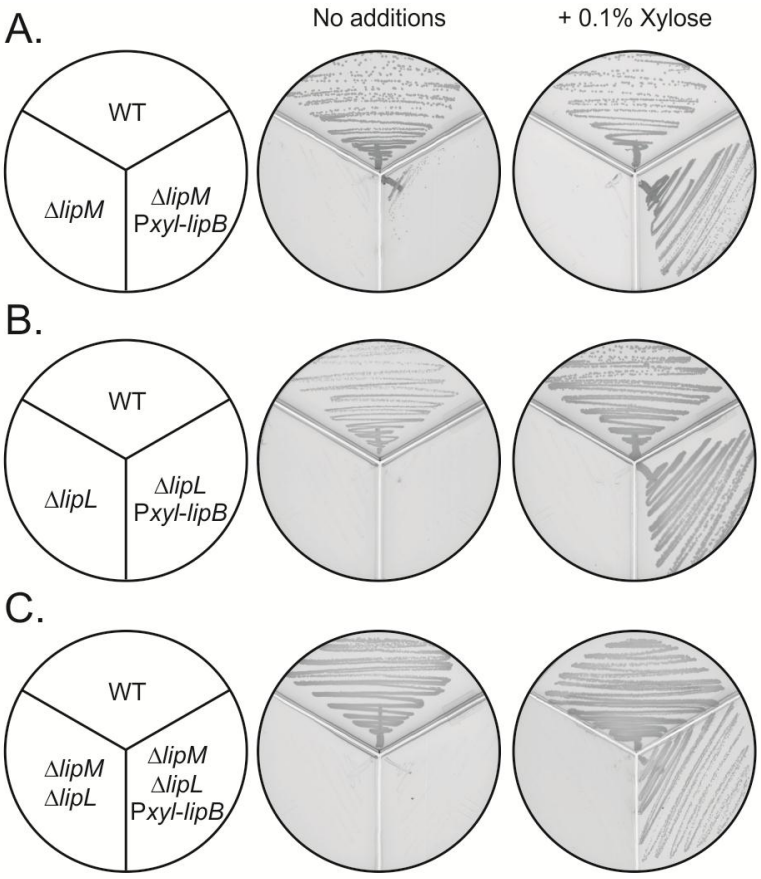


Figure 4-6. Complementation of *B. subtilis* mutants deficient in lipoate synthesis by expression of *E. coli* LipB. *B. subtilis* strains JH642 (wild type), NM57 (Δ*lipM),* NM11 (Δ*lipM amyE*::P*xyl*-*lipB*), NM51 (Δ*lipL),* NM12 (Δ*lipL amyE*::P*xyl*-*lipB*), CM28 (Δ*lipL ΔlipM*) and NM14 (Δ*lipL ΔlipM amyE*::P*xyl*-*lipB*) were streaked onto minimal medium-glycerol plates with or without the addition of 0.1% xylose and incubated for 24 h at 37°C. These data were produced by Natalia Martin.

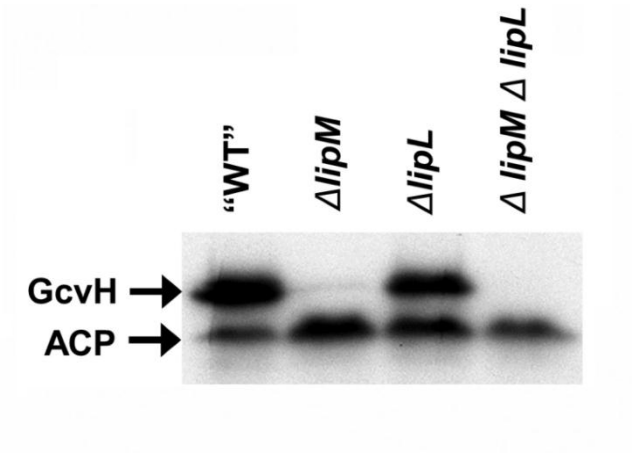


Figure 4-7. LipM acts before LipL. Autoradiogram of an SDS-PAGE gel of the products of octanoyltransfer assays from octanoyl-ACP (synthesized with AasS) to GcvH. The enzyme source were crude extracts of the indicated *B. subtilis* strains. All strains, including the "WT", carried an Δ*lplJ* lesion to prevent possible complications by ligation of octanoate.

A.

Strain	Peptide Sequences	Modification	Residue
NM60 (Δ*lplJ*)	WFVKPNDEVDEDDVLAEVQNDKAVVEIPSPVK	None Lipoylation Octanoylation	K43
NM65 (Δ*lipM ΔlplJ*)	WFVKPNDEVDEDDVLAEVQNDK WFVKPNDEVDEDDVLAEVQNDKAVVEIPSPVK WFVKPNDEVDEDDVLAEVQNDKAVVEIPSPVKGK	None	K43
NM67 (Δ*lipL ΔlplJ*)	WFVKPNDEVDEDDVLAEVQNDK WFVKPNDEVDEDDVLAEVQNDKAVVEIPSPVK WFVKPNDEVDEDDVLAEVQNDKAVVEIPSPVKGK	None	K43
NM68 (Δ*lipL ΔlipM ΔlplJ*)	WFVKPNDEVDEDDVLAEVQNDK WFVKPNDEVDEDDVLAEVQNDKAVVEIPSPVK WFVKPNDEVDEDDVLAEVQNDKAVVEIPSPVKGK	None	K43

B.

1 MAFEFKLPDI GEGIHEGEIV K**WFVKPNDEV DEDDVLAEVQ NDKAVVEIPS**
51 **PVKGK**VLELK VEEGTVATVG QTIITFDAPG YEDLQFKGSD ESDDAKTEAQ

Figure 4-8. Liquid chromatography-tandem mass spectrometric analysis of PDH lipoyl domain tryptic peptides. Panel A. The modification states of PDH residue K43 detected. Panel B. The sequence of the PDH lipoyl domain is given. The sequences of the peptides detected that contained K43 are shown in bold type. "None" denotes that no distinct peaks for modified forms were observed. These data were produced primarily by Natalia Martin with my guidance.

CHAPTER 5

A NOVEL AMIDOTRANSFERASE REQUIRED FOR LIPOIC ACID COFACTOR ASSEMBLY IN *BACILLUS SUBTILIS*

INTRODUCTION

Comparative genomics allows for detection of incomplete metabolic models. Analysis of discrepancies in our knowledge provides opportunities for new discoveries. Here I describe the analysis of an "extra" protein required for lipoic acid metabolism in *Bacillus subtilis*, homologues of which are found in nearly all sequenced Firmicutes genomes. The Firmicutes, also known as the low G+C gram-positive bacteria, are a phylum present throughout the environment and includes many pathogens. *B. subtilis* is the best characterized of this group and has been subject to intense genetic study since the discovery of its natural competence in 1958 (Spizizen,1958). *B. subtilis* is a facultative spore-forming bacterium that grows in chemically defined medium lacking lipoic acid (Spizizen,1958). In general, lipoic acid is required for aerobic, energy-conserved metabolism of 2-oxoacids and glycine, pyruvate dehydrogenase being the canonical lipoate-requiring enzyme (Perham,2000). In *B. subtilis*, pyruvate dehydrogenase activity is required for growth under both aerobic and anaerobic conditions (Nakano *et al.*, 1997), and thus, it is clear that *B. subtilis* must synthesize the cofactor (Spizizen,1958).

The lipoyl domain modifying enzymes are grouped with those that modify biotinyl domains into Pfam PF03099. There are very few residues that are absolutely conserved within the family, a lysine residue being the sole conserved residue (Reche, 2000). However, there is sufficient transitive homology throughout the sequences to allow grouping (Reche,2000). Despite this lack of sequence conservation, the enzymes have considerable structural similarity including a conserved folding pattern (Ma *et al.*, 2006). Lipoyl ligases have an additional accessory domain, which has been shown to be required for adenylation, but not for acyltransfer (Chapter 2). Comparisons of the crystal structures of LplA obtained with and without the acyl adenylate intermediate show that

the accessory domain rotates away from the active site following adenylate formation, thereby allowing interaction with the substrate lipoyl domain (Fujiwara *et al.*, 2010). Interestingly, many proteins of the LplA family lack a C-terminal accessory domain. This may be due to the domain being a separate protein, as is the case in Archaea (Chapter 2). One of these single domain LplA homologues, *B. subtilis* LipM, has been shown to be a new ACP:GcvH octanoyltransferase isozyme (Chapter 3).

GcvH is a lipoyl acceptor protein that consists of a single lipoyl domain and is a key component of the glycine cleavage system. Although the glycine cleavage system is lipoic acid dependent, its inactivation is not known to cause a growth defect in any bacterium. The well-characterized glycine cleavage systems consist of four proteins, GcvH, GcvT, GcvP and GcvL. In *B. subtilis* expression of only the T and P subunits is under control of a glycine-activated riboswitch (Mandal *et al.*, 2004). No dedicated GcvL subunit is apparent, but this function (oxidation of the lipoate moiety) may be provided by the E3 subunit that is shared among the 2-oxoacid dehydrogenases, as is the case in *E. coli* (Steiert *et al.*, 1990). Given its key role in transfer of the methylamine group of glycine from the P subunit to the T subunit, it is surprising that GcvH is not encoded together with the T and P subunits, but rather at a remote genomic location where no recognizable riboswitch is detected (Mandal *et al.*, 2004). In this chapter I demonstrate that *B. subtilis* GcvH is both a substrate and a required component of the lipoic acid biosynthesis pathway.

Based on the *E. coli* model, the characterized LipA lipoyl synthase (Martin *et al.*, 2009) and the newly described LipM octanoyltransferase (Chapter 2) should suffice for lipoic acid synthesis and attachment in *B. subtilis.* However, a third gene, *lipL,* is also required for lipoyl domain modification. LipL belongs to the Pfam PF03099 family and lacks an accessory domain. In this chapter I describe the novel biosynthetic requirement for LipL and the unexpected reaction catalyzed by this new enzyme. Based on these data I present a novel pathway for lipoic acid biosynthesis.

EXPERIMENTAL PROCEDURES

Media and Chemicals

E. coli strains were grown on LB rich or M9 minimal media (Sambrook *et al.*, 2001) under aerobic conditions at 37^{o}C unless otherwise indicated. Antibiotics (Sigma Chemical Co) were used at the following concentrations for *E. coli (*in μg/ml): sodium ampicillin, 100; kanamycin sulfate, 50; spectinomycin sulfate, 50; chloramphenicol, 12; gentamycin sulfate, 25 and tetracycline hydrochloride, 12. 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 [α-^{32}P]ATP. The pH of buffers and solutions is reported at room temperature.

Bacterial Strains and Plasmids

Strains and plasmids used are shown on Table 4-1 and Table 4-2 respectively. Primers used in this chapter are given in Table 4-3. Standard techniques for DNA manipulation and cloning were employed unless otherwise indicated (Sambrook *et al.*, 2001). PCR amplification was performed using Taq (New England Biolabs) or Pfu (Stratagene) polymerase according to the manufacturer's recommendation, except with the addition of 5% dimethyl sulfoxide to Pfu reactions.

The genes encoding proteins containing lipoyl domains were placed under the control of a T7 promoter. The lipoyl domain from *B. subtilis* PdhC ($E2_{PdhC}$) was identified by alignment with the *B. stearothermopolis* lipoyl domain isolated by V8 protease digestion (Packman *et al.*, 1988) and this portion of the *pdhC* gene was PCR amplified using primers Q030 and Q031 (the latter primer added a termination codon). After digestion with NcoI and BamHI this product was inserted into pET28b+ cut with the same enzymes to give pQC023. The full-length pyruvate dehydrogenase E2p subunit (*pdhC*) was PCR amplified using primers Q095 and Q096 (which added a C-terminal hexahistidine tag) and inserted into pCR2.1 using the TA cloning kit. The *pdhC* insert was then moved into pRSF-1b using the NcoI and BamHI sites to give pQC047. The biotin/lipoic acid protein (BLAP) was PCR amplified using primers Q034 and Q042 (which added a C-terminal hexahistidine tag) and inserted into pCR2.1 using the TA cloning kit.

The BLAP insert was then moved into pET28b+ using the NcoI and BamHI sites to give the pQC026.

In order to minimize proteolysis of the full length PdhC, the protein was expressed in a strain lacking the OmpT protease, which cleaves the flexible linker region (Lessard *et al.*, 1998). The *ompT*::Km mutation was constructed as described (Baba *et al.*, 2006) to give strain RMK98. The *ompT*::Km mutation was transduced to strain QC146 with a phage P1 stock grown on strain RMK98 to give strain QC171. The kanamycin cassette was removed (Datsenko *et al.*, 2000) to give strain QC172 and the deletion was confirmed by PCR using primers Q113 and Q114.

Mutants of LipL were produced by modification of pQC033 using QuickChange mutagenesis as per the manufacturers recommendations (Agilent). Primers were used for the corresponding mutations were: Q147/Q148 for K148A, Q149/Q150 for K148R, Q151/Q152 for C150A, Q153/Q154 for C150R, Q171/Q172 for C39A, Q173/Q174 for C39S, Q175/Q176 for Y180A, Q177/Q178 for Y180F.

B. subtilis mutant strains were constructed by Natalia Martin. The *gcvH* deletion mutant, strain NM20, was obtained by transformation of strain JH642 with plasmid pNM67. This plasmid was constructed as follows. A 418 bp fragments from the 5' end and upstream region of the gene was PCR amplified with oligonucleotides L and LI (Table 5-3)and inserted into BamHI and EcoRI sites of pJM114 (Perego,1993). In the same manner a 434 bp fragment containing the 3' end and downstream region of the gene was PCR amplified with oligonucleotides LII and LIII and cloned into XhoI and KpnI sites from the previously generated plasmid, yielding plasmid pNM67. This plasmid was linearized and used to transform JH642.

Strain NM20 was complemented with an ectopic wild type copy of *gcvH* under an IPTG dependent promoter (P*spac*) integrated at the *amyE* gene. To this end plasmid pNM70 was constructed by cloning of a 456 bp PCR-amplified fragment generated with primers XLVI and XLVII into the HindIII and BamH sites of pGES485 (Schujman GE, unpublished). Plasmid pNM70 was linearized and used to transformed strain NM20,

yielding strain NM21. Transformants were selected on kanamycin plus spectinomycin plates and screened for *amyE* phenotype.

Purification of Lipoyl Domains

Purification of GcvH. Hexahistidine-tagged *B. subtilis* GcvH was heterologously expressed in *E. coli* and purified by nickel affinity and anion exchange chromatographic steps as previously described (Chapter 3). GcvH was verified to be in the unmodified form lacking the N-terminal methionine residue by electrospray mass spectrometry as previously described (Chapter 3) and was quantified by absorbance at 280 nm using a calculated extinction coefficient of 16,960 M^{-1} cm^{-1}.

The *E. coli* E2p (1,3) hybrid lipoyl domain (LD_{AceF}) was purified by acid treatment followed by ion exchange chromatography as described previously (Zhao *et al.*, 2003). Mass spectroscopy analysis was performed as described for GcvH (Chapter 3). The $E2_{AceF}$ was in the unmodified form and lacked the N-terminal methionine residue. The $E2_{AceF}$ lacks tryptophan and tyrosine residues, it was quantified using the Bradford assay reagent (Bio-Rad) with bovine gamma globulin (Pierce) as a standard.

To purify $E2_{PdhC}$, strain QC063 was grown in LB with kanamycin to an OD_{600} of 0.6. Protein expression was induced with 1 mM IPTG and the culture was incubated for another 2 h before the cells were pelleted by centrifugation and frozen at -80C. The cells were lysed by passage through a French pressure cell and the $E2_{PdhC}$ was purified by pH precipitation and anion exchange chromatography and prepared for mass spectrometry as described (Zhao *et al.*, 2003). The anion exchange protocol allows resolution of the unmodified and modified forms of the domain as shown by 20% native PAGE. The modification state of the $E2_{PdhC}$ was verified to be in the unmodified form without the N-terminal methionine by electrospray mass spectrometry as described above for ACP (Chapter 3). $E2_{PdhC}$ was quantified by absorbance at 280 nm using an extinction coefficient of 6,990 M^{-1} cm^{-1}.

The *B. subtilis* BLAP protein was expressed using strain QC152 and purified by nickel affinity and anion exchange chromatographic steps as described for LplA (Chapter 2). BLAP was verified to be in the unmodified form lacking the N-terminal methionine

by electrospray mass spectrometry as described for ACP (Chapter 3) and was quantified by absorbance at 280 nm (extinction coefficient of 5,500 M^{-1} cm^{-1}). *B. subtilis* PdhC was expressed using strain QC191 and purified by nickel affinity and anion exchange as described for LplA (Chapter 2). PdhC was quantified by absorbance at 280 nm using an extinction coefficient of 18,450 M^{-1} cm^{-1}. To determine if the C-terminal hexahistidine tag interfered with complex formation, I subjected pure PdhC to size exclusion chromatography as described for LipM (Chapter 3).

Purification of Lipoate Metabolism Enzymes

LipB was purified by nickel affinity and anion exchange as described (Jordan *et al.*, 2003). LipB was quantified by absorbance at 280 nm using an extinction coefficient of 22,920 M^{-1} cm^{-1}. LplJ was purified from strain QC103 by nickel affinity and anion exchange as described for *T. acidophilum* LplA (Chapter 2). LplJ was quantified by absorbance at 280 nm using an extinction coefficient of 34,380 M^{-1} cm^{-1}. LipM and LipL were also purified from the *E. coli fabA* strains QC142 and QC143, respectively, as described for LipM and analyzed by MALDI MS as previously described (Chapter 3). LipL was also purified from strain QC083 grown in the absence of triclosan. LipM and LipL were quantified by absorbance at 280 nm using extinction coefficients of 45,380 and 25,900 M^{-1} cm^{-1}, respectively. The purified proteins were concentrated with Vivaspin centrifuge concentrators (GE Healthcare) and flash frozen for storage in as above except that the buffer contained 100 mM sodium chloride.

Trypsin Digestion and LC-MS/MS Analysis of LipL

Trypsin digestion and LC-MS/MS analysis of LipL was performed as previously described for LipM (Chapter 3). Analysis was performed on LipL from QC083 grown without triclosan and from the *fabA* strain QC143 grown with triclosan.

Lipoic Acid Ligation Assay

Octanoyl ligase activity was assayed to determine the LD substrate specificity of LplJ. The reaction contained 100 mM sodium phosphate (pH 7.0), 50 mM sodium chloride, 5 mM TCEP, 0.25 mM sodium [1-^{14}C]octanoate, 1 mM magnesium chloride, 1 mM ATP, 20 µM lipoyl domain and 1 µM LplJ. The reaction was analyzed using a

modification of the method of Laskey and Mills (Laskey *et al.*, 1975) in which 10 µl of the reactions were subjected to SDS-PAGE on 4-20% gradient gels which were soaked in Amplify fluorographic reagent (GE Healthcare), dried, and exposed to preflashed Biomax XAR film (Kodak) at -70°C for 24 h.

Assay of octanoyl-[Acyl Carrier Protein]:Protein N-octanoyltransfer

Octanoyltransfer by LipM and LipB was assayed in order to determine the lipoyl domain substrate specificity of these enzymes. Assays using AasS were performed as previously described (Chapter 3) with different lipoyl domains substituted for GcvH. Reactions were analyzed as described above with LplJ.

Assay of amidotransfer

Amidotransfer was assayed by coupling to lipoyl ligation by LplJ. The reactions contained 100 mM sodium phosphate (pH 7.0), 50 mM sodium chloride, 5 mM TCEP, 0.25 mM sodium [1-^{14}C]octanoate, 1 mM magnesium chloride, 1 mM ATP, 20 µM GcvH, 20 µM PdhC, 1 µM LipL, and 1 µM LplJ. The reactions were analyzed as described above for the LplJ assays.

Amidotransfer was also assayed by coupling to octanoyltransfer by LipM. The reactions contained 100 mM sodium phosphate (pH 7.0), 50 mM sodium chloride, 5 mM TCEP, 0.25 mM sodium [1-^{14}C]octanoate, 1 mM magnesium chloride, 1 mM ATP, 50 µM holo-AcpP, 2 µM AasS, 20 µM GcvH, 20 µM PdhC, 10 µM LipL and 10 µM LipM. Reactions were analyzed as described for the LplJ assays above.

B. subtilis crude extracts were also assayed for LipM and LipL activities. The cultures were grown in Spizizens minimal salts (Spizizen,1958) supplemented with 0.5% glucose, 10 mM sodium acetate, 10 mM sodium succinate, 0.1 mM sodium isovalerate, 0.1 mM sodium isobutyrate, and 0.1 mM sodium 2-methylbutyrate. Cultures were grown to an OD_{600} of 0.6, pelleted by centrifugation, frozen at -80°C, and lysed in 100 mM sodium phosphate (pH 7.0) by sonication. The reaction conditions were the same as the LipM coupled assay with enzyme or 50 µg of extract protein added where indicated. Reactions were analyzed as described for the LplJ assays above.

In order to directly assay amidotransfer [1-^{14}C]octanoyl labeled GcvH was prepared using LplJ. The reaction contained 100 mM sodium phosphate (pH 7.0), 50 mM sodium chloride, 5 mM TCEP, 1 mM sodium [1-^{14}C]octanoate, 2 mM ATP, 0.5 mM GcvH, and 10 µM LplJ. The reaction was allowed to proceed at 37°C for 2 h. Then the reaction was diluted 20-fold in 25 mM sodium MES buffer (pH 6.1) and purified using anion exchange chromatography using AKTA purifier10 (GE Healthcare) with a 1.8 ml POROS QE anion exchange column with a flowrate of 5 ml per minute. Protein was bound with 25 mM sodium MES buffer (pH 6.1) and eluted with a 0-1 M gradient of lithium chloride, where octanoyl GcvH eluted at 480 mM. Purified lipoyl domains were stored as above except 5 mM TCEP was the reducing agent. Amidotransfer from purified ^{14}C-octanoyl GcvH to various LDs was directly assayed. The reactions contained 100 mM sodium phosphate (pH 7.0), 50 mM sodium chloride, 5 mM TCEP, 20 µM [1-^{14}C]octanoyl-GcvH, 20 µM of different lipoyl domains, and 1 µM LipL. Amidotransfer reactions with mutant LipL proteins were performed with 10 µM LipL or mutant LipL plus $E2_{PdhC}$ domain as an additional lipoyl domain. The LipL acyl-enzyme intermediate was visualized using the same assay conditions lacking an acceptor domain and with 10 µM LipL or mutant LipL. The reactions were analyzed as described for the LplJ assays above.

Immunoblotting Analysis

Immunoblotting of crude extract was performed by Natalia Martin as described in Chapter 4.

Phylogenetic Analysis and Bioinformatics

I determined the phylogeny of select amino acid sequences of the LipB/LplA/BirA superfamily (PF03099), the biotin_lipoyl_domain superfamily (PF00364), and the glycine cleavage system H protein family (PF01597) (Finn *et al.*, 2010). Alignment was performed using Dialign-TX with low scoring region length 4, low quality region length 40, and unlimited sensitivity (Subramanian *et al.*, 2008). The edges of the alignment were trimmed using Jalview (Waterhouse *et al.*, 2009) so only the relevant domains remained. A minimum evolution tree was constructed with bootstrap analysis of 1000 replicates using Mega4 (Tamura *et al.*, 2007) using the default settings. All positions

containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 63 positions in the final dataset comparing lipoyl domains. There were a total of 57 positions in the final dataset that compared catalytic domains.

Display of protein structures and structural alignments were performed using the Swiss PDB viewer (Kaplan *et al.*, 2001). Protein extinction coefficients and theoretical masses were calculated using Protparam at the EXPASY website (Gasteiger *et al.*, 2003) and used to determine molarity. The extinction coefficients were calculated on the assumption that all cysteine residues were in the reduced state.

RESULTS

Expression, Purification and Modification of LipL

LipL (YwfL) was readily expressed in *E. coli* and purified as a soluble hexahistidine-tagged protein (Figure 5-1A). However, upon expression in *E. coli* *Mycobacterium tuberculosis* LipB and *B. subtilis* LipM are modified by attachment of acyl moieties (Ma *et al.*, 2006) (Chapter 3). To determine if LipL purified from *E. coli* was modified, the protein was subjected to trypsin digestion and the resulting peptides were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). LipL was unusually amenable to this analysis, giving 88% coverage (Figure 5-1A). Two sites of modification were found: cysteine residues C39 and C150. Lipoyl and octanoyl modifications were present at both sites (Figure 5-1B) indicating that LipL accepted octanoate and lipoate from sources in *E. coli*. Assuming the reaction catalyzed by this enzyme is the same in *B. subtilis*, the substrates must be common to both organisms. The two cysteine residues are located on opposite sides of the LipL active site cleft (see below). Unlike LipM and *M. tuberculosis* LipB, no decanoyl Michael adduct was found when LipL was expressed in a wild type strain of *E. coli*.

LipM is Specific for GcvH

Although LipM readily catalyzed octanoyltransfer from ACP to GcvH (Chapter 3), it was puzzling to find that the lipoyl domain of the *B. subtilis* pyruvate dehydrogenase PdhC subunit ($E2_{PdhC}$) was a poor octanoyl acceptor substrate (Figure 2A). This was first

considered to be an artifact caused by using overly truncated lipoyl domains. However, the full length PdhC subunit was also a poor substrate. PdhC was purified in good yield and eluted slightly before catalase by size exclusion, indicating an octahedral complex. This was expected and indicates that the C-terminal hexahistidine tag does not interfere with complex formation. Moreover, *E. coli* LipB was able to modify all of the *B. subtilis* lipoyl domains tested indicating that the acceptor proteins were properly folded. However, the enigmatic biotin lipoyl acceptor protein (BLAP), a protein previously reported to be biotinylated and lipoylated when expressed in *E. coli* (Cui *et al.*, 2006) (Figure 5-2) is not modified by any enzyme tested.

These data led to the hypothesis that LipL is essential for PdhC modification and hence growth. To test this scenario octanoyltransfer assays were done with octanoyl-ACP (generated *in situ*) as the acyl donor substrate (Chapter 3). Similar to previous results (Figure 5-2) addition of only LipM and LipL failed to result in octanoylation of either $E2_{PdhC}$ or LipL (Figure 5-3A, lane 3). It was only in the presence of GcvH that octanoylation of LipL and $E2_{PdhC}$ proceeded (Figure 5-3A, lane 6).

A similar phenomenon was also seen for the LplJ, which was identified and well characterized as a lipoyl ligase (Chapter 4). Although LplJ was able to ligate octanoate to the *E. coli* lipoyl domain ($E2_{AceF}$), no modification of either *B. subtilis* $E2_{PdhC}$ or of full-length *B. subtilis* PdhC was seen *in vitro* (Figure 5-2B). Addition of LipL to the LplJ reaction failed to give modification of $E2_{PdhC}$ (Figure 5-3B, lane 3). Only when GcvH, LipL and LplJ were all present did modification of $E2_{PdhC}$ proceed (Figure 5-3B, lane 5). These observations argued that modified GcvH was an obligate intermediate in the modification of $E2_{PdhC}$. It also showed that LipL is required for PdhC modification.

<u>LipL is a Novel Amidotransferase</u>

Since an amide linkage attaches the octanoyl moiety of octanoyl-GcvH, LipL seemed to catalyze a novel amidotransfer reaction in which the active site cysteine thiol of LipL would attack the octanoyl-GcvH amide linkage to form octanoyl-S-LipL and would subsequently transfer the octanoyl moiety to $E2_{PdhC}$ to form a new amide moiety (Figure 5-4D). To test if LipL was a novel GcvH:E2 amidotransferase LipL activity was assayed

using purified [1-^{14}C]octanoyl-GcvH as the donor substrate. Transfer of the octanoyl moiety to each of the PDH domains tested was observed whereas BLAP was not modified (Figure 5-4A). Transfer from GcvH to LipL was also observed (Figure 5-4C).

I performed site-directed mutagenesis of residues conserved in the PF03099 family to explore the LipL reaction mechanism. LipM K162 is a residue that is strictly conserved in PF03099 whereas Y180 is conserved in the LipL transamidase subfamily (Figure 5-5). Unfortunately, when expressed in *E. coli* both K162 substitutions (K162R and K162A) resulted in low levels of insoluble proteins, whereas substitutions of alanine or phenylalanine for Y180 had no effect on catalysis. I also mutated residues C39 and C150, both of which had been found to be modified by LC-MS/MS (Figure 5-1A). Replacement of C39 with alanine or serine had no effect on catalysis whereas replacement of C150 with either alanine or serine resulted in loss of transfer to $E2_{PdhC}$ (Figure 5-4B) and the inability to form an octanoyl-enzyme intermediate (Figure 5-4C). Given the above observations, I propose a mechanism for lipoyl domain amidotransfer by LipL (Figure 5-4D).

Amidotransfer was also assayed in crude extracts of various *B. subtilis* mutant strains. Although LipM activity was observed, no amidotransfer by LipL was seen unless the extracts were supplemented with purified LipL and LipM (Figure 5-6). The reason for this discrepancy is not clear. Perhaps higher concentrations of octanoyl-GcvH are required to detect LipL activity, although transfer was not seen using purified [1-^{14}C]octanoyl-GcvH as a substrate (data not shown). Additionally, LipL may be present in low amounts under the growth conditions used or LipL may become inactivated during extract preparation.

GcvH is Required for Protein Lipoylation *In Vivo*

Our *in vitro* results argued that GcvH was an essential intermediate in lipoylation of PdhC and possibly BkdB. To test the model for lipoic acid biosynthesis in *B. subtilis*, Natalia Martin disrupted *gcvH*. The *ΔgcvH* strain was a lipoic acid auxotroph, the same phenotype shown by *ΔlipA* and *ΔlipM* strains. As observed in the other lipoate auxotrophs, the lipoate requirement of the *ΔgcvH* strain was bypassed by addition of

acetate and BCFA precursors. *In vivo* results showing that bypass of a *ΔlipL* mutation requires both acetate and branched chain fatty acid (BCFA) precursors indicates that LipL is also required for lipoic acid modification of the BkdB subunit of the essential branched chain dehydrogenase. Lipoic acid also restored growth suggesting that LplJ ligated lipoic acid to $E2_{PdhC}$ in the absence of GcvH (Figure 5-7A). Expression of a functional ectopic copy of the *gcvH* gene under the control of an inducible promoter allowed growth of the Δ*gcvH* strain when induced with IPTG (Figure 5-7B). This proves that the growth deficiency was due to the absence of a functional copy of the *gcvH* gene. When uninduced, growth is impaired although some is observed, likely due to basal expression from the spac promoter. Moreover, the expression of the *E. coli lipB* gene in a Δ*gcvH* mutant strain restored growth in minimal medium (data not shown). These results indicated that the LipB octanoyl transferase activity was sufficient to overcome the absence of GcvH, confirming its role in the octanoyl transfer from ACP to the lipoyl domains.

LipM octanoyltransferase activity with GcvH as the acceptor was previously demonstrated *in vitro* (Chapter 3). Western blotting analysis with anti-lipoic acid antibodies showed a lipoylated protein of apparent mass of 20 kDa (Figure 5-7C). As shown in the companion paper, the GcvH band was detected in extracts of strains in which *lipM* was active. The gel migration of the band is consistent with the previously reported migration rate of GcvH (Chapter 3) and disruption of *gcvH* resulted in loss of the 20 kDa band (Fig 5-3C) thereby confirming its identity. Disruption of *gcvH* resulted in a complete loss of biosynthetic lipoic acid assembly (Figure 5-7C), confirming that GcvH was required in *B. subtilis* lipoic acid biosynthesis. Upon supplementation with lipoic acid the Δ*gcvH* strain showed strong lipoylation of the dehydrogenase E2 subunits (Figure 5-7C) consistent with the robust growth seen in this medium (Figure 5-7A). These results confirm that the results of our biochemical assays are physiologically relevant.

LipL and LipB are Mechanistically Convergent

Phylogenetic analysis of LipL compared to other members of PF03099 shows significant, albeit modest, support for *B. subtilis* LipL as part of a deeply branching clade related to lipoate protein ligases (Figure 5-8A). LipL is also closely related to LipM octanoyltransferases, which branch off later from the same lipoate protein ligase clade (Chapter 3). Indeed, the unpublished *B. halodurans* LipL structure (PDB entry 2P5I) is the closest available structure to LipM. The positions of the catalytic cysteine residues (C150 in both proteins) are very similar in LipM and LipL. Upon structural alignment of LipL with *M. tuberculosis* LipB (PDB 1W66) the catalytic cysteine residues are located only 2.34 Å apart (Figure 5-9). This was unexpected because the residues are contributed by different loops that are ~45 residues removed in sequence alignments (Chapter 3). Therefore, it appears that LipL and LipB have undergone mechanistic convergence within their active sites using the same protein scaffold.

DISCUSSION

I report delineation of a new pathway of lipoic acid synthesis in which octanoyl moieties are transferred from the ACP of fatty acid synthesis to the GcvH protein of the glycine cleavage system by LipM. I characterized the pathway in *B. subtilis*, although orthologous genes are present in other Firmicutes which synthesize lipoic acid. The octanoyl moieties are then transferred by LipL, a novel GcvH:E2 amidotransferase, from GcvH to E2 subunits to form active dehydrogenase complexes (Figure 5-10). Note that at this stage of our investigations it is not clear at what step(s) LipA acts. It is possible that LipA inserts the sulfur groups before LipL transfer, after LipL transfer, or at both steps.

The requirement for LipL is due to the specificity of the LipM for GcvH. I find significant *in vitro* modification of the $E2_{AceF}$ by LplJ, but not by LipM (Figure 5-2) suggesting LplJ may be less specific than LipM. However, LipM complements an *E. coli* lipoic acid auxotroph (Chapter 3), which demonstrates modification of $E2_{AceF}$. These data indicate that restoration of growth is a much more sensitive assay than the *in vitro*

radiolabel assay. This is expected since growth results from protein lipoylation which results in dehydrogenase catalysis with a concomitant amplification of the signal. Another difference is that lipoylation of PdhC may occur *in vivo* after the protein has been assembled into the PDH complex rather than as the free subunit I tested *in vitro*. The $E2_{AceF}$ and $E2_{PdhC}$ domains clearly form different clades indicating different conserved residues (Figure 5-8B). This may explain why LplJ is able to modify $E2_{AceF}$ but not $E2_{PdhC}$. The residues responsible for the observed differences in substrate recognition will require further study.

The amidotransfer reaction (also called transamidation) catalyzed by LipL was unexpected, but not unprecedented. The sortase transpeptidases that anchor proteins to the outer surfaces of various gram-positive bacteria use a Cys-His-Asn triad in their amidotransfer reaction (Clancy *et al.*, 2010). Moreover, peptide bond synthesis catalyzed by cysteine proteases (Morihara *et al.*, 1981) employs this same catalytic triad. The DD-transpeptidase/carboxypeptidases of bacterial cell wall biosynthesis employ a Ser-Lys-Tyr catalytic triad (Silvaggi *et al.*, 2003) and substitution of cysteine for the nucleophilic serine residue in closely related enzymes often results in active enzymes (Hadonou *et al.*, 1992). In our work, LipL residues C39 and C150 were found to be acylated. However, C39 was not essential for catalysis (Figure 5-4B) and is not conserved in more diverse members of the LipL clade (Figure 5-5). (Acylation of C39 may have resulted by trans-thioesterification from C150 during the overnight trypsin digestion.) Mutagenesis of LipL C150 resulted in loss of enzymatic activity and the inability to form an acyl-enzyme intermediate (Figure 5-4), which is the same result seen upon mutagenesis of the active site LipM cysteine residue (Chapter 3). Based on the *B. halodurans* LipL crystal structure K162 and Y180 are less than 3 Å from C150 (Figure 5-10). I found that these residues are conserved in all members of the LipL clade (Figure 5-9). From these data, it seemed likely that LipL employed a Cys-Lys-Tyr catalytic triad similar to those seen in other amidotransferases. However, I found that Y180 was not required for amidotransfer as the Y180F and Y180A proteins was active. Therefore, I propose that LipL amidotransferases utilize a Cys-Lys dyad as do the LipB and LipM ACP-

octanoyltransferases (Ma *et al.*, 2006) (Chapter 2). If so, this would a novel active site configuration for an amidotransferase (transamidase).

The *B. subtilis* biotin-lipoyl acceptor protein (BLAP) was reported to be at least partially modified with lipoate when expressed in *E. coli* (Cui *et al.*, 2006). In that work the concentration of lipoic acid added to the culture medium corresponded to >60,000-fold excess over that needed for half-maximal growth of a *lipA* strain (Reed *et al.*, 1993). As previously reported (Cui *et al.*, 2006), I found that most of the BLAP was in the unmodified form after two hours of expression. It was thought that BLAP might a good candidate for intermediate carrier in lipoic acid biosynthesis. However, I assayed BLAP as an octanoyl acceptor and found it was not modified by any enzyme tested (Figure 5-2, 5-3A). The role of BLAP in *B. subtilis* remains unknown, but it does not appear to be involved in lipoic acid metabolism.

Although the data presented in these papers provide a good working model of lipoic acid biosynthesis, a conflicting view of lipoic acid scavenging emerges. As shown in the companion paper, addition of external lipoic acid only partially restored growth of a Δ*lipL* mutant and modification of $E2_{PdhC}$ by LplJ was not seen *in vitro* (Figure 5-2B) whereas growth of the Δ*gcvH* strain was fully restored by lipoic acid (Figure 5-5A) and the dehydrogenase E2 subunits became highly modified (Figure 5-5C). One possibility is LplJ activity may be lower in a *lipL* strain or higher in a *gcvH* strain. If there is an allosteric or regulatory effect, it would have to be large, as Natalia Martin finds the phenotypes are the same over a wide range of lipoic acid concentrations (0.5 nM-0.5 mM) (data not shown). An alternative explanation would be that deletion of *lipL* results in global changes in gene expression making lipoyl scavenging insufficient to restore the required enzymatic activities. This may be why an unusual E2 lipoylation pattern was seen in the western blot of a Δ*lipL* strain (Chapter 4).

A similar mechanism based on genetic analyses has been proposed for the yeast, *Saccharomyces cerevisiae*, where it was found that the glycine cleavage system H protein Gcv3 is not only a target for lipoylation, but also is necessary for the lipoylation of the other lipoate-modified proteins, the 2-oxoacid dehydrogenases (Schonauer *et al.*,

2009). These workers favor a model of complex formation and allosteric interactions. A model in which Lip2, the yeast LipB homolog, transfers the octanoyl moiety from octanoyl-ACP to Gcv3, where two sulfur atoms are inserted into the acyl chain by Lip5 (the yeast LipA homolog) was also proposed (Hiltunen *et al.*, 2010). The lipoyl moiety would then be transferred to the 2-oxoacid dehydrogenases by a LplA homologue called Lip3. It is tempting to speculate that Lip3 might perform the same reaction as *B. subtilis* LipL.

It is not clear why *B. subtilis* routes the synthesis of the essential lipoate cofactor through GcvH. Given that this bacterium has a predominately aerobic life style, if routing were needed, PDH would seem more the likely intermediate especially since the role of the glycine cleavage system in bacterial metabolism is obscure. It should be noted that GcvH lipoyl domains are highly divergent from the dehydrogenase E2 lipoyl domains (Figure 5-8B). This divergence may explain how GcvH behaves as a distinct acceptor protein. In *B. subtilis,* strains carrying a *gcvH* disruption grow well when provided with lipoic acid or the products of the inactivated dehydrogenases (Figure 5-7A), which indicates that GcvH does not play an essential role in amino acid or single carbon metabolism. Natalia Martin showed that *E. coli lipB* complements both Δ*lipM* and Δ*lipL* mutant strains (data not shown). Some evolutionary pressure may exist for *B. subtilis* to maintain the specificity of LipM for GcvH. Instead of a rational phenomenon, it may be the result of constructive neutral evolution (Gray *et al.*, 2010). It may be that LipM coevolved with the glycine cleavage system and LipL provided a means for it to be used for other dependent complexes. A more careful bioinformatic analysis may reveal the ancestral states of the pathway.

B. subtilis is predicted to encode all five known lipoic acid dependent complexes: pyruvate dehydrogenase, 2-oxoacid dehydrogenase, branched keto acid dehydrogenase, acetoin dehydrogenase and glycine cleavage system. Amidotransfer of octanoyl/lipoyl moieties may provide *B. subtilis* with the ability to quickly adapt to changing environments by transferring lipoic acid among different enzymes. Alternatively, the extra step in the biosynthetic pathway may provide additional

opportunities for regulatory control. *B. subtilis* has previously been shown to utilize lipoic acid as a sulfur source (Mansilla *et al.*, 1997). Although it is unlikely that lipoic acid would be a significant source of sulfur in most environments, lipoylation could be used as an indirect readout of sulfur availability. No transcriptional regulation of lipoic acid biosynthesis has been found in *E. coli* (Cronan unpublished) or *Salmonella enterica* (Smith *et al.*, 1991). However, *B. subtilis lplJ* has been shown to be induced during spore outgrowth (Keijser *et al.*, 2007) and *lipM* and *lplJ* are induced in response to valine (Ye *et al.*, 2009). Constitutive expression of GcvH would allow it to be always available to act as a lipoate transfer protein regardless of glycine supplementation. Clearly, there is much to be learned about the physiological and ecological implications of this pathway.

TABLES

Table 5-1. Strains used in this chapter.

Strain	Genotype	Source
	Escherichia coli:	
MG1655	*rph-1*	CGSC*
RMK98	MC1061 *fadK*::his6-*cat* Δ*ompT*::FRT	This chapter
QC146	*rph-1* Δ*lplA*::FRT Δ*lipB*::FRT	(Chapter 2)
QC152	*rph-1* Δ*lplA*::FRT Δ*lipB*::FRT / pTARA, pQC026	This chapter
QC172	*rph-1* Δ*lplA*::FRT Δ*lipB*::FRT Δ*ompT*::FRT	This chapter
QC191	*rph-1* Δ*lplA*::FRT Δ*lipB*::FRT Δ*ompT*::FRT / pQC047, pTARA	This chapter
QC222	*rph-1* IN (*rrnD-rrnE*)1 ϕ (fabA-lacZ)1 (Hyb)*cat* / pQC077, pCY598	This chapter
QC223	*rph-1* IN (*rrnD-rrnE*)1 ϕ (fabA-lacZ)1 (Hyb)*cat* / pQC078, pCY598	This chapter
QC224	*rph-1* IN (*rrnD-rrnE*)1 ϕ (fabA-lacZ)1 (Hyb)*cat* / pQC079, pCY598	This chapter
QC225	*rph-1* IN (*rrnD-rrnE*)1 ϕ (fabA-lacZ)1 (Hyb)*cat* / pQC080, pCY598	This chapter
QC255	*rph-1* IN (*rrnD-rrnE*)1 ϕ (fabA-lacZ)1 (Hyb)*cat* / pQC086, pCY598	This chapter
QC256	*rph-1* IN (*rrnD-rrnE*)1 ϕ (fabA-lacZ)1 (Hyb)*cat* / pQC087, pCY598	This chapter
QC257	*rph-1* IN (*rrnD-rrnE*)1 ϕ (fabA-lacZ)1 (Hyb)*cat* / pQC088, pCY598	This chapter
QC258	*rph-1* IN (*rrnD-rrnE*)1 ϕ (fabA-lacZ)1 (Hyb)*cat* / pQC089, pCY598	This chapter
	Bacillus subtilis:	
JH642	*trpC2 pheA1*	Laboratory stock
NM20	JH642 *gcvH*:: Km	This chapter
NM21	NM20 *amyE*::Pspac-gcvH	This chapter

*CGSC, *E. coli* genetic stock center.

Table 5-2. Plasmids used in this chapter.

Plasmids	**Relevant Properties**	**Source**
Ptara	pACYC origin, arabinose inducible T7 polymerase	(Wycuff *et al.*, 2000)
pCY598	RSF origin, arabinose inducible T7 polymerase	(Cronan,2003)
pCR2.1	TOPO TA cloning vector	Invitrogen
pSJ120	*E. coli* LipB expression vector	(Jordan *et al.*, 2003)
pQC023	*B. subtilis* E2p lipoyl domain expression vector	This chapter
pQC026	C-terminal hexahistidine BLAP expression vector	This chapter
pQC033	N-terminal hexahistidine LipL expression vector	Chapter 4
pQC047	C-terminal hexahistidine PdhC expression vector	This chapter
pQC077	N-terminal hexahistidine LipL K162R mutant expression vector	This chapter
pQC078	N-terminal hexahistidine LipL K162A mutant expression vector	This chapter
pQC079	N-terminal hexahistidine LipL C150A mutant expression vector	This chapter
pQC080	N-terminal hexahistidine LipL C150S mutant expression vector	This chapter
pQC086	N-terminal hexahistidine LipL C39A mutant expression vector	This chapter
pQC087	N-terminal hexahistidine LipL C39S mutant expression vector	This chapter
pQC088	N-terminal hexahistidine LipL Y180A mutant expression vector	This chapter
pQC089	N-terminal hexahistidine LipL Y180F mutant expression vector	This chapter
pGES485	Integrational vector; Sp	This chapter
pJM114	Integrational vector; Km	(Perego,1993)
pNM67	pJM114 containing gcvH interrupted with a kanamycin cassette	This chapter
pNM70	pGES485 containing gcvH cloned into HindIII-BamHI sites	This chapter

Table 5-3. Primers used in this chapter.

Oligonucleotides	**Sequence 5'-3'**
Q030	ttaaccatggcatttgaatttaaacttcc
Q031	aatggatccttaaaattgaagatcttcgtaacc
Q034	gaaacagcacctataaaggaggca
Q042	ttaatgatgatgatgatgttgagtggaattgctcagttcg
Q095	taaggtaccaaggagatataccatggcatttgaatttaaacttc
Q096	attggatccttagtgatggtgatggtgatgcgcctccattaaaattaattg
Q113	aacggataagacgggcataa
Q114	gggatgaaggaacgtcattt
Q147	ctatgacctcagtataaatggcaaagcgttcgccggcatct
Q148	agatgccggcgaacgctttgccatttatactgaggtcatag
Q149	cagctatgacctcagtataaatggcaaacgtttcgccggcatctct
Q150	agagatgccggcgaaacgtttgccatttatactgaggtcatagctg
Q151	ctatgaaattgaagggtcttattctcccggcagcta
Q152	tagctgccgggagaataagacccttcaatttcatag
Q153	agcctatgaaattgaagggtcttatgctcccggcagctat
Q154	atagctgccgggagcataagacccttcaatttcataggct
Q0171	gcaatggatgatacgctagccatgtccgtcggaaaagg
Q0172	ccttttccgacggacatggctagcgtatcatccattgc
Q0173	cgcaatggatgatacgctatccatgtccgtcggaaa
Q0174	tttccgacggacatggatagcgtatcatccattgcg
Q0175	gcggcggagtcgctgttcaaattgctctctgtgcagata
Q0176	tatctgcacagagagcaatttgaacagcgactccgccgc
Q0177	cggcggagtcgctgttcaaatttttctctgtgcaga
Q0178	tctgcacagagaaaaatttgaacagcgactccgccg
L	agtggatccaagagcatgggaaag
LI	ttgtcgactcgtgttctcctgagtaa
LII	gagtcgacacaagaagactaaaacag
LIII	aaagactgcagtgaaattcaccgcca

A.

Peptide Sequence	Residue	ΔMass	Modification	Score
QSFAMDDTL**C**MSVGK	C40	0	None	60
QSFAMDDTL**C**MSVGK	C40	188	Lipoylation	34
QSFAMDDTL**C**MSVGK	C40	126	Octanoylation	41
IEAYEIEGSY**C**PGSYDLSINGK	C150	188	Lipoylation	57
IEAYEIEGSY**C**PGSYDLSINGK	C150	126	Octanoylation	58

B.

```
  1 MANQPIDLLM QPKWRVIDQS SLGPLFDAKQ SFAMDDTLCM SVGKGVSPAT
 51 ARSWVHHDTI VLGIQDTRLP FLQDGISLLE SEGYRVIVRN SGGLAVVLDD
101 GVLNISLIFE DEKKGIDIDK GYEAMVELMR RMLRPYNAKI EAYEIEGSYC
151 PGSYDLSING KKFAGISQRR VRGGVAVQIY LCADKSGSER ADLIRRFYQA
201 ALKDKQNDKK GVYPEIRPET MASLSELLQK DISVQDLMFA
```

Figure 5-1. Panel A: Modifications of LipL detected after trypsin digestion followed by LC-MS/MS analysis. The theoretical peptides are shown with the modified cysteine residues in bold and underlined. The mass difference of the modification is given. The ion score is equal to -10log (P) where P is the probability the result is random chance. Panel B: Sequence coverage of LipL by LC-MS/MS analysis. The LipL peptide sequences detected are underlined.

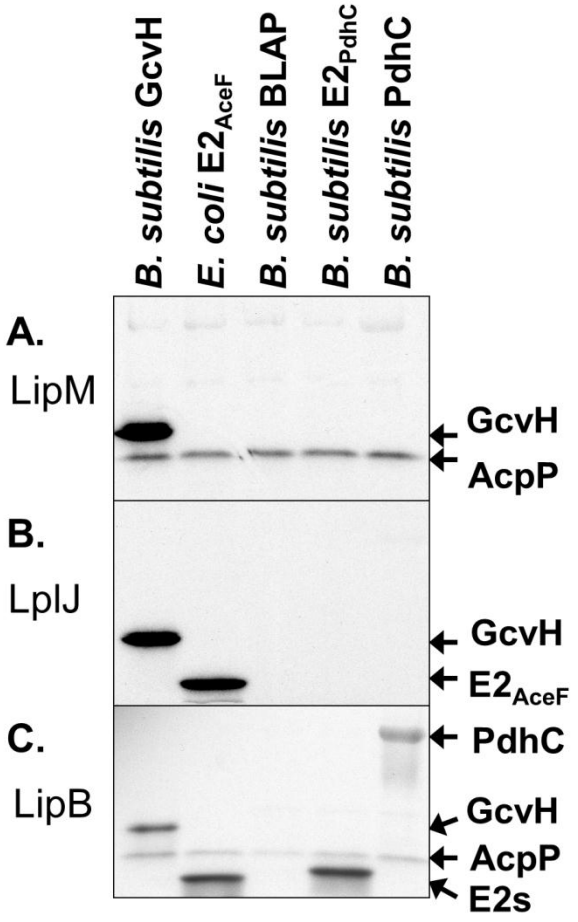
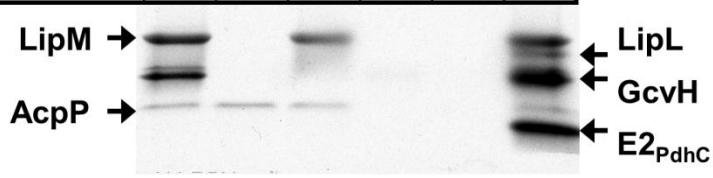


Figure 5-2. Specificity of *B. subtilis* LipM, *B. subtilis* LplJ, and *E. coli* LipB for various lipoyl domains. The lipoyl domain tested as a substrate is indicated at the top of each lane and by an arrow. The [1-^{14}C]octanoyl-ACP substrate is indicated by and arrow (for A and C). Autoradiograms of SDS-PAGE gels separating lipoyl domain substrate specificity assays are shown. The substrate [1-^{14}C]octanoyl-ACP is indicated by an arrow. Free sodium [1-^{14}C]octanoate was used as a substrate for LplJ. Panel A: LipM octanoyltransfer to lipoyl domains from [1-^{14}C]octanoyl-ACP generated using AasS. Panel B: Ligation of [1-^{14}C]octanoate by LplJ to lipoyl domains. Panel C: LipB octanoyltransfer to lipoyl domains from [1-^{14}C]octanoyl-ACP generated using *Vibrio harveyi* acyl-ACP synthetase (AasS).

A.

	1	2	3	4	5	6
LipL	-	+	+	+	+	+
LipM	+	-	+	+	+	+
GcvH	+	+	-	+	+	+
Holo-AcpP	+	+	+	-	+	+
AasS	+	+	+	+	-	+
$E2_{PdhC}$	+	+	+	+	+	+



B.

	1	2	3	4	5
LipL	-	+	+	+	+
LplJ	+	-	+	+	+
GcvH	+	+	-	+	+
$E2_{PdhC}$	+	+	+	-	+

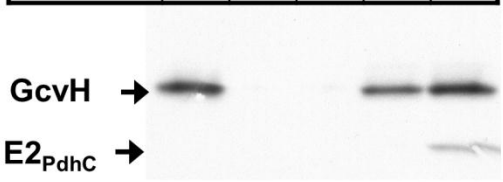


Figure 5-3. Panel A: Component controls for a coupled AasS/LipM/LipL assay starting with [1-^{14}C]octanoate, which becomes ligated to ACP by AasS. Following the reaction the proteins were resolved by SDS-PAGE and visualized by autoradiography. The identities of the [1-^{14}C]octanoylated proteins are indicated. Panel B: Component controls for a coupled LplJ/LipL assay as in Panel A. The starting substrates for ligation by LplJ in every condition are [1-^{14}C]octanoate and ATP.

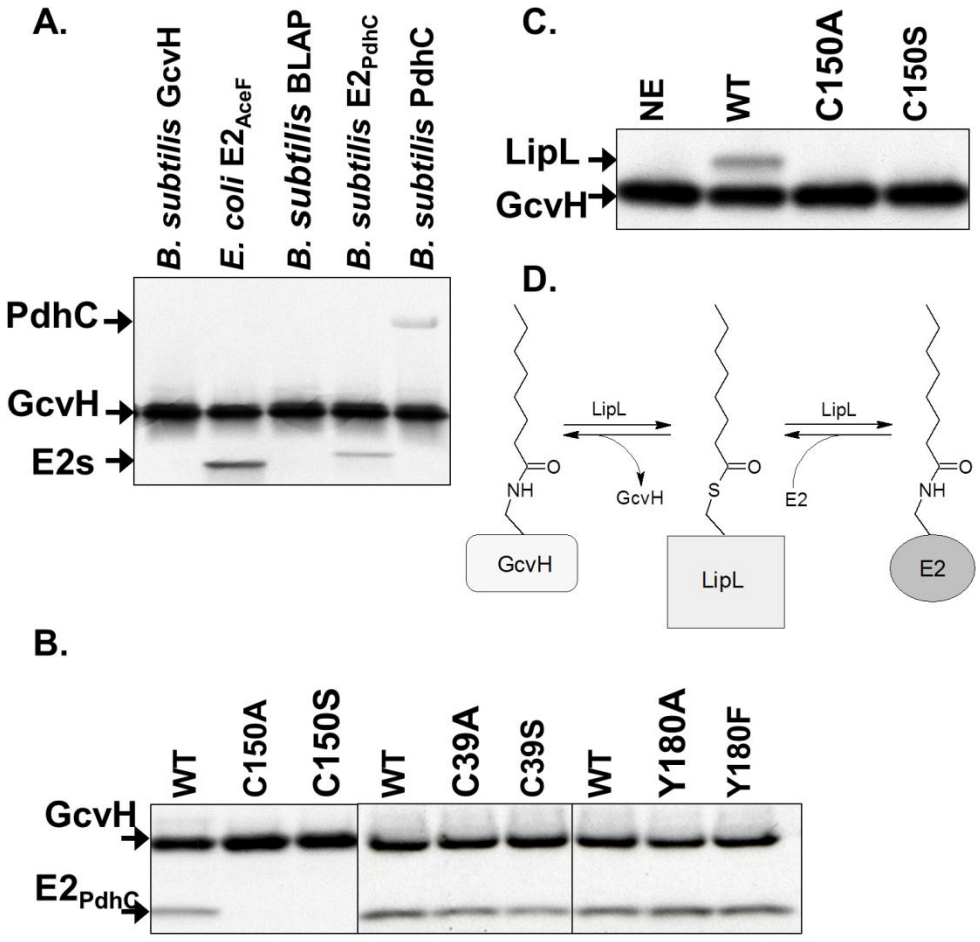


Figure 5-4. Autoradiograms of SDS-PAGE gels of assays for LipL-catalyzed amidotransfer from purified [1-^{14}C]octanoyl-GcvH to lipoyl domains. Panel A: Amidotransfer of the [1-^{14}C]octanoyl moiety from purified octanoyl-GcvH to the unmodified lipoyl domain indicated. Each reaction contained purified WT LipL. Panel B: Amidotransfer from purified [1-^{14}C]octanoyl-GcvH to the $E2_{PdhC}$. The purified LipL wild type (WT) or point mutant proteins indicated were used as enzyme sources. Panel C: Additional enzyme was added to facilitate detection of the octanoyl-LipL intermediate. Wild type (WT), C150A point mutant, C150S point mutant LipL were assayed as well as a control lacking LipL (NE). Panel D: Schematic of the LipL amidotransfer reaction is shown with the acyl-LipL intermediate.

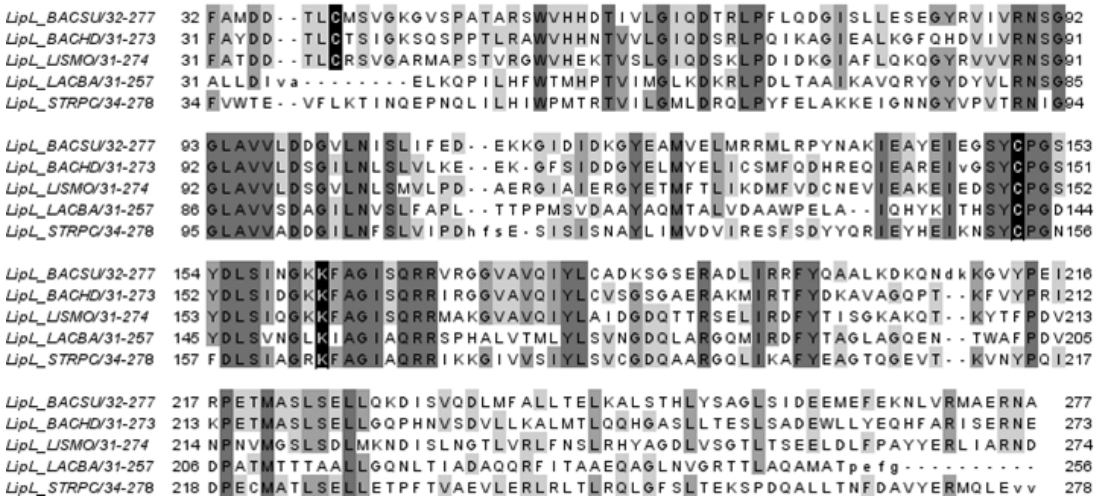


Figure 5-5. Alignment of members of the LipL clade. Positions having 50% or greater similarity are highlighted in grey. The catalytic cysteine residue (C150 in *B. subtilis* LipL), the other modified cysteine residue (C39 in *B. subtilis* LipL), and the conserved PF03099 lysine residue are highlighted in black. BACSU is *Bacillus subtilis* 168, BACHD is *Bacillus halodurans*, LISMO is *Listeria monocytogenes*, LACBA is *Lactobacillus brevis* and STRPC is *Streptococcus pyogenes* serotype M12 (strain MGAS9429). This alignment is part of the larger one used to create Figure 8A.

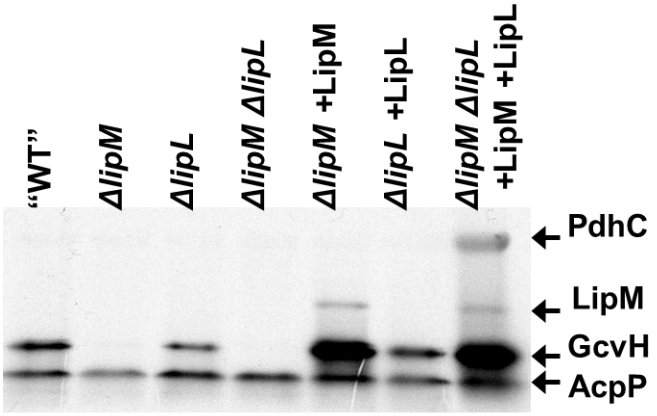


Figure 5-6. Autoradiograph of an SDS-PAGE gel of octanoyltransfer assays using AasS generated [1-^{14}C]octanoyl-ACP as a substrate. Extracts of the mutant strains indicated were used as a source of enzyme for transfer to GcvH and PdhC. All strains also contain a disruption in *lplJ*. Purified LipM and LipL were added to replace the missing protein(s) where indicated with a plus sign.

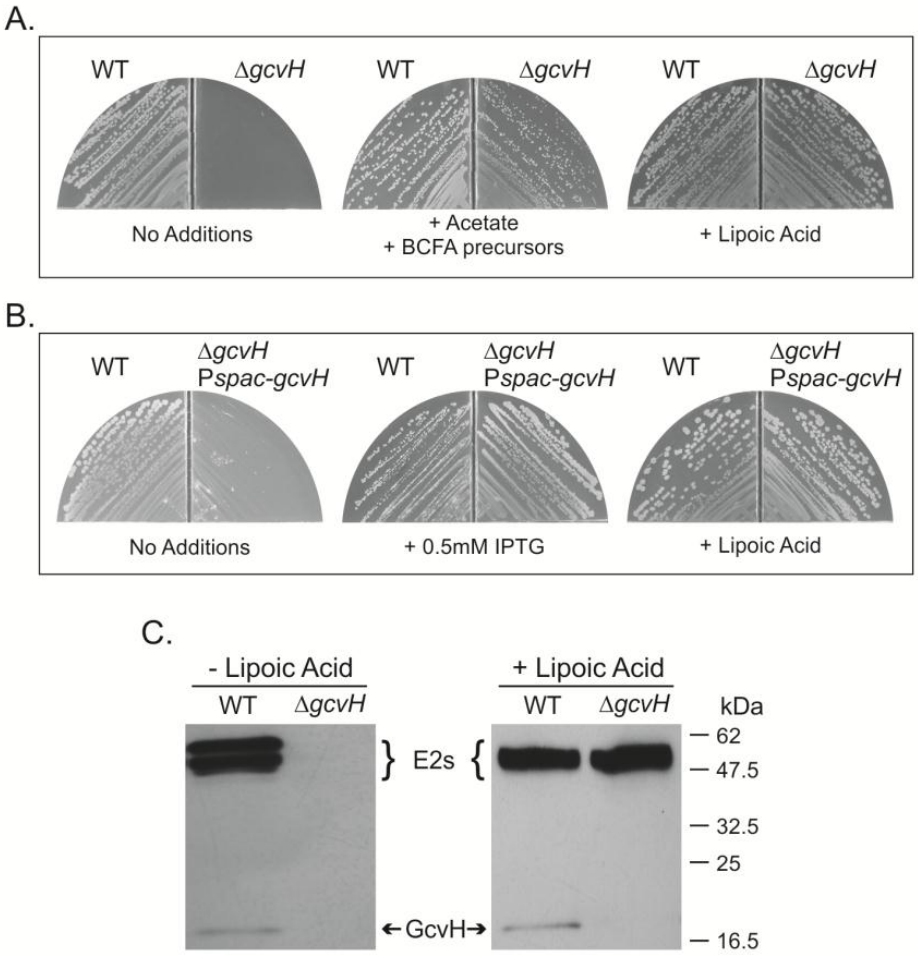


Figure 5-7. Growth and lipoylation phenotypes of a *B. subtilis* Δ*gcvH* strain. Growth of strains on minimal glucose media plates with the supplements indicated after 48 hours at 37°C. LA denotes lipoic acid. Panel A: Growth of strains JH642 (wild type) and NM20 (Δ*gcvH*). Panel B: Growth of strains JH642 (wild type) and NM21 (Δ*gcvH amyE::* P*spac-gcvH*). Panel C: Lipoylation of GcvH in *B. subtilis* crude extracts. SDS-PAGE and immunoblotting with anti-LA antibody was done as described in Experimental Procedures. Cells were grown 22 hours in minimal media with or without lipoate added as indicated. Crude extracts of *B. subtilis* wild type (WT) and Δ*gcvH* strains were used as indicated. Data collected from experiments performed by Natalia Martin.

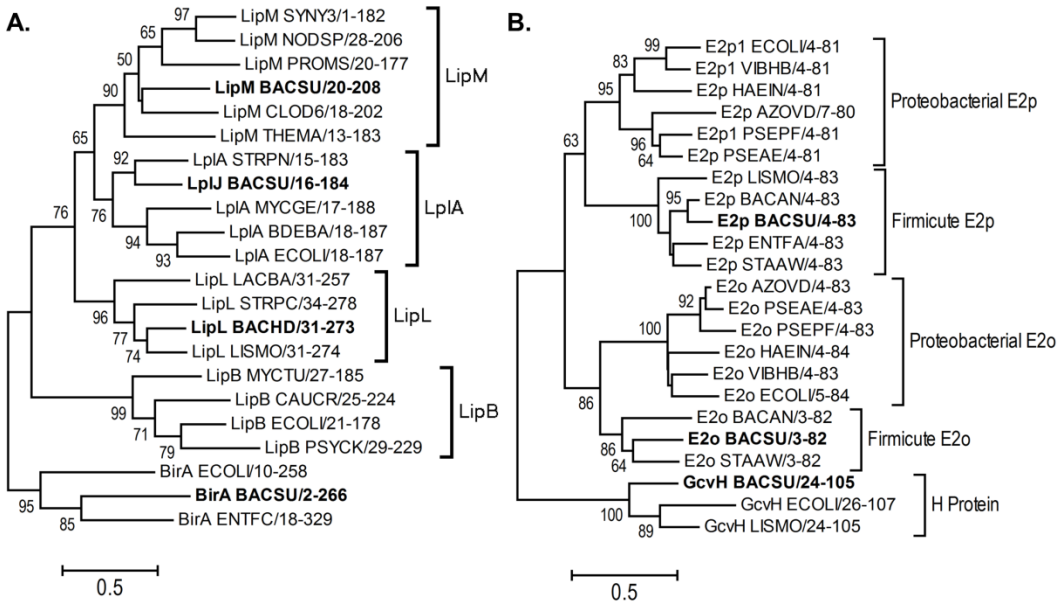


Figure 5-8. Minimum evolution phylogenetic analysis of alignments with bootstrap percentage values shown for each branch. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The scale represents a 50% difference in compared residues per length. Panel A: Analysis of selected catalytic domains from PF03099 the structurally related biotin ligases are used as the out-group. Panel B: Selected lipoyl domains involved in lipoic acid metabolism are shown.

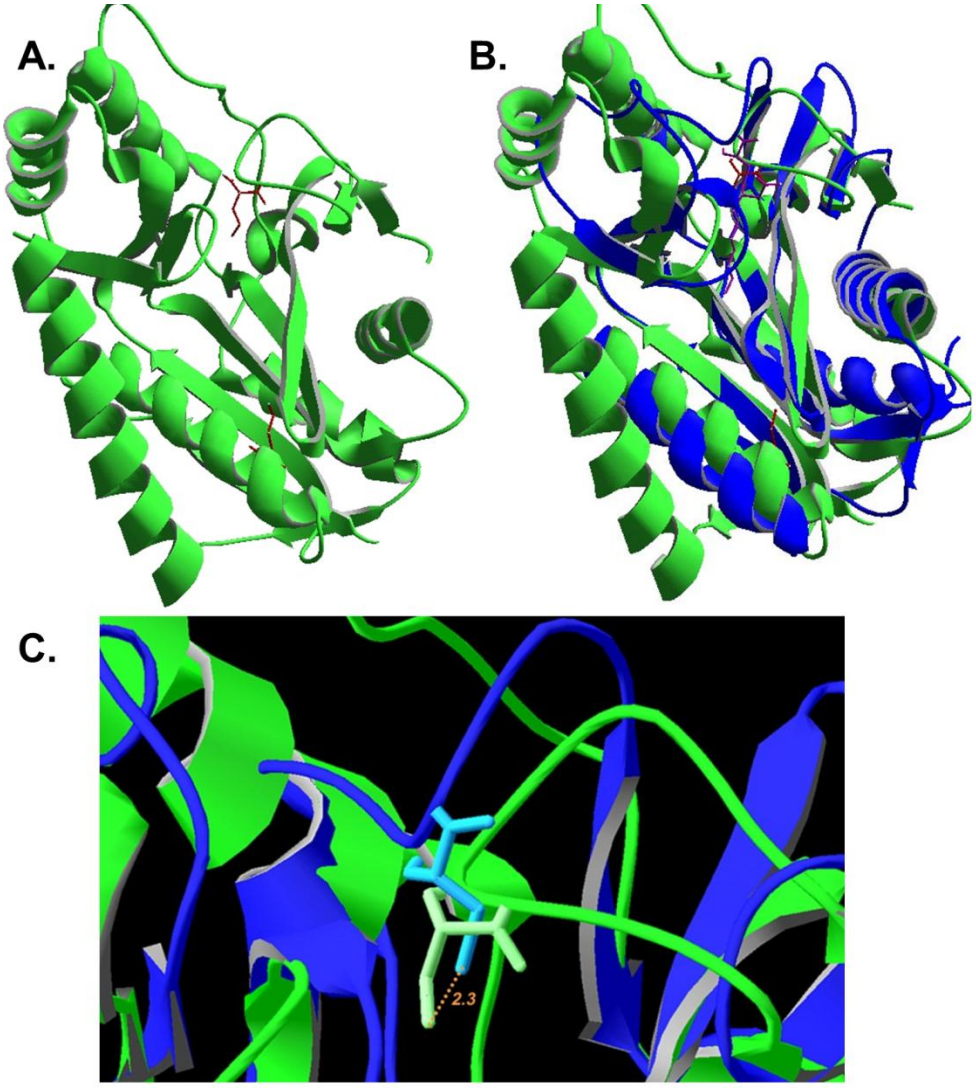


Figure 5-9. Structure of LipL and comparison with that of LipB. Panel A: The unpublished structure of LipL from *B. halodurans* (PDB 2P5I). The modified cysteine residues, C39 and C150, detected by LC-MS/MS are shown in red. Panel B: Structural alignment of LipL from *B. halodurans* with LipB from *M. tuberculosis (*PDB 1W66) (Ma *et al.*, 2006). The active site adduct is shown in purple. Panel C: Close up view of the structural overlay with the LipB decanoyl adduct removed. The active site cysteine sulfur atoms are colored orange whereas carbon atoms are white. The distance between the two sulfur atoms is 2.4 Å.

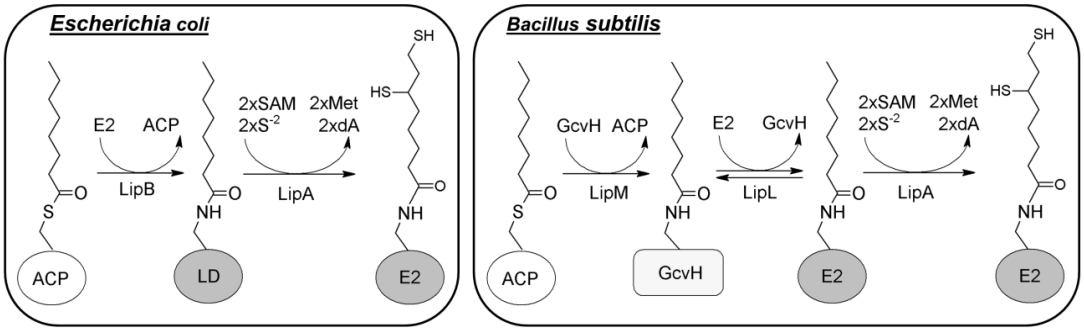


Figure 5-10. Current models for lipoic acid biosynthesis in *E. coli* and *B. subtilis*. Lipoic acid synthesis in *E. coli* is accomplished by two enzymes whereas *B. subtilis* requires three enzymes. *B. subtilis* also requires GcvH as an intermediate carrier where *E. coli* does not. Data are lacking to determine whether LipA acts before or after LipL. I propose that *B. subtilis*, LipA uses octanoyl-LD (both octanoyl-GcvH and octanoyl-E2) as substrates, and therefore can act before or after LipL.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

SUMMARY AND NARRATIVE OF FINDINGS

In this Thesis I describe three new enzymes that make post-translational modifications of proteins for lipoic acid metabolism, adding to our knowledge of physiologically relevant lipoylation pathways (Figure 6-1). The first enzyme, the heterodimeric lipoyl ligase LplA-LplB, was well characterized structurally. The previously published suggestion (McManus *et al.*, 2006) that this lipoyl ligase requires a small protein upstream was correct. This was also verified by a study concluded at the same time as mine (Posner *et al.*, 2009). The study by Posner *et al.* also demonstrated that the E2 is lipoylated in *T. acidophilum* extracts. Some spots at the bottom of the lane may be the H protein, which I and Natalia Martin have found is not amendable to western blotting (Posner *et al.*, 2009). My study in Chapter 2 goes further to find that this small protein, which is homologous to the C-terminal domain of other characterized ligases, and is required for adenylation but not lipoyl transfer. I call this the accessory domain. This finding was later supported by structural work that revealed that the C-terminal domain is in one conformation for adenylation but then rotates away from the active site for transfer (Fujiwara *et al.*, 2010). This structural study casts doubt on the notion of a terinary complex composed of LplA, LplB, and lipoyl domain, as suggested by size exculsion (Posner *et al.*, 2009). In Chapter 2, I found that LplA and LplB migrate anomalously by size exclusion, so they would co-migrate with the lipoyl domain. Futher experimental tests of the association of LplB (or the C-terminal domain) and the catalytic subunit in the presence of their substrates would be informative. It appears an Asp residue is conserved in the accessory domain (Figure 2-7). This residue is near others that contact the catalytic domain in structural studies of *E. coli* LplA (Fujiwara *et al.*, 2010). The contacting residues revealed by the structure are not conserved, however. Further experimental work could also explore residues important for

association of these two domains, and by extension for enzymatic activity. By surveying the LplA catalytic domain protein family, I find many members are not predicted to have a C-terminal domain, or a candidate for a separate one, in the same genome. This casts doubt on the annotation of other single domain proteins homologous to *T. acidophilum* LplA as lipoyl ligases.

The second enzyme I characterize in Chapter 3 is one such single-domain ligase homologue, LipM. I identified this gene using a complementation strategy which is unbiased by sequence similarity. Although the only gene found to be capable of complementing an *E. coli lipB lplA* mutant proved to be one of three genes in the genome with similarity to previously characterized proteins, this type of complementation strategy is a powerful and informative one. I have not been the first, nor will I be the last to use it. Although the complementation studies *per se* were straightforward, I had problems demonstrating activity with heterologously expressed, purified protein. Years went by where I tried looking for activity by various methods with various components added. Through careful analysis by mass spectrometry I found the mass was too large. Out of desperation I analyzed pure LipM by trypsin digest and LC-MS/MS, which was possible due to the Protein Sciences Center across the street. Although the technique is prone to false positives I found convincing evidence that LipM was modified by octanoate (the expected substrate) and decanoate (a covalent adduct). As a result I recommend that any protein purified should be subject to mass spectrometric analysis. MIT Professor of Biochemistry, Joanne Stubbe, goes further to recommend that proteins should be purified and studied from the native host at native expression levels (Cotruvo *et al.*, 2011). From purification and derivitization I found that about 2% of the active site was modified with octanoate. This supports the finding of Bachar Hassan (Hassan *et al.*, 2011) that octanoyl-ACP, not lipoyl-ACP, is the physiologically relevant substrate. This also supports the assumption that LipM and LipB are largely irreversible. Spurred on by the LC-MS/MS result I cloned and purified different lipoyl domains to test as a substrate. As reported in Chapter 3, the glycine cleavage system H protein worked well. The first four lipoyl domains I tried as substrates

did not work. The initial survey of the LplA family was interesting so I decided to do a more careful phylogenetic analysis of the family. I verified that LipM is more closely related to lipoyl ligases than to LipB octanoyltransferases. This is interesting because it doesn't fit the definition of a non-homologous isofunctional enzyme (Omelchenko *et al.*, 2010). Although it is homologous to LipB, it is clear that it follows a different evolutionary path. Therefore, it appears that LipB and LipM are functionally convergent. This assumes the common ancestor of LipB and LipM was not an octanoyltransferase. It would be difficult to account for the sequence divergence if this were not the case. Because LipM catalyses the same reaction as LipB, it is a novel isozyme. A structure of LipM is lacking and efforts to obtain one are underway by the de Mendoza lab and others in Argentina. Orthologs of both LipM and LipB are encoded by cyanobacteria in the same genomes. It would be interesting to determine the interplay of these two enzymes in a photosynthetic organism. Although the role of lipoic acid biosynthesis in virulence has been determined for acomplexan parasites, no studies have been conducted in bacteria.

In Chapter 4 and 5, Natalia Martin and I combine our efforts to figure out lipoic acid biosynthesis in *B. subtilis*. It is certain I wouldn't have gotten this far in this investigation without this collaboration, and I am fortunate things worked out for the best. Members of Diego de Mendoza's lab had been musing about *lipL* for some time and they called my attention to it early in our collaboration. I added *lipL* to the list of genes I would study, while Martin knocked out everything predicted to be involved in lipoic acid biosynthesis. Our first results conflicted: Martin found that *lipM* and *lipL* were required for lipoic acid biosynthesis, while I found only *lipM* complemented *E. coli*. To make matters worse, I could not detect *in vitro* activity from either protein. This was unexpected because *lipM* could complement *lipB,* leaving us in an uncomfortable position for a couple years. It caused me to reevaluate everything, which reinforces that in biochemistry especially "the devil is in the details". My first clue to what the problem was came from some early western blots I performed, where a small lipoylated protein showed up on some of my blots. It wasn't reproducible so I dismissed it. The second clue

came when I assayed octanoylation of the full length E2 subunit by LipL and LipB with radiolabeled octanoyl-ACP as a substrate. Making octanoyl-ACP in the reaction with AasS was a convenience that improved signal and made the assay easier. I saw no activity until I added *B. subtilis* crude extract. It was then that I saw a faint small band. It seemed this was an intermediate protein substrate. I guessed it was GcvH and am fortunate that I was right, because identifying it through purification would have been much more work. I demonstrated activity of LipM. I tried "one last" series of experiments with LipL, but this time with GcvH added as well. Less than two months later I had activity for LipL as well. It was a new enzyme. Other amidotransfer reactions had been described. This one used novel substrates, and LipL does not resemble other amidotransferases by any metric.

A more careful analysis of lipoic acid scavenging and LipL should be done. Early in my studies I was puzzled by the distribution of LipL homologues in all Firmicutes that use lipoic acid, including ones that are natural auxotrophs. My data suggest that LplJ is fairly specific, while Martin's data show that ligation depends on *lipL* but not *gcvH.* This is confusing, as one would have predicted from the ligation deficiency of a *lipL* strain that a *gcvH* would also be deficient in lipoic acid scavenge. A natural lipoic acid auxotroph would be ideal to determine the importance of LipL in scavenging. Of natural auxotrophs, *Listeria* is also interesting because of the uncertainty regarding the mechanisms for utilization of lipoyl peptides (Keeney *et al.*, 2007).

OBSERVATIONS ON PHYLOGENY

The metabolism of biotin and lipoic acid share common ancestry, although biotin biosynthesis requires additional enzymes. The enzymes responsible for sulfur insertion are homologous. The domains modified by lipoic acid and biotin, the lipoyl domain and biotin carboxyl carrier protein, respectively, are homologous. The enzymes that modify them are homologous as well. All such modification enzymes discovered so far belong to the BPL-LipB-LplA superfamily. Although it is a single Pfam entry (PF03099), it contains members catalyzing different reactions by the same mechanism, so it is best considered

a superfamily (Gerlt *et al.*, 2001). The conserved reaction throughout the superfamily is the acylation of substrate proteins. LipL is the first member characterized that additionally catalyzes the reverse of this reaction. It should also be possible for a LipL-like enzyme to transfer the acyl-enzyme intermediate to water instead of to another lipoyl domain, therefore acting as a lipoamidase. The only lipoamidase characterized so far is not in PF03099 (Jiang *et al.*, 2005). Three major clades comprise PF03099, originally exemplified by biotin ligases, ACP:LD octanoyltransferases, and lipoate ligases (Figure 6-2). This study has characterized three novel enzymes from the lipoate ligase family. After these findings it is clear that the members of the LplA clade catalyze different reactions that are mechanistically similar, so it can also be considered a superfamily (Figure 6-3).

From examining the phylogeny of the lipoate ligase family we can draw some interesting conlcusions. Both the mammalian lipoyltransferases and yeast LIP3 are part of the same clade, shown in Figure 6-3. This suggests that LIPT and LIP3 are performing the same reaction, and that the transfer reaction of LIPT studied *in vitro* is not the physiologically relevant reaction. As suggested in Chapter 4, amidotransfer catalyzed by LIP3 would be consistent with the current data. The LIPT clade derives from within the major lipoate protein ligase clade near the proteobacterial LplA's. This is consistent with the idea that LIPT and LIP3 derived from the mitochondrial predecessor before the endosymbiotic event.

The lipoyl ligase clades are distributed throughout the tree. Also, the earliest clade characterized so far contains the *T. acidophilum* lipoyl ligase. Both of these properties suggest that the LplA family ancestor may have been a ligase. The more deeply branching clades characterized also include LipL and LipM clades, which do not have an accessory domain. This suggests the common ancestor lacked an attached accessory domain. So the best guess is the LplA family common ancestor was a bipartite lipoyl ligase or of a similar function predispositioning it to becoming a ligase. It would be interesting to assay the *T. acidophilum* LplA subunit for amidotransfer activity. It is tempting to speculate some members of this family are bifunctional.

PHYLOGENETIC DISTRIBUTION OF ENZYMES

The distribution of lipoic acid metabolic enzymes in selected organisms is shown in Table 6-1. By reevaluating the components of lipoic acid metabolism in different organisms, some discrepancies are apparent. Both *Picrophilus torridus* and *Ferroplasma acidarmus* have a putative *lipA* and two *lipBs* in their genome. However, they do not have anything resembling an acyl carrier protein. Are they making lipoic acid, and if so how?

The members of the LplA family are subdivided into clades as shown in figure 6-3. Enzymes that have been characterized experimentally are in bold. The LplA clade 2 contains three studied members, although they are not well characterized. This clade has LplA2 (or LipL2) from *Plasmodium falciparium*, which weakly complements an *E. coli lipB lplA* when lipoic acid is added (Allary *et al.*, 2007); LplA from rice (*Oryza sativa*), which complements *E. coli lipB lplA* with improved complementation when lipoic acid is added (Kang *et al.*, 2007) ; and *C. trachomatis* LplA, which was found to be inactive (Ramaswamy *et al.*, 2010). The lack of lipoyl ligase activity of the *Chlamydia trachomatis* member can be attributed to a missing accessory domain, although it is not known if the missing domain exists. The lack of activity of some of these ligases may be due to lipoyl domain specificity as described for *P. falciparum* LplA2 during complementation studies in *E. coli* (Allary *et al.*, 2007). It is likely this clade consists of lipoyl ligases although the lack of activity of some members means further work is required to definitively know the function of members of this clade.

We characterized *B. subtilis* LipM in Chapters 3, 4, and 5. Sequenced cyanobacterial genomes including *Prochlorococcus marinus* are predicted to encode a LipB and LipM octanoyltransferase. It would be interesting to examine their roles in metabolism and photosynthesis. The *C. trachomatis* LipM homologue (LplA1) has been recently characterized and has detectable lipoyl ligase activity, while the LplA and LipA lack activity (Ramaswamy *et al.*, 2010). It is unexpected that the LipM has ligase activity and it should be studied further to see if it is an ACP:LD octanoyltransferase as well. This enzyme may be a novel example of a bifunctional enzyme.

It is clear that our understanding of lipoic acid metabolism and the LplA family is much improved by the findings of this Thesis. We were previously ignorant of additional functions, so our uncertainty is also correspondingly increased. This is similar to what was found in other superfamilies (Gerlt *et al.*, 2001). It seems that protein families could differ in the amount of functional diversity, although it is difficult to be sure, as families lacking diversity may appear so from ignorance. Lack of understanding of diverse protein functions is a significant hurdle to gene annotation. From study of diverse proteins functions and their mechanisms we gain greater predictive power.

TABLES

Table 6-1. Distribution of enzymes and homologues.

	LipA	LipB	LipM	LIPT	LplA C1	LplA C2	LplA C3	LipL
Arabidopsis thaliana	**X**	**X**				X		
***Bacillus subtilis* 168**	**X**		**X**		**X**			**X**
***Chlamydia trachomatis* L2**	X		**X**			X		
***Escherichia coli* K12**	X	X			**X**			
Enterococcus faecalis					XX			X
Homo sapiens	**X**	**X**		**X**				
***Listeria monocytogenes* EDGe**					**XX**			X
***Picrophilus torridus* DSM9790**	X	XX						
***Plasmodium falciparum* 3D7**	**X**	**X**			**X**	**X**		
***Prochlorococcus marinus* MED4**	X	X	X					
***Pseudomonas aeruginosa* PA01**	X	X						X
Saccharomyces cerevisiae	**X**	**X**		**X**				
***Thermoplasma acidophilum* DSM1728**							**X**	

FIGURES

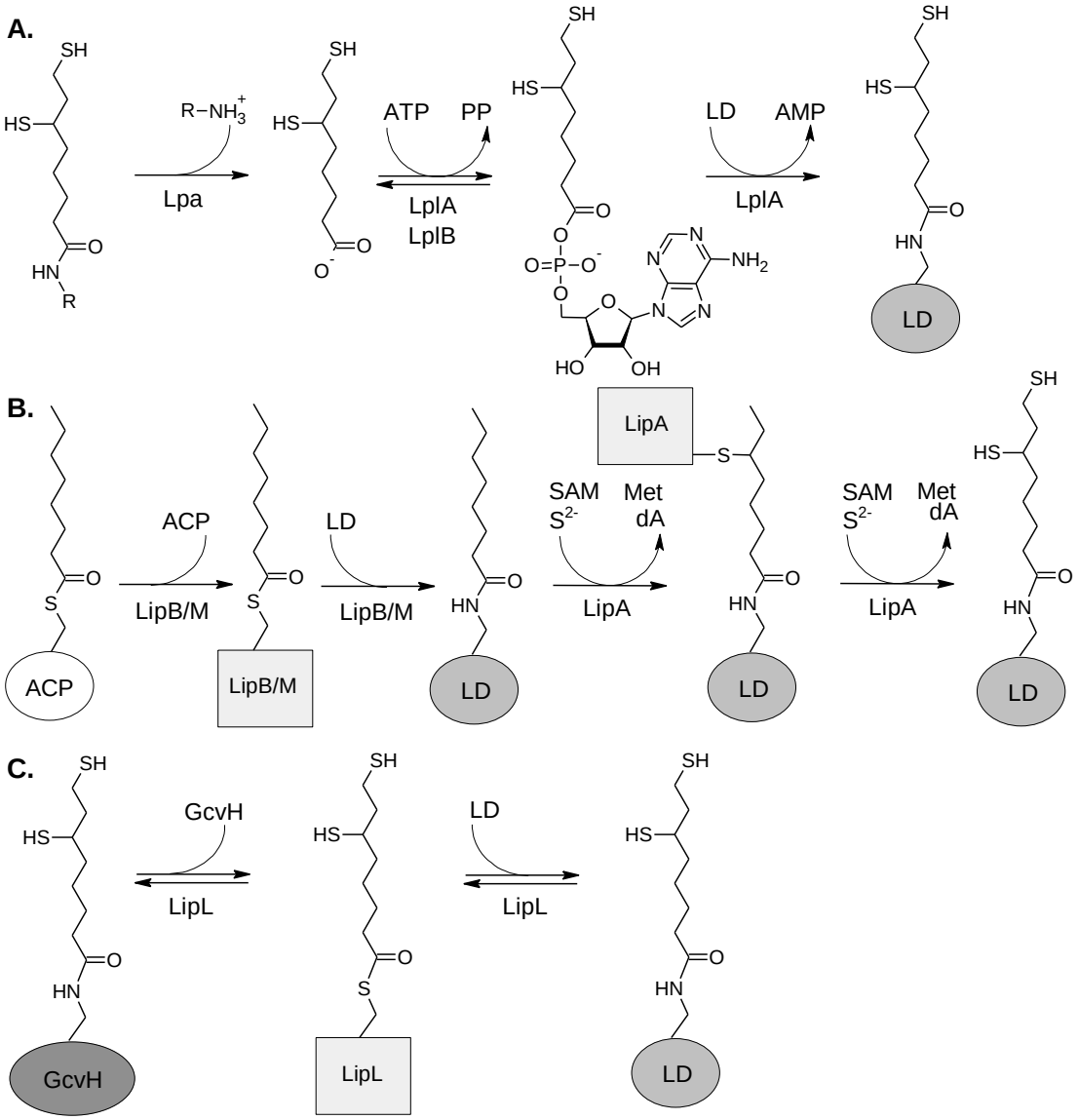


Figure 6-1. Lipoic acid metabolism and lipoyl domain modification. Four different types of enzymes modify the lipoyl domain. Panel A: Lipoyl ligases are capable of utilizing free lipoic acid, which is produced by lipoamidase, Lpa, and perhaps by other processes. Panel B: The ACP:LD octanoytransferases LipB or LipM work with the lipoyl synthase, LipA, to make lipoic acid on the lipoyl domain. Panel C: The novel lipoylamidotransferase, which can shuffle lipoyl and octanoyl groups to different lipoyl domains.

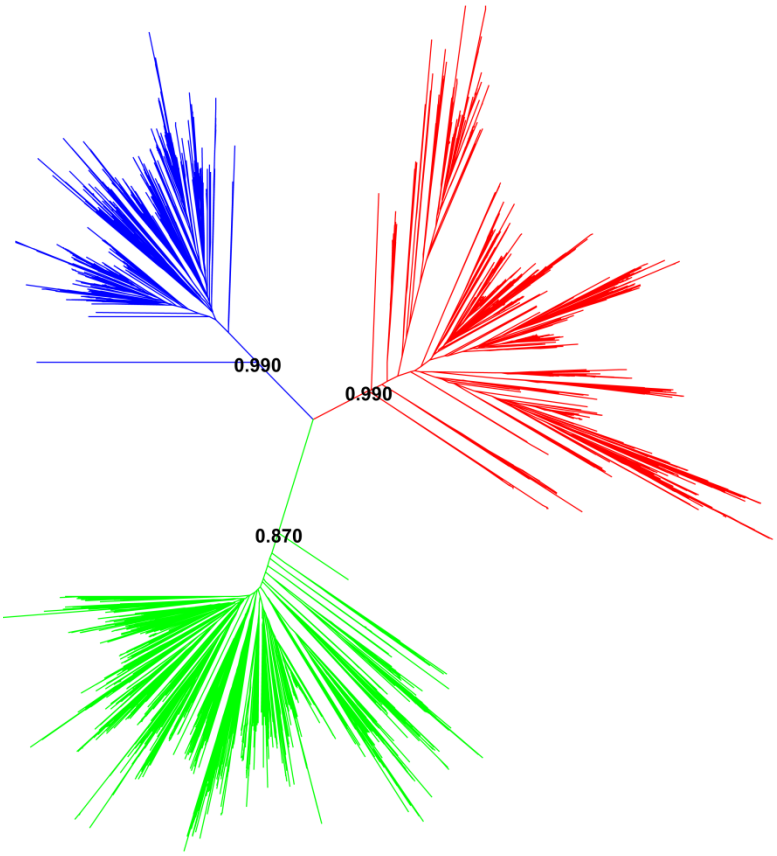


Figure 6-2. The BPL-LipB-LplA protein superfamily pylogenetic tree obtained from Pfam PF03099. The tree was inferred by the neighbor-joining method using FastTree. The frequency each branch occurred during bootstrap resampling with 100 replicates is shown. The blue clade contains characterized LipB ACP:LD octanoyltransferases. The green clade contains characterized BirA and BPL biotin-protein ligases. The red clade contains characterized lipoate protein ligases as well as other enzyme characterized in this Thesis.

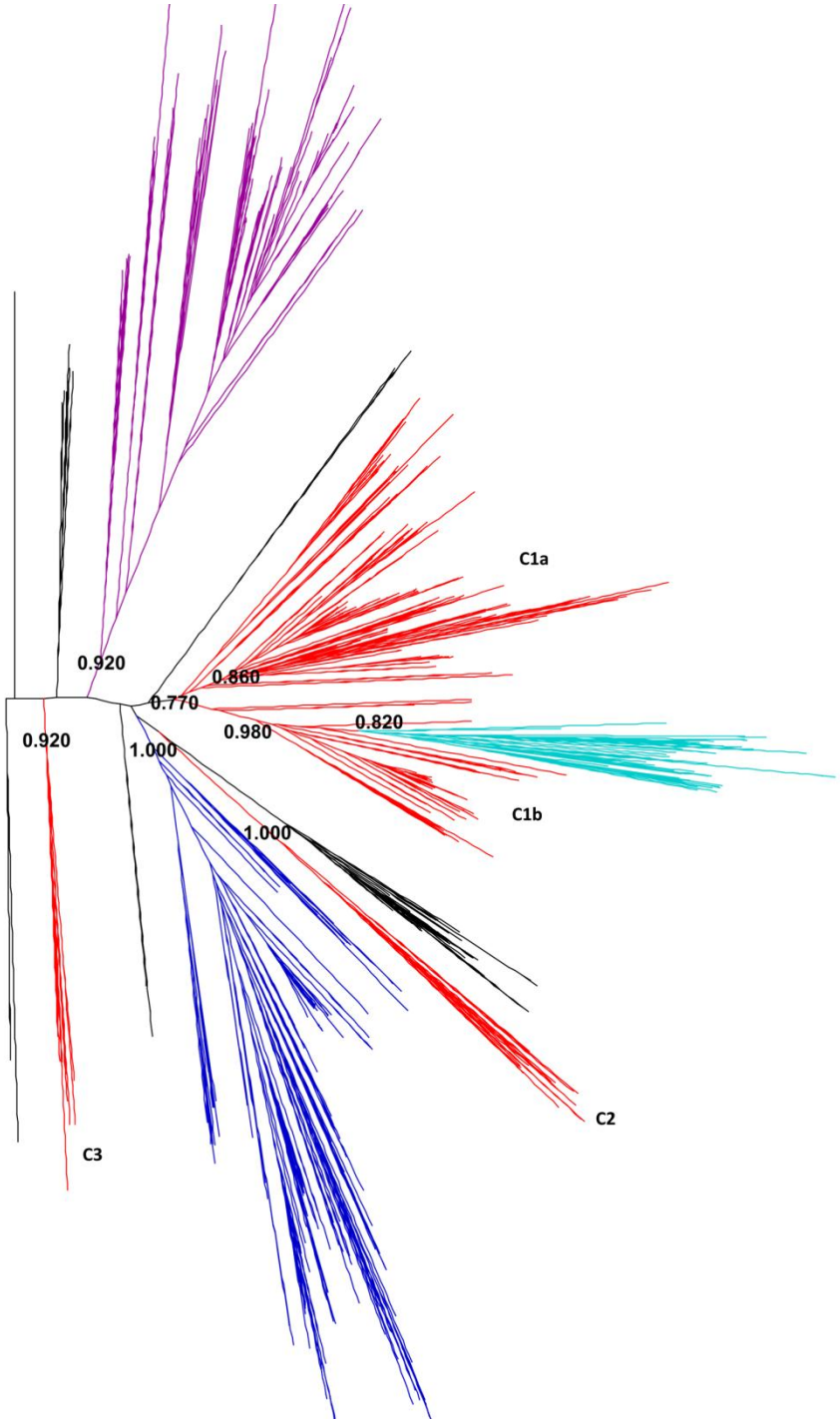


Figure 6-3. The LplA family tree extracted from the full tree in figure 6-2. The frequency each branch occurred during bootstrap resampling with 100 replicates is shown. Clades that contain one member with lipoate-protein ligase activity are colored red and individually numbered. The clade contining *B. subtilis* LipL is colored purple. The clade containing *B. subtilis* LipM is colored blue. The turquoise clade contains both mammalian lipoyltransferases and LIP3p from *S. cerevisiae*.

CHAPTER 7

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CURRICULUM VITAE

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Education:

Postdoctoral Research: University of Washington, Seattle. Starting May 2011
Ph.D. Microbiology: University of Illinois at Urbana-Champaign. Spring 2011
M.S. Microbiology: University of Illinois at Urbana-Champaign. Spring 2007
B.S. Bacteriology: University of Wisconsin, Madison. Spring 2005

Publications:

Christensen QH, Martin N, Mansilla MC, de Mendoza D, Cronan JE. A Novel Amidotransferase Required for Lipoic Acid Biosynthesis by *Bacillus subtilis*. Mol Microbiol. 2011 Apr 80(2):350-363

Martin N, **Christensen QH,** Cronan JE, Mansilla MC, de Mendoza D. A Two Gene Requirement for Octanoyltransfer for Lipoic Acid Synthesis by *Bacillus subtilis*. Mol Microbiol. 2011 Apr 80(2):335-349

Christensen QH, Cronan JE. Lipoic acid synthesis: A New Octanoyltransferase Family Generally Annotated as Lipoic Acid Ligases. Biochemistry. 2010 Nov 23;49(46):10024-36. PMID: 20882995

Christensen QH, Cronan JE. The *Thermoplasma acidophilum* LplA-LplB complex defines a new class of bipartite lipoate-protein ligases. J Biol Chem. 2009 Aug 7;284(32):21317-26. PMID: 19520844

Berti AD, Greve NJ, **Christensen QH**, Thomas MG. Identification of a biosynthetic gene cluster and the six associated lipopeptides involved in swarming motility of *Pseudomonas syringae* pv. tomato DC3000. J Bacteriol. 2007 Sep;189(17):6312-23. PMID: 17601782

Awards and Honors:

Interdisciplinary Training Program in Bacterial Pathogenesis Fellowship 2011-2012

- With the possibility for an additional one year extension

Chemical Biology Interface Training Program Fellowship 2006-2008

- Participation in the program from 2005-2011
- Attended monthly meetings to discuss research
- With the possibility for an additional one year extension

Ira L. Baldwin Research Scholarship 2004

- For undergraduate research in the laboratory of Michael G. Thomas

Research Experience:

Laboratory of **E. Peter Greenberg,** Postdoctoral Research Fellow,
University of Washington, 2011-

- To perform independent research on homoserine lactone signaling in bacteria

Laboratory of **John E. Cronan,** Graduate Research Fellow,
University of Illinois, 2005-2011

- Engaged in independent research on lipoic acid metabolism
- Discovered a new amidotransferase enzyme and octanoyltransferase isozyme
- Extended findings using gene phylogeny and comparative genomics

Laboratory of **Michael G. Thomas,** Undergraduate Researcher,
University of Wisconsin, 2003-2005

- Cataloged putative non-ribosomal peptide synthetases in Pseudomonads
- Made gene deletions and fusions in *P. syringae* and *P. aeruginosa*

Laboratory of **Ann E. Macguidwin**, Research Scholars Program,
University of Wisconsin, 2002

- Examined nematode and fungi pathogenesis of the Russet Burbank potato

Presentations and Meetings Attended:

Boston Bacterial Meeting, Boston, MA, 2010

- Poster: "A Novel Pathway for Lipoic Acid Biosynthesis in Bacilli"

American Society for Microbiology: General Meeting, San Diego, CA, 2010

- Poster: "Lipoic Acid Biosynthesis in *Bacillus subtilis* 168: A Novel Octanoyltransferase Isozyme"

American Society for Microbiology: General Meeting, Philadelphia, PA, 2009

- Poster: "Structural Diversity of Lipoic Acid Ligases Reveal a Function for the Accessory Domain"

Archaea and Evolution Symposium, Urbana, IL, 2007

Gordon Conference on Enzymes, Biddeford, ME, 2007

- Poster: "Lessons from the Lipoyl Ligase of *Thermoplasma acidophilum*"

American Society for Microbiology: North Central Branch Meeting, Madison, WI, 2004

Teaching Experience:

Molecular Genetics, MCB250, Teaching Assistant, University of Illinois, Fall 2008 and Spring 2009.

- Responsible for four discussion sections, 60-80 students, each semester
- Discussed and clarified lecture material to students
- Organized and led small group learning exercises

Professional Societies:

American Society for Microbiology, 2004 to Present