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## A Novel Amidotransferase Required for Lipoic Acid Cofactor Assembly in *Bacillus subtilis*

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### SUMMARY

In the companion paper (Martin *et al.*, 2011) we reported that *Bacillus subtilis* requires three proteins for lipoic acid metabolism, all of which are members of the lipoate protein ligase family. Two of the proteins, LipM and LplJ, have been shown to be an octanoyltransferase and a lipoate:protein ligase, respectively. The third protein, LipL, is essential for lipoic acid synthesis, but had no detectable octanoyltransferase or ligase activity either *in vitro* or *in vivo*. We 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). We 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,  $\Delta gcvH$  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.

### Keywords

Post-Translational Modification; Lipoic Acid; *Bacillus subtilis*

### Introduction

Comparative genomics allows for detection of incomplete metabolic models. Analysis of these discrepancies in our knowledge provides opportunities for new discoveries. Here we 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 that includes many pathogens. *B. subtilis* is the best

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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.

The current model for lipoic acid metabolism is based on *Escherichia coli* (Cronan *et al.*, 2005, Rock, 2009). Enzymes that require lipoic acid for activity become modified by covalent attachment of the coenzyme to the  $\epsilon$ -amino group of a specific lysine residue of conserved ~80 residue domains called lipoyl domains (LDs). Lipoate biosynthesis begins by transfer of octanoate from octanoyl-acyl carrier protein (ACP), a transient intermediate of fatty acid biosynthesis, to the LD by the ACP:LD octanoyltransferase LipB (Zhao *et al.*, 2003). After the LD has been octanoylated, hydrogen atoms attached to the octanoate C6 and C8 carbons atoms are replaced with two sulfur atoms by lipoyl synthase, an *S*-adenosyl-L-methionine radical enzyme (Booker *et al.*, 2007) to give lipoic acid. Exogenous free lipoic acid can also be scavenged by lipoyl ligase, LplA (Green *et al.*, 1995, Morris *et al.*, 1995), which is also active with octanoate (Green *et al.*, 1995, Jordan & Cronan, 1997). Octanoate can also come from intracellular sources and thereby bypass a biosynthesis requirement at the expense of ATP (Hermes & Cronan, 2009).

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 (Christensen & Cronan, 2009). Comparisons of the crystal structures of LplA obtained with and without the acyl adenylyl intermediate show that the accessory domain rotates away from the active site following adenylyl 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 can be due to the domain being a separate protein, as is the case in archaea (Christensen & Cronan, 2009). More recently, one of these single domain LplA homologues, *B. subtilis* LipM, have been shown to be a new ACP:GcvH octanoyltransferase isozyme (Christensen & Cronan, 2010).

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 glycine cleavage system is well characterized in *Arabidopsis* and other plants, where it is known as glycine decarboxylase (Bauwe & Kolukisaoglu, 2003). There it serves a vital role in the C2 pathway (dark cycle), where it concentrates the carbon dioxide released by glycine degradation. In *E. coli* the glycine cleavage system is one of two synthetic routes to serine and 5, 10-methylenetetrahydrofolate. Disruption of the glycine cleavage system in *E. coli* has no growth phenotype, although *gcv* mutants accumulate and excrete glycine (Plamann *et al.*, 1983). Disruption results in an inability to make serine from glycine, which creates a serine auxotrophy in the presence of glycine when the primary serine synthetic pathway is inactivated as well (Vanden Boom *et al.*, 1991). 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). Recently, the glycine cleavage system H protein of yeast, Gcv3, has been shown to be required for lipoic acid biosynthesis and RNA processing (Schonauer *et al.*, 2009). In this paper we 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 (Christensen & Cronan, 2010) should suffice for lipoic acid synthesis and attachment in *B. subtilis*. However, as reported in the companion paper (Martin *et al.*, 2011) 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 paper we describe the novel biosynthetic requirement for LipL and the unexpected reaction catalyzed by this new enzyme. Based on these data we present a novel pathway for lipoic acid biosynthesis.

## RESULTS

### Expression, purification and modification of LipL

LipL (YwfL) was readily expressed in *E. coli* and purified as a soluble hexahistidine-tagged protein which lacked precedent catalytic activities (Martin *et al.*, 2011). *Mycobacterium tuberculosis* LipB and *B. subtilis* LipM have been shown to become modified by attachment of acyl moieties in *E. coli* (Christensen & Cronan, 2010, Ma *et al.*, 2006), so we wanted to determine if LipL also became modified. To this end 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 (Fig. 1A). Two sites of modification were found: cysteine residues C39 and C150. Lipoyl and octanoyl modifications were present at both sites (Fig. 1B) indicating that LipL accepted octanoate and lipoate from *E. coli* sources. Assuming that the reaction catalyzed by this enzyme remains the same in *B. subtilis*, the substrates must be common to both organisms. The two modified 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 (Christensen & Cronan, 2010), we were puzzled by the finding that the lipoyl domain of the *B. subtilis* pyruvate dehydrogenase PdhC subunit (E2<sub>PdhC</sub>) was a poor octanoyl acceptor substrate (Fig. 2A). This was first considered to be an artifact of use of 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 upon size exclusion chromatography consistent with an octahedral complex (data not shown). This indicates that the C-terminal hexahistidine tag does not interfere with multimerization of the protein. 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)(Fig. 2) was 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 (Christensen & Cronan, 2010). Similar to previous results (Fig. 2) addition of only LipM and LipL failed to result in octanoylation of either E2<sub>PdhC</sub> or LipL (Fig. 3A, lane 3). It was only in the presence of GcvH that octanoylation of LipL and E2<sub>PdhC</sub> proceeded (Fig. 3A, lane 6).

A similar phenomenon was also seen for the LplJ, which was identified and demonstrated to be a lipoyl ligase in the companion paper (Martin *et al.*, 2011). Although LplJ was able to ligate octanoate to the *E. coli* lipoyl domain (E2<sub>AccF</sub>), no modification of either *B. subtilis* E2<sub>PdhC</sub> or of full-length *B. subtilis* PdhC was seen *in vitro* (Fig. 2B). Addition of LipL to the LplJ reaction failed to give modification of E2<sub>PdhC</sub> (Fig. 3B, lane 3). Only when GcvH, LipL and LplJ were all present did modification of E2<sub>PdhC</sub> proceed (Fig. 3B, lane 5). These observations argued that modified GcvH was an obligate intermediate in the modification of E2<sub>PdhC</sub>. It also showed that LipL was required for PdhC modification.

### LipL is a novel amidotransferase

Since the octanoyl moiety of octanoyl-GcvH is attached by an amide linkage, 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 thioester-bound octanoyl moiety to E2<sub>PdhC</sub> to give a new amide-linked moiety (Fig. 4D). To test if LipL was indeed a GcvH:E2 amidotransferase, LipL activity was assayed using purified [1-<sup>14</sup>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 (Fig. 4A). Transfer from GcvH to LipL was also observed (Fig. 4C).

To explore the LipL reaction mechanism site-directed mutagenesis of residues conserved in the PF03099 family was performed. LipM K162 is a strictly conserved residue in PF03099 whereas Y180 is conserved in the LipL amidotransferase subfamily (Fig. 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. We also mutated residues C39 and C150, both of which had been found to be modified by LC-MS/MS (Fig. 1B). 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<sub>PdhC</sub> (Fig. 4B) and the inability to form an octanoyl-enzyme intermediate (Fig. 4C). Given the above observations, we propose a mechanism for amidotransfer by LipL (Fig. 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 (Fig. 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-<sup>14</sup>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*, we disrupted *gcvH*. The  $\Delta gcvH$  strain was auxotrophic for lipoic acid, the same phenotype shown by  $\Delta lipA$  and  $\Delta lipM$  strains. As observed in the other lipoate auxotrophs, the lipoate requirement of the  $\Delta gcvH$  strain was bypassed by addition of acetate and BCFA precursors.

*In vivo* results showing that bypass of a  $\Delta lipL$  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<sub>PdhC</sub> in the absence of GcvH (Fig. 7A). Expression of a functional ectopic copy of the *gcvH* gene under the control of an inducible promoter allowed growth of the  $\Delta gcvH$  strain when induced with IPTG (Fig. 7B). This demonstrates that the growth deficiency was due to the absence of a functional copy of the *gcvH* gene. Residual growth of the uninduced cells was observed, which can be attributed to the known basal expression of the *spac* promoter. Moreover, the expression of the *E. coli lipB* gene in a  $\Delta gcvH$  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* (Christensen & Cronan, 2010). Western blotting analysis with anti-lipoic acid antibodies showed a lipoylated protein of apparent mass of 20 kDa (Fig. 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 (Christensen & Cronan, 2010) and disruption of *gcvH* resulted in loss of the 20 kDa band (Fig 7C) thereby confirming its identity. Disruption of *gcvH* resulted in a complete loss of biosynthetic lipoic acid assembly (Fig. 7C), confirming that GcvH was required in *B. subtilis* lipoic acid biosynthesis. Upon supplementation with lipoic acid the  $\Delta gcvH$  strain showed strong lipoylation of the dehydrogenase E2 subunits (Fig. 7C) consistent with the robust growth seen in this medium (Fig. 7A). These results confirm the physiological relevance of the *in vitro* biochemical assays and show GcvH is required not only for biosynthetic lipoylation of PdhC, but other E2 subunits as well.

### 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 (Fig. 8A). LipL is also closely related to LipM octanoyltransferases, which branch off later from the same lipoate protein ligase clade (Christensen & Cronan, 2010). 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 (Fig. 9). This was unexpected because the residues are contributed by different loops that are ~45 residues removed in sequence alignments (Christensen & Cronan, 2010). Therefore, it appears that LipL and LipB have undergone mechanistic convergence within their active sites using the same protein scaffold.

## DISCUSSION

We 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. The octanoyl moieties are then transferred by LipL, a novel GcvH:E2 amidotransferase, from GcvH to E2 subunits to form active dehydrogenase complexes (Fig. 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 either step. Note that although we characterized the *B. subtilis* pathway due to the sophisticated genetics available in this bacterium, paralogous genes are present in other Firmicutes proficient in lipoic acid synthesis.

The requirement for LipL is due to the specificity of the LipM for GcvH. We find significant *in vitro* modification of the E2<sub>AceF</sub> by LplJ, but not by LipM (Fig. 2) suggesting LplJ may be less specific than LipM. However, LipM complements an *E. coli lipB* lipoic acid auxotroph (Christensen & Cronan, 2010) which demonstrates modification of E2<sub>AceF</sub>. 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 in turn 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 we tested *in vitro*. The E2<sub>AceF</sub> and E2<sub>PdhC</sub> domains clearly form different clades indicating different conserved residues (Fig. 8B). This may explain why LplJ is able to modify E2<sub>AceF</sub> but not E2<sub>PdhC</sub>. Identification of 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 (Moriyama & Oka, 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 (Fig. 4b) and is not conserved in more diverse members of the LipL clade (Fig. 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 (Fig. 4) which is the same result seen upon mutagenesis of the active site LipM cysteine residue (Christensen & Cronan, 2010). Based on the *B. halodurans* LipL crystal structure K162 and Y180 are less than 3 Å from C150 (Fig. 10). We found that these residues are conserved in all members of the LipL clade (Fig. 9). From these data, it seemed likely that LipL employed a Cys-Lys-Tyr catalytic triad similar to those seen in other amidotransferases. However, we found that Y180 was not required for amidotransfer as the Y180F and Y180A proteins were active. Therefore, we propose that LipL amidotransferases utilize a Cys-Lys dyad as do the LipB and LipM ACP-octanoyltransferases (Christensen & Cronan, 2010, Ma *et al.*, 2006). If so, this would be a novel active site configuration for an amidotransferase (transamidase). Note that LipL shares no detectable overall sequence or structural similarity to other known amidotransferases.

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 & Cronan, 1993). As previously reported (Cui *et al.*, 2006), we found that most of the BLAP was in the unmodified form after two hours of expression. It was thought that BLAP might be a good candidate for intermediate carrier in lipoic acid biosynthesis. However, we assayed BLAP as an octanoyl acceptor and found it was not modified by any enzyme tested (Fig. 2, 4A). 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  $\Delta lipL$

mutant and modification of E2<sub>PDHC</sub> by LplJ was not seen *in vitro* (Fig. 2B) whereas growth of the  $\Delta gcvH$  strain was fully restored by lipoic acid (Fig. 5A) and the dehydrogenase E2 subunits became highly modified (Fig. 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 we find 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  $\Delta lipL$  strain in the companion paper (Martin *et al.*, 2011).

A similar mechanism for lipoic acid synthesis 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, although 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 subsequently 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 given the obscure role of the glycine cleavage system in bacterial metabolism. It should be noted that GcvH lipoyl domains are highly divergent from the dehydrogenase E2 lipoyl domains (Fig. 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 (Fig. 7A) which indicates that GcvH does not play an essential role in amino acid or single carbon metabolism. In the companion paper we show that *E. coli lipB* complements both  $\Delta lipM$  and  $\Delta lipL$  mutant strains. Some evolutionary pressure may exist for *B. subtilis* to maintain the specificity of LipM for GcvH. However, instead of a phenomenon that can be rationalized, constructive neutral evolution (Gray *et al.*, 2010) may be involved. LipM may have coevolved with the glycine cleavage system and LipL provided a means for it to be used for other lipoate dependent enzymes. A more extensive 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-chain 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 & de Mendoza, 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.

## EXPERIMENTAL PROCEDURES

### Media and Chemicals

*Escherichia coli* strains were grown on LB rich or M9 minimal media (Sambrook & Russell, 2001) under aerobic conditions at 37°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-<sup>14</sup>C]Octanoic acid and [1-<sup>14</sup>C]octanoyl-CoA were purchased from Moravек. American Radiolabeled Chemicals provided [α-<sup>32</sup>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 1. Primers used in this study are given in Table 2. Standard techniques for DNA manipulation and cloning were employed unless otherwise indicated (Sambrook & Russell, 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<sub>PdhC</sub>) was identified by alignment with the *B. stearothermophilis* 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 using the primers described (Baba *et al.*, 2006) to give strain RMK98. Strain QC146 was transduced with a phage P1 stock grown on strain RMK98 to give strain QC171. The kanamycin cassette was removed (Datsenko & Wanner, 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. Mutant LipL proteins were expressed and purified as described in the companion paper (Martin *et al.*, 2011).

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 2) 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 (Table 2) 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 (*Pspac*). To this end plasmid pNM70 was constructed by cloning of a 456 bp PCR-amplified fragment generated with primers XLVI and XLVII (Table 2) into the HindIII and BamH sites of pGES485 (Schujman GE, unpublished). Plasmid pNM70 was linearized and used to transform strain NM20, yielding strain NM21. Transformants were selected on kanamycin plus spectinomycin plates and screened for *amyE* phenotype.

### Purification of Lipoyl Domains

The *E. coli* E2<sub>p(1,3)</sub> hybrid lipoyl domain (LD<sub>AceF</sub>) 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 (Christensen & Cronan, 2010). The E2<sub>AceF</sub> was in the unmodified form and lacked the N-terminal methionine residue. The E2<sub>AceF</sub> 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<sub>PdhC</sub> strain QC063 was grown in LB with kanamycin to an OD<sub>600</sub> 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<sub>PdhC</sub> 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<sub>PdhC</sub> was verified to be in the unmodified form without the N-terminal methionine by electrospray mass spectrometry as described above for ACP (Christensen & Cronan, 2010). E2<sub>PdhC</sub> was quantified by absorbance at 280 nm using an extinction coefficient of 6,990 M<sup>-1</sup> cm<sup>-1</sup>.

The *B. subtilis* BLAP protein was expressed using strain QC152 and purified by nickel affinity and anion exchange chromatographic steps as described for LplA (Christensen & Cronan, 2009). BLAP was verified to be in the unmodified form lacking the N-terminal methionine by electrospray mass spectrometry as described for ACP (Christensen & Cronan, 2010) and was quantified by absorbance at 280 nm (extinction coefficient of 5,500 M<sup>-1</sup> cm<sup>-1</sup>). *B. subtilis* PdhC was expressed using strain QC191 and purified by nickel affinity and anion exchange as described for LplA (Christensen & Cronan, 2009). PdhC was quantified by absorbance at 280 nm using an extinction coefficient of 18,450 M<sup>-1</sup> cm<sup>-1</sup>. To determine if the C-terminal hexahistidine tag interfered with complex formation, we subjected pure PdhC to size exclusion chromatography as described for LipM (Christensen & Cronan, 2010).

### 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 (Christensen & Cronan, 2010). 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-<sup>14</sup>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 & Mills, 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 LD substrate specificity of these enzymes. Assays using AasS were performed as previously described (Christensen & Cronan, 2010) with different lipoyl domains substituted for GcvH. Reactions were analyzed as described above with LplJ.

## Assay of amidotransfer

Amidotransfer was assayed coupled 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-<sup>14</sup>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-<sup>14</sup>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<sub>600</sub> 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-<sup>14</sup>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-<sup>14</sup>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 an AKTA purifier10 (GE Healthcare) with a 1.8ml POROS QE anion exchange column with a flow rate 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 was directly assayed from purified <sup>14</sup>C-octanoyl GcvH to various LDs. The reactions contained 100 mM sodium phosphate (pH 7.0), 50 mM sodium chloride, 5 mM TCEP, 20 μM [1-<sup>14</sup>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<sub>PdhC</sub> 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  $\mu$ M LipL or mutant LipL. The reactions were analyzed as described for the LplJ assays above.

### Immunoblotting analysis

Lipoylation of crude extracts of *B. subtilis* strains was assayed with anti-lipoic acid antibody (Calbiochem) as described in the companion paper (Martin *et al.*, 2011)

### Phylogenetic Analysis and Bioinformatics

We 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 & Littlejohn, 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.

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**A.**

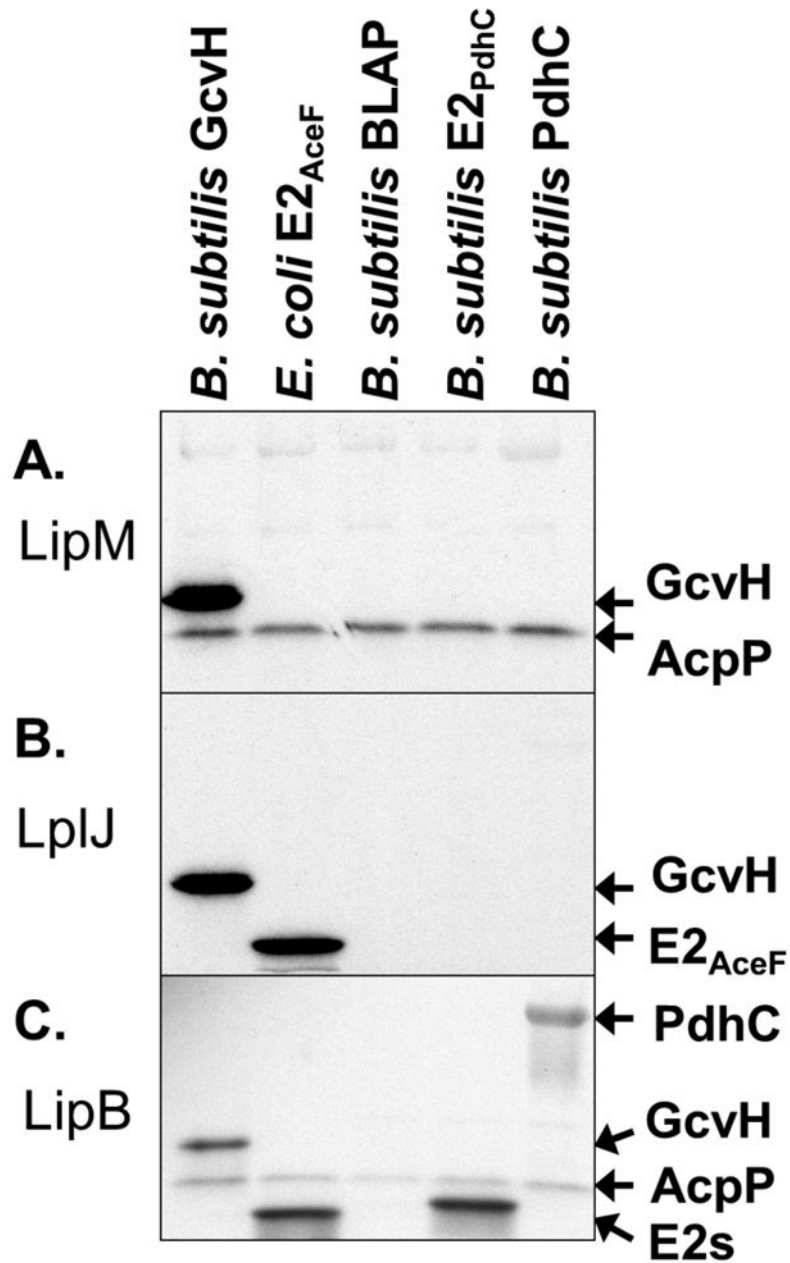
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 51 ARSWVHHTI VLGIQDTRLP FLQDGISLLE SEGYRVIVRN SGGLAVVLDD  
 101 GVLNISLIFE DEKKGIDIDK GYEAMVELMR RMLRPYNAKI EAYEIEGSYC  
 151 PGSYDLSING KKFAGISQRR VRGGVAVQIY LCADKSGSER ADLIRRFYQA  
 201 ALKDKQNDKK

**B.**

Peptide Sequence	Residue	$\Delta$ Mass	Modification	Score
QSFAMDDTL <u><b>C</b></u> MSVGK	C40	0	None	60
QSFAMDDTL <u><b>C</b></u> MSVGK	C40	188	Lipoylation	34
QSFAMDDTL <u><b>C</b></u> MSVGK	C40	126	Octanoylation	41
IEAYEIEGSY <u><b>C</b></u> PGSYDLSINGK	C150	188	Lipoylation	57
IEAYEIEGSY <u><b>C</b></u> PGSYDLSINGK	C150	126	Octanoylation	58

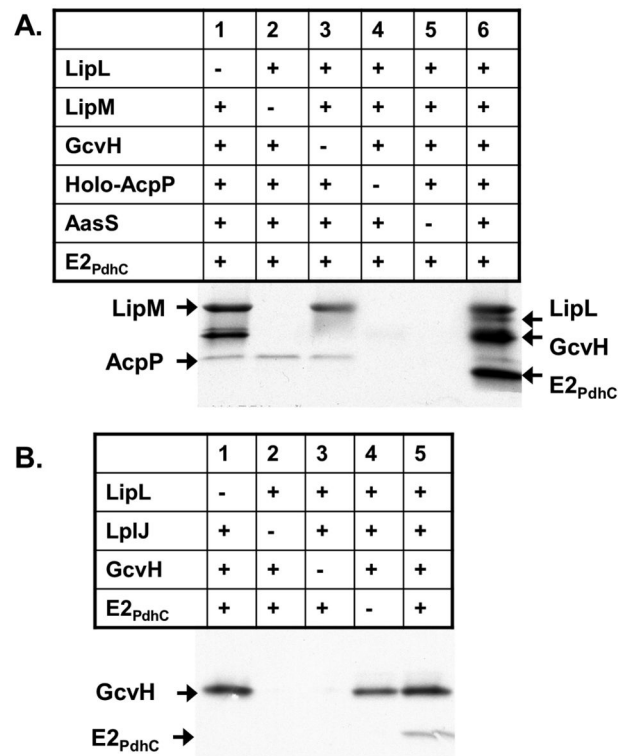
**Fig. 1.**

Panel A: Sequence coverage of LipL by LC-MS/MS analysis of LipL derived peptides. The LipL peptide sequences detected are underlined. Panel B: 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 differences of the modification are given. The ion score is equal to  $-10\log(P)$  where P is the probability the result is random chance

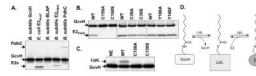


**Fig. 2.** Specificity of *B. subtilis* LipM, *B. subtilis* LplJ, and *E. coli* LipB for various lipoyl domains. In panels A and C the lipoyl domain tested as a substrate is indicated at the top of each lane and by an arrow. The [<sup>1-14</sup>C]octanoyl-ACP substrate is indicated by an arrow. Autoradiograms of SDS-PAGE gel separations of lipoyl domain substrate specificity assays are shown. Sodium [<sup>1-14</sup>C]octanoate was used as a substrate for LplJ. Panel A: LipM octanoyltransfer to lipoyl domains from [<sup>1-14</sup>C]octanoyl-ACP generated using AasS. Panel B: Ligation of [<sup>1-14</sup>C]octanoate by LplJ to lipoyl domains. Panel C: LipB octanoyltransfer to lipoyl domains from [<sup>1-14</sup>C]octanoyl-ACP generated using *Vibrio harveyi* acyl-ACP synthetase (AasS). The two E2 domains migrate similarly and are denoted by E2s.



**Fig. 3.**

Panel A: Component controls for a coupled AasS/LipM/LipL assay starting with [1-<sup>14</sup>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-<sup>14</sup>C]octanoylated proteins are indicated. Panel B: Component controls for a coupled LplJ plus LipL assay as in Panel A. The starting substrates for LplJ-catalyzed ligation reactions were [1-<sup>14</sup>C]octanoate and ATP.

**Fig. 4.**

Autoradiograms of SDS-PAGE gels of assays for LipL-catalyzed amidotransfer from purified [ $1\text{-}^{14}\text{C}$ ]octanoyl-GcvH to lipoyl domains. Panel A: Amidotransfer of the [ $1\text{-}^{14}\text{C}$ ]octanoyl moiety from purified octanoyl-GcvH to the unmodified lipoyl domain indicated. Each reaction contained purified wild type (WT) LipL. The two E2 domains migrate similarly and are denoted by E2s. Panel B: Amidotransfer from purified [ $1\text{-}^{14}\text{C}$ ]octanoyl-GcvH to the E2<sub>PdhC</sub>. The purified wild type LipL or point mutant proteins indicated were used as enzyme sources. Panel C: Additional enzyme was added to allow detection of the octanoyl-LipL intermediate. Wild type (WT), C150A point mutant, C150S point mutant LipL were assayed in addition to a control (NE) lacking LipL. Panel D: Schematic of the LipL amidotransfer reaction is shown with the acyl-LipL intermediate.

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LipL_BACSU/32-277 32 FAMD--TLCMSVGGKVPATARSWVHDTIVLGIQDTRLRFLQDGLSLESEGYRIVRNSG92
LipL_BACHD/31-273 31 FAYDD--TLCTSIGKSQSPPTLRAWVHNTVVLGIQDLSRLRQIKAGIEALKGFQHDVIVRNSG91
LipL_USMO/31-274 31 FATDD--TLCRSVGARMAPSTVRGWVHEKTVSLGIQDLSKLRDIDKGIAFLQKQGYRNVVRS91
LipL_LACBA/31-257 31 ALLDiva-----ELKQPIHLFWTMHPTVIMGLKDKRLRDLTAAIKAVQRYGYDYLVRNSG85
LipL_STRPC/34-278 34 FVWTE--VFLKTIINQEPNQLILHIMPMTRTVILGMLDRQLRYFELAKKEIGNNGYVPVTRNIG94

LipL_BACSU/32-277 93 GLAVVLDGVLNLSLIFED--EKKGIDIDKGYEAVVELMRRMLRPYNAKIEAYEIEGSCPGS153
LipL_BACHD/31-273 92 GLAVVLDGVLNLSLVLKE--EKGFSDDGVELMYELICSMFQDHRQIEAREVVGSCPGS151
LipL_USMO/31-274 92 GLAVVLDGVLNLSMVLDP--AERGIAIERGYETMFTLIKDMFVDCNEVIEAKEIEDSCPGS152
LipL_LACBA/31-257 86 GLAVVSDAGILNVSLFAPL--TTPPMSVDAAYAQMTALVDAAWPELA--IQHYKITHSYCPGD144
LipL_STRPC/34-278 95 GLAVVADGILNLSLVIPDhfseSISISNAALIVDVVIRESFSDYYQRIEYHEIKNSYCPGN156

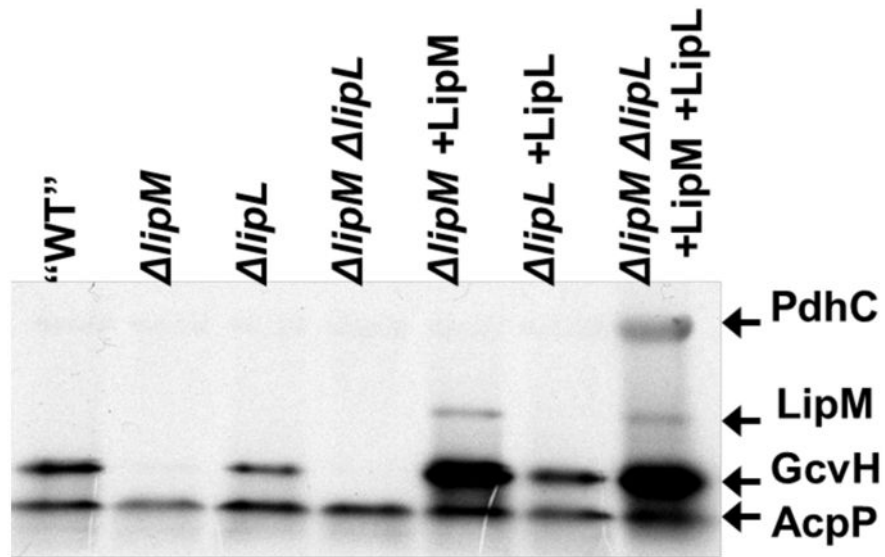
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LipL_USMO/31-274 153 YDLSIQDKKFAGISQRRMAKGVAVQIYLAIDGQDQTTSELIRDFYTI S GKAKQT--KYTFRDV213
LipL_LACBA/31-257 145 YDLSVNLKLIAGIARRSPHALVTMLYLSVNGDQLARQGMIRDFYTAGLAGQEN--TWAFRDV205
LipL_STRPC/34-278 157 FDLSIARRKFAGIARRIKKGI VMSIYLSVCGDQAARGLLKAFFEAGTQGEVTT--KVNYSQI217

LipL_BACSU/32-277 217 RPEMASLSELLQKDISVQDLMFALLTELKALSTHLYSAGLSIDEEMEFKKNLVRMAERNA 277
LipL_BACHD/31-273 213 KPEMASLSELLGQPHNVSDVLLKALMTLQHGASLLTESLDAEWLLYEQHFARISERNE 273
LipL_USMO/31-274 214 RPNVMSLSDLMKNDISLNGTLVRLFNLSRHYAGDLVSGTLTSEELDLFPAYYERLIARND 274
LipL_LACBA/31-257 206 DPATMTTAAALGQNLTIAAQQRFI TAAEQAGLNVGRITTAQAMATpfg----- 256
LipL_STRPC/34-278 218 DEECMATLSELLLETPFTVAEVLRLRLRLRQLGFSLITEKSPDQALLTNF DAVYERMQLEvv 278

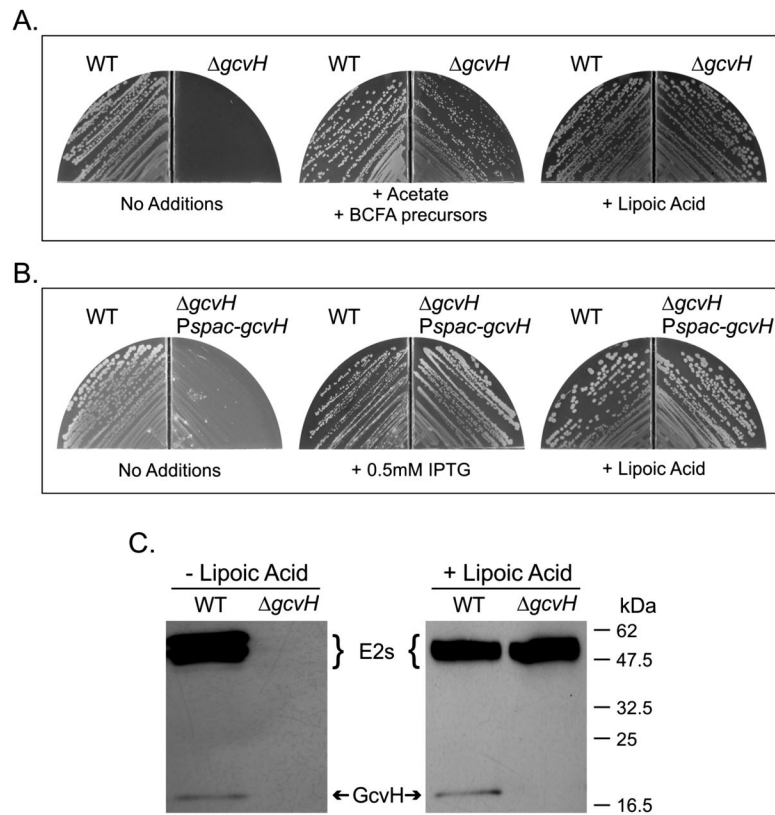
```

**Fig. 5.**

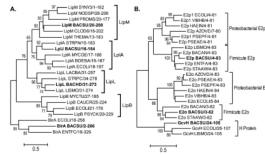
Alignment of members of the LipL clade. Positions having 50% or greater similarity are highlighted in grey. The catalytic cysteine residue (C150 in *B. subtilis* LipL), the other modified cysteine residue (C39 in *B. subtilis* LipL), and the conserved PF03099 lysine residue are highlighted in black. 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 Fig. 8A.



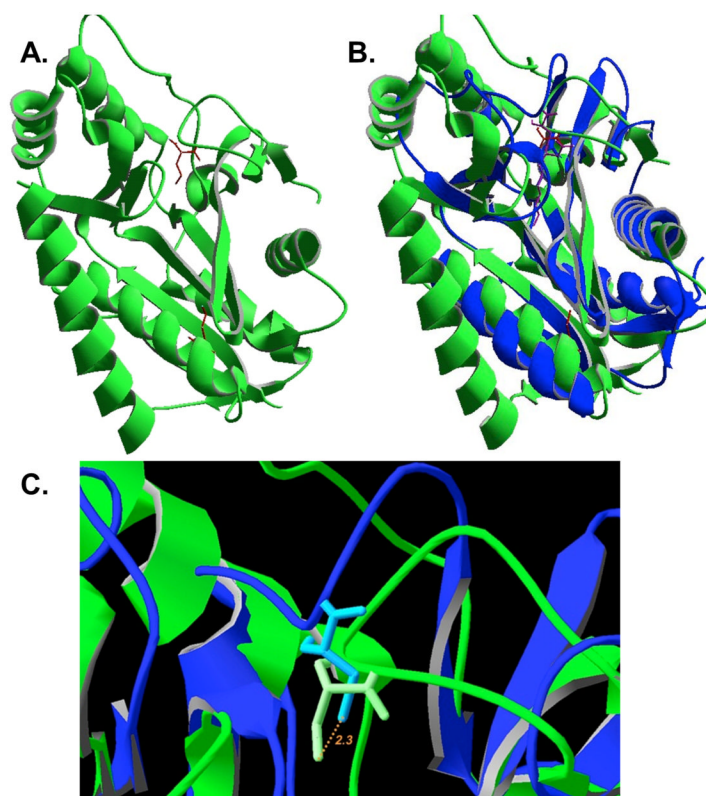
**Fig. 6.**  
 Panel A: Autoradiograph of an SDS-PAGE gel of octanoyltransfer assays using AasS generated  $[1-^{14}\text{C}]$ octanoyl-ACP as a substrate. Extracts of the mutant strains indicated were used as a source of enzyme for transfer to GcvH and PdhC. All strains also carried a disruption in *lplJ*. When purified LipM and LipL were added to replace the missing protein(s) this is indicated with a plus sign.



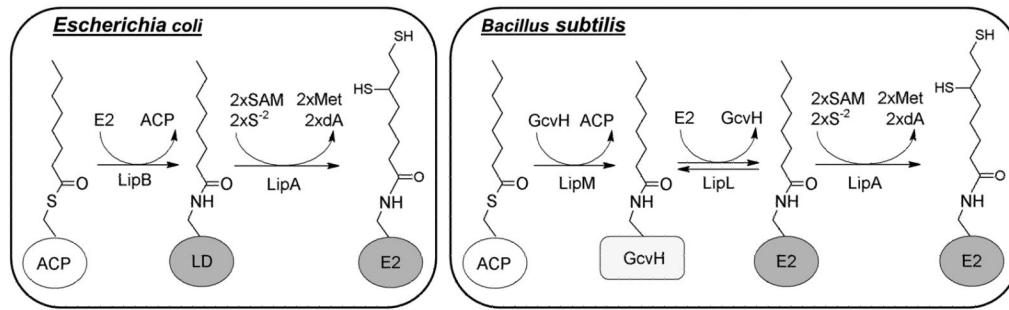
**Fig. 7.** Growth and lipoylation phenotypes of a *B. subtilis*  $\Delta gcvH$  strain. Growth of strains on minimal glucose media plates with the supplements indicated after 48 h at 37°C. LA denotes lipoic acid. Panel A: Growth of strains JH642 (wild type) and NM20 ( $\Delta gcvH$ ). Panel B: Growth of strains JH642 (wild type) and NM21 ( $\Delta gcvH$  amyE:: Pspac-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 h in minimal media with or without lipoate added as indicated. Crude extracts of *B. subtilis* wild type (WT) and  $\Delta gcvH$  strains were used as indicated.



**Fig. 8.** Minimum evolution 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. Branches representing *Bacillus* proteins are indicated in bold. 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.



**Fig. 9.** Structure of LipL and comparison with that of LipB. Panel A: The unpublished structure of *B. halodurans* LipL (PDB 2P51). The modified cysteine residues, C39 and C150, detected by LC-MS/MS of the *B. subtilis* protein are shown in red. Panel B: Structural alignment of LipL from *B. halodurans* with *M. tuberculosis* LipB (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.



**Fig. 10.**

Current models for lipoyl synthase in *E. coli* and *B. subtilis*. Lipoyl synthase in *E. coli* is accomplished by two enzymes whereas *B. subtilis* requires three enzymes. *B. subtilis* also requires GcvH as an intermediate carrier whereas *E. coli* does not. We lack data to determine if LipA acts before or after LipL. We propose that *B. subtilis*, LipA uses octanoyl-LD (both octanoyl-GcvH and octanoyl-E2) as substrates, and therefore can act before or after LipL.



Table 1

Strain	Genotype	Source
<b><i>Escherichia coli</i>:</b>		
MG1655	<i>rph-1</i>	CGSC <sup>a</sup>
RMK98	MC1061 <i>fadK::his6-cat ΔompT::FRT</i>	this study
QC146	<i>rph-1 ΔlplA::FRT ΔlipB::FRT</i>	(Christensen & Cronan, 2009)
QC152	<i>rph-1 ΔlplA::FRT ΔlipB::FRT/pTARA</i> , pQC026	this study
QC172	<i>rph-1 ΔlplA::FRT ΔlipB::FRT ΔompT::FRT</i>	this study
QC191	<i>rph-1 ΔlplA::FRT ΔlipB::FRT ΔompT::FRT/pQC047</i> , pTARA	this study
QC222	<i>rph-1 IN(rrnD-rrnE)1 φ(fabA-lacZ)1(Hyb)cat/pQC077</i> , pCY598	this study
QC223	<i>rph-1 IN(rrnD-rrnE)1 φ(fabA-lacZ)1(Hyb)cat/pQC078</i> , pCY598	this study
QC224	<i>rph-1 IN(rrnD-rrnE)1 φ(fabA-lacZ)1(Hyb)cat/pQC079</i> , pCY598	this study
QC225	<i>rph-1 IN(rrnD-rrnE)1 φ(fabA-lacZ)1(Hyb)cat/pQC080</i> , pCY598	this study
QC255	<i>rph-1 IN(rrnD-rrnE)1 φ(fabA-lacZ)1(Hyb)cat/pQC086</i> , pCY598	this study
QC256	<i>rph-1 IN(rrnD-rrnE)1 φ(fabA-lacZ)1(Hyb)cat/pQC087</i> , pCY598	this study
QC257	<i>rph-1 IN(rrnD-rrnE)1 φ(fabA-lacZ)1(Hyb)cat/pQC088</i> , pCY598	this study
QC258	<i>rph-1 IN(rrnD-rrnE)1 φ(fabA-lacZ)1(Hyb)cat/pQC089</i> , pCY598	this study
<b><i>Bacillus subtilis</i>:</b>		
JH642	<i>trpC2 pheA1</i>	Laboratory stock
NM20	JH642 <i>gcvH::Km</i>	This study
NM21	NM20 <i>amyE::Pspac-gcvH</i>	This study
<b>Plasmids</b>	<b>Relevant Properties</b>	<b>Source</b>
pTARA	pACYC origin, arabinose inducible T7 polymerase	(Wycuff & Matthews, 2000)
pCY598	RSF origin, arabinose inducible T7 polymerase	(Cronan, 2003)
pCR2.1	TOPO TA cloning vector	Invitrogen
pSJ120	<i>E. coli</i> LipB expression vector	(Jordan & Cronan, 2003)
pQC023	<i>B. subtilis</i> E2p lipoyl domain expression vector	this study
pQC026	C-terminal hexahistidine BLAP expression vector	this study
pQC033	N-terminal hexahistidine LipL expression vector	(Martin <i>et al.</i> , 2011)
pQC047	C-terminal hexahistidine PdhC expression vector	this study
pQC077	N-terminal hexahistidine LipL K162R mutant expression vector	this study
pQC078	N-terminal hexahistidine LipL K162A mutant expression vector	this study
pQC079	N-terminal hexahistidine LipL C150A mutant expression vector	this study
pQC080	N-terminal hexahistidine LipL C150S mutant expression vector	this study
pQC086	N-terminal hexahistidine LipL C39A mutant expression vector	this study
pQC087	N-terminal hexahistidine LipL C39S mutant expression vector	this study
pQC088	N-terminal hexahistidine LipL Y180A mutant expression vector	this study
pQC089	N-terminal hexahistidine LipL Y180F mutant expression vector	this study
pGES485	Integrational vector; Sp	(GE Schujman, unpublished)

Strain	Genotype	Source
pJM114	Integrational vector; Km	(Perego, 1993)
pNM67	pJM114 containing <i>gcvH</i> interrupted with a kanamycin cassette	This study
pNM70	pGES485 containing <i>gcvH</i> cloned into HindIII-BamHI sites	This study

<sup>a</sup>CGSC, E. coli Genetic Stock Center

Table 2

Oligonucleotides	Sequence 5'-3'
Q030	ttaacatggcatttgaatttaaactcc
Q031	aatggatcctaaaattgaagatctctgaacc
Q034	gaaacagcacctataaaggaggca
Q042	ttaatgatgatgatgatgttgagtgaattgctcagttcg
Q095	taaggtaccaaggagatataccatggcatttgaatttaaactc
Q096	attggatccttagtgatgtgatggatgctccattaaaattaattg
Q113	aacggataagacgggcataa
Q114	gggatgaaggaacgtcattt
Q147	ctatgacctcagtataaatggcaaagcttccggcatct
Q148	agatgccggcgaacgcttgcatttatactgaggtcatag
Q149	cagctatgacctcagtataaatggcaaagcttccggcatctct
Q150	agagatgccggcgaacgcttgcatttatactgaggtcatagctg
Q151	ctatgaaattgaagggtcttattctcccgcagcta
Q152	tagctgccgggagaataagaccttcaatttcatag
Q153	agcctatgaaattgaagggtcttattctcccgcagctat
Q154	atagctgccgggagcataagaccttcaatttcatagct
Q0171	gcaatggatgatacgtagccatgtccgtcggaaaagg
Q0172	cctttccgacggacatgctagcgtatcatccattgc
Q0173	cgcaatggatgatacgtatccatgtccgtcggaaa
Q0174	tttccgacggacatgtagcgtatcatccattgc
Q0175	gcggcggagtcgctgttcaaattgctctctgtgcagata
Q0176	tatctgcacagagacaatttgaacagcgactccgccgc
Q0177	cggcggagtcgctgttcaaatttctctgtgcaga
Q0178	tctgcacagagaaaaatttgaacagcgactccgccg
L	agtggatccaagagcatgggaaag
LI	ttgtcactcgtttctctgagtaa
LII	gagtcgacacaagaagactaaaacag
LIII	aaagactgcagtgaaattcaccgcca