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A novel two-gene requirement for the octanoyltransfer reaction of *Bacillus subtilis* lipoic acid biosynthesis

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Summary

The *Bacillus subtilis* genome encodes three apparent 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 *Escherichia coli*, *B. subtilis* requires a third protein for lipoic acid assembly, LipL. *B. subtilis* Δ*lipL* strains are unable to synthesize lipoic acid despite the presence of LipM and the sulphur insertion enzyme, LipA, which should suffice for lipoic acid biosynthesis based on the *E. coli* model. 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 together with those of the companion article provide evidence that LipM and LipL catalyse sequential reactions in a novel pathway for lipoic acid biosynthesis.

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Introduction

Lipoic acid is a sulphur-containing coenzyme found in all domains of life that is required for the function of several key multienzyme complexes involved in oxidative and one-carbon metabolism. Five lipoate-dependent multienzyme complexes have been characterized. Three are closely related 2-oxoacid dehydrogenases: pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (OGDH) and branched-chain 2-oxoacid dehydrogenase (BKDH). These complexes are composed of multiple copies of each of three catalytic components referred as E1 (often produced as two proteins), E2 and E3. A fourth complex, acetoin dehydrogenase, is highly homologous to PDH and shares the three-subunit architecture of the 2-oxoacid dehydrogenases but produces acetaldehyde instead of carbon dioxide. The fifth complex, the glycine cleavage complex, has a markedly different architecture and is composed of four loosely associated proteins, called P, H (GcvH), T and L proteins (Perham, 2000). Lipoic acid is linked through an amide bond to a specific lysine residue of the lipoyl domains of the E2 and GcvH proteins where it acts as a swinging arm in transfer of covalently attached reaction intermediates among the multiple active sites of the enzyme complexes (Perham, 2000).

In *Escherichia coli* lipoylation is directly carried out by lipoyl protein ligase A (LplA) which uses exogenous lipoic acid in an ATP-dependent reaction (Morris *et al.*, 1994; 1995). However, this is a scavenging rather than a biosynthetic pathway. The *de novo* synthesis pathway proceeds by two consecutive reactions. In the first step the octanoyl-acyl carrier protein (ACP):protein-*N*-octanoyltransferase (LipB) transfers endogenously produced octanoyl moieties to the target proteins (Morris *et al.*, 1994; 1995; Jordan and Cronan, 2003). In the second step the octanoyl moieties are converted to lipoic acid by the *S*-adenosyl-L-methionine (SAM) radical enzyme, lipoyl synthase (LipA) which replaces a hydrogen atom on each of the octanoate C6 and C8 carbon atoms with a sulphur atom (Zhao *et al.*, 2003; Cicchillo and Booker, 2005) (Fig. 1).

Homologues of the *E. coli* lipoic acid metabolism proteins are found in all domains of life and thus unravelling the pathways by which this cofactor is synthesized and transferred to lipoate-dependent proteins is of broad

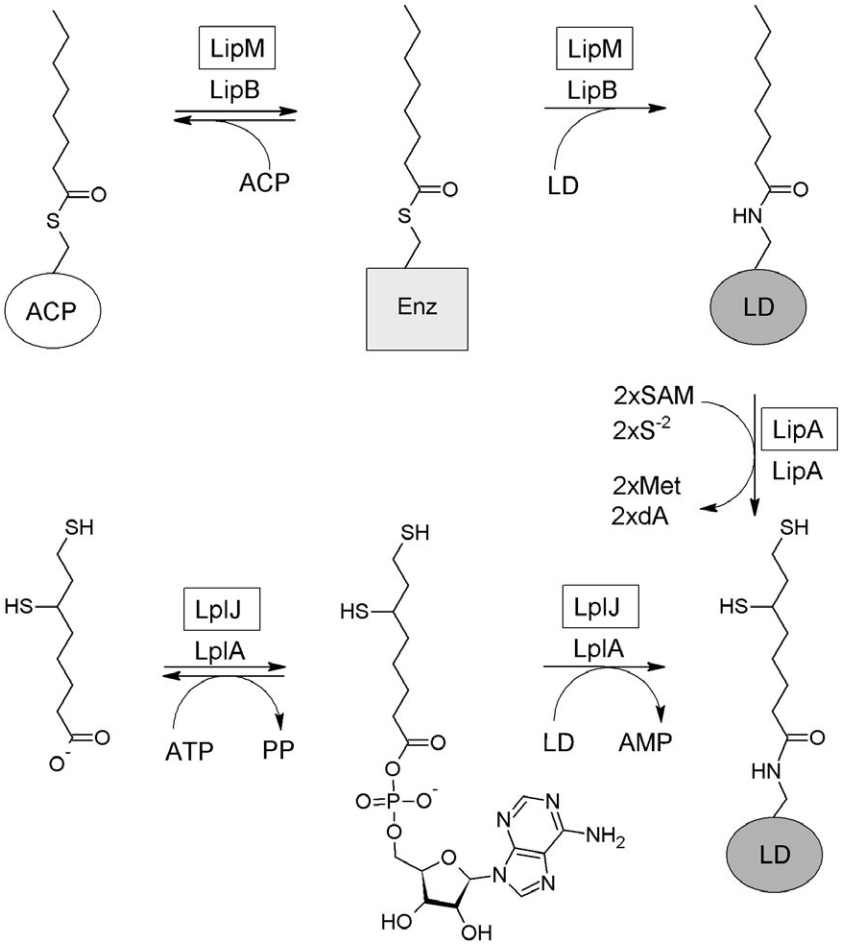

Fig. 1. Current model for lipoic acid synthesis and scavenging in *E. coli*. *E. coli* uses two independent pathways for lipoic acid synthesis and scavenging. The endogenous synthesized octanoate is transferred from acyl carrier protein (ACP) to apoproteins by LipB, an octanoyl-ACP:protein-*N*-octanoyltransferase. The octanoylated domains then become substrates for sulphur insertion by LipA. Exogenous lipoate (or octanoate) is transferred to unmodified acceptor lipoyl domains (LD) in an ATP-dependent two-step reaction catalysed by LplA. The dA designation denotes 5′-deoxyadenosine. The *B. subtilis* protein that catalyses the same reaction as the *E. coli* protein is given in a box above the *E. coli* enzyme.

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 (O'Riordan *et al*., 2003). 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 (Fujiwara *et al*., 1999; Marvin *et al*., 2001; Thomsen-Zieger *et al*., 2003).

Since *Bacillus 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* (Christensen and Cronan, 2010). 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; Christensen and Cronan, 2010). 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. We have now tested the role of LipM in *B. subtilis* and confirmed its proposed role as octanoyltransferase *in vivo*. 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 study we 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 and the resulting data demonstrate that *B. subtilis* synthesizes lipoic acid by a novel mechanism.

Results

LipM is responsible for octanoyl transfer in vivo

Bacillus 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 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 article we have renamed *yhfJ* and *ywfL* as *lplJ* and *lipL*, respectively, whereas *yqhM* was previously renamed *lipM* (Christensen and Cronan, 2010).

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 (Christensen and Cronan, 2010). 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 catalyse octanoyl transfer *in vitro* by the same general acyl-enzyme intermediate mechanism used by LipB (Christensen and Cronan, 2010). 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 (Christensen and Cronan, 2010). 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 and performed its physiological and biochemical characterization.

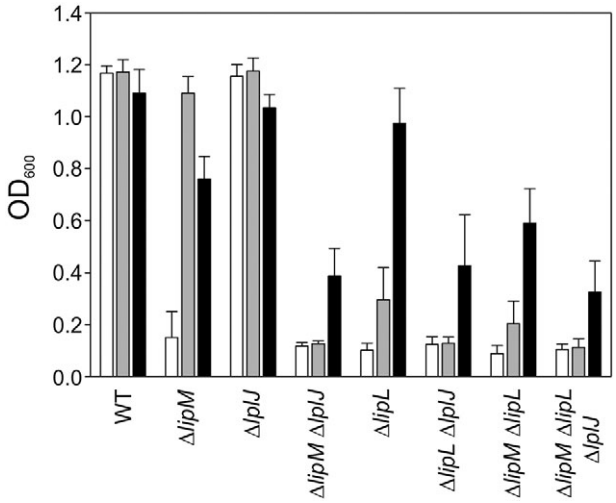

Fig. 2. Growth phenotypes of *B. subtilis* mutant strains. The strains were grown overnight in minimal medium supplemented with acetate and branched-chain fatty acid (BCFA) precursors. The cultures were centrifuged and the cells resuspended in minimal medium without supplements (white bars); minimal 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.

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 (Figs 2 and 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 (Figs 2 and 3A), which upon conversion to their CoA esters are the products of the two lipoylated *B. subtilis* enzymes PDH and BKDH required for growth in minimal medium. Cells grown in this medium were devoid of lipoylated proteins detectable by Western blotting (Fig. 4). 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 (Fig. 3A). Moreover, LipM could be functionally replaced by expression of *E. coli lipB*. The *lipB*

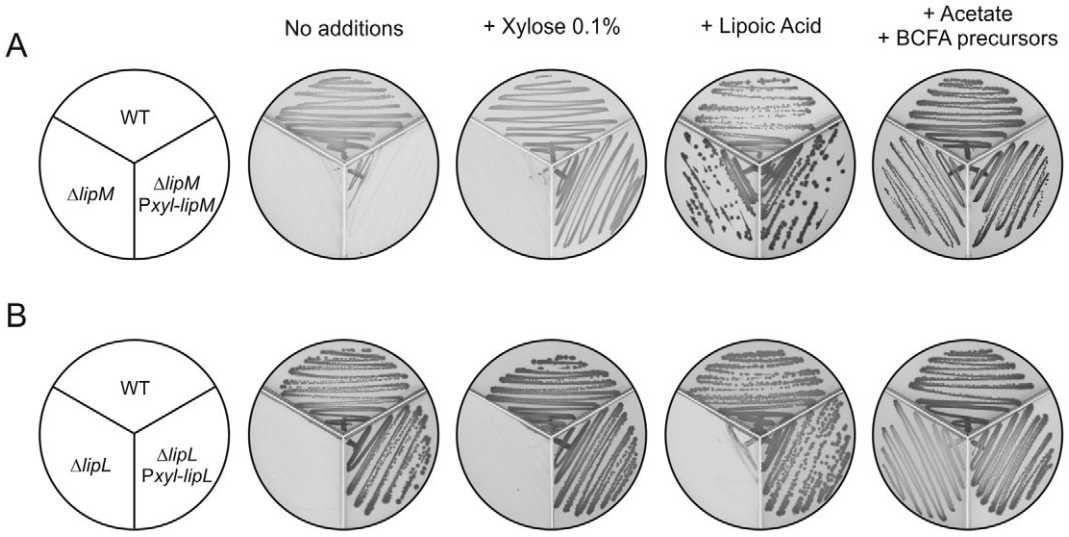

Fig. 3. Characterization of *B. subtilis* strains blocked in lipoic acid synthesis.
A. Growth of bacterial strains JH642 (wild type), NM57 (Δ*lipM*) and NM08 (Δ*lipM amyE*::P*xyl-lipM*).
B. Growth of wild type (JH642), NM51 (Δ*lipL*) and NM13 (Δ*lipL amyE*::P*xyl-lipL*).
The strains were streaked onto minimal medium-glycerol plates containing the supplements indicated above and incubated for 24 h at 37°C.

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 Δ*lipL* strain (see below). These results together with those of Christensen and Cronan (2010) demonstrate that LipM functions as the sole *B. subtilis* octanoyltransferase.

B. 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

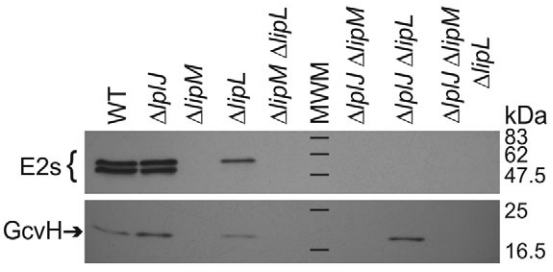

Fig. 4. Immunoblotting analyses of mutant strains with an anti-lipoic acid antibody. The strains were grown overnight in minimal medium supplemented with acetate and BCFA precursors. The cells were diluted in fresh medium of the same composition and grown for 22 h before analysis. The black bars denote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

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 (Fig. 5A). Since prototrophy could result from either octanoyltransfer or ligation of traces of endogenous octanoate (Hermes and Cronan, 2009), we also tested complementation of an *E. coli lplA lipA* strain in the presence of lipoate. Growth was also restored in this strain (Fig. 5B) and it was accompanied by activation (hence lipoate modification) of the *E. coli* 2-oxoacid dehydrogenases (Fig. 5C). 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 (Fig. 2) and had a wild-type pattern of lipoylated proteins (Fig. 4). This behaviour was expected because Δ*lplA* 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; 1995) (Fig. 1). Based on this precedent the Δ*lipM* Δ*lplJ* 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 in the absence of lipoic acid (Fig. 2), 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 catalyse octanoyl transfer, it could not be assumed that the *B. subtilis* lipoate salvage enzyme catalysed a ligase reaction. Therefore, we purified the hexahistidine-tagged protein to homogeneity (Fig. 6A) and assayed it for both the overall and first partial reaction of the ligase reaction. In the absence of an

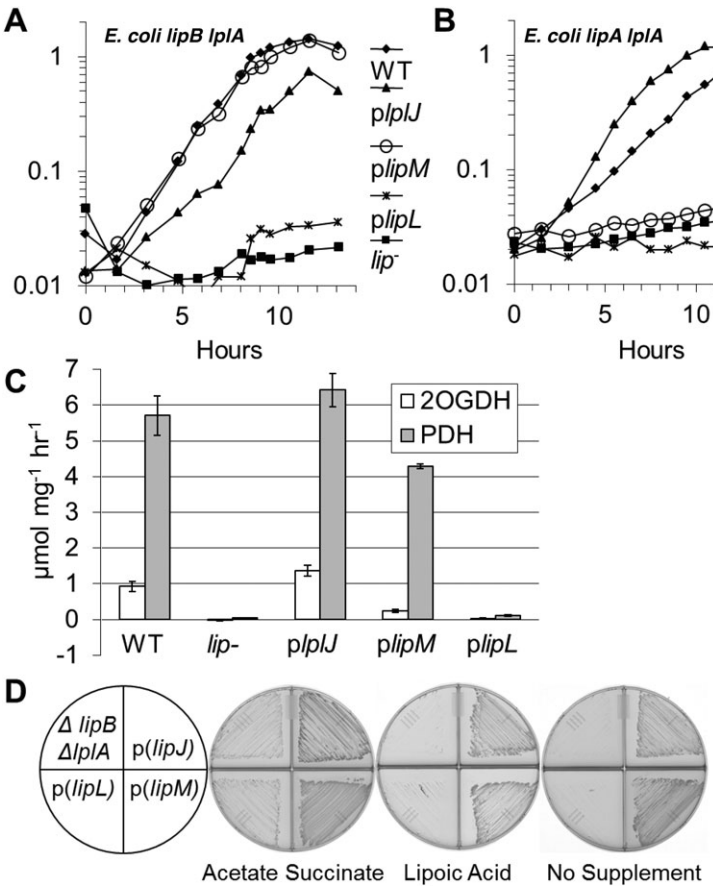

Fig. 5. Complementation of *E. coli* lipoic acid auxotrophs with *B. subtilis* genes as described in *Experimental procedures*.
A. Growth of the *lipB lplA* strain TM136 containing plasmids expressing the genes indicated on the figure. Culture absorbance at 600 nm is reported.
B. Growth of *lipA lplA* strain TM131 containing plasmids expressing genes indicated as in (A).
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.
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 and Cronan, 2009).

acceptor domain, synthesis of both octanoyl-adenylate and lipoyl-adenylate intermediates was readily demonstrated by use of ATP labelled in the α-phosphate. Moreover, upon addition of an acceptor lipoyl domain the adenylate intermediates were hydrolysed to AMP (Fig. 6B). 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 (Fig. 6C). Finally, modification of GcvH was confirmed by electrospray mass spectrometry of the reaction products (Fig. 6D). From these data we conclude that *B. subtilis* LplJ catalyses 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; Christensen and Cronan, 2010), indicated that *B. subtilis* like *E. coli* has an octanoyltransferase (LipM), a sulphur 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* Δ*lipL* strains. Strains devoid of LipL were unable to grow in minimal medium as observed for strains lacking either *lipM* (Fig. 3) 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 Δ*lipL* strain NM51. The growth behaviour of the Δ*lipL* strain denoted a block in the endogenous lipoylation pathway because, like the *lipA* conditionally mutant strain and Δ*lipM* strains, the Δ*lipL* strain grew as well as its wild-type parent in minimal medium supplemented with acetate and BCFA precursors.

The residual growth of the Δ*lipL* strain in minimal medium containing lipoic acid was due to LplJ activity because the Δ*lipL* Δ*lplJ* double mutant strain, NM67, failed to grow in this medium. Hence the Δ*lipL* 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 (Fig. 3B) indicating that

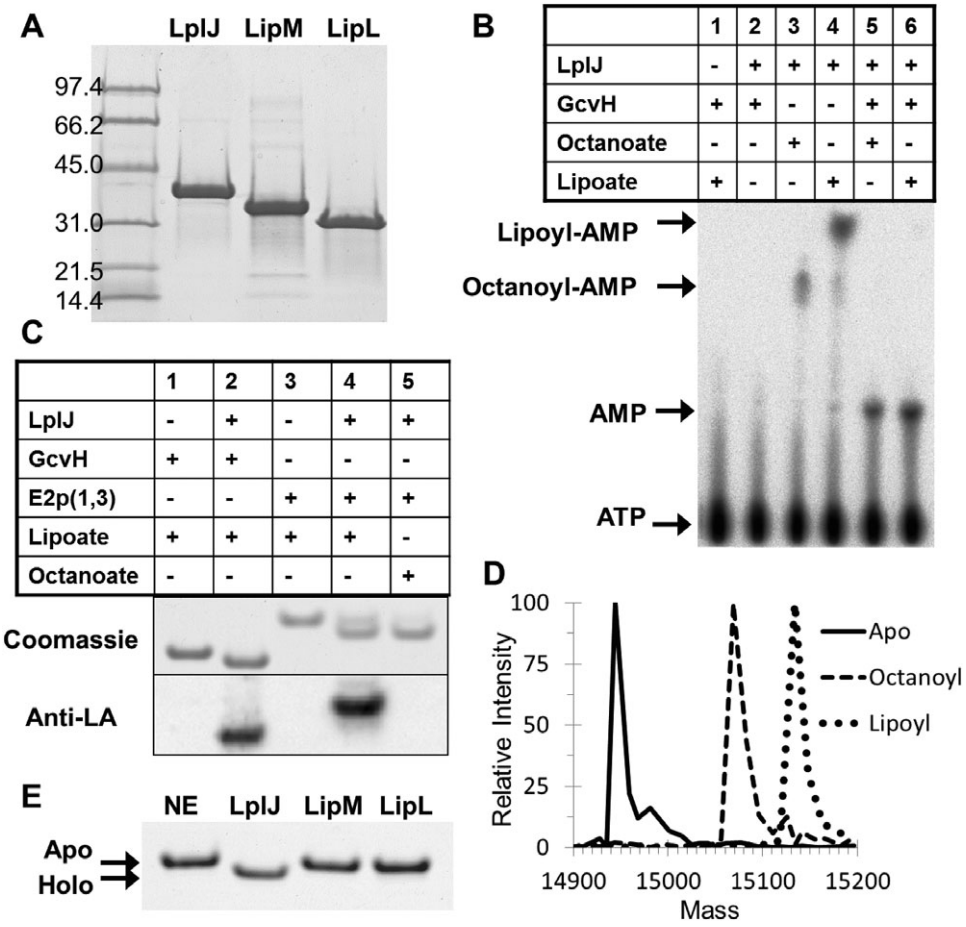

Fig. 6. The *B. subtilis* lipoate salvage enzyme is a lipoate-protein ligase.
A. An SDS-PAGE gel (4–20% acrylamide) containing ~2 μg of each of the indicated purified proteins is shown. The molecular weight marker standards in kilodaltons are given.
B. Formation of the acyl adenylate intermediates by 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.
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 either by Coomassie R250 staining or by Western blotting with anti-lipoic acid antibody (Anti-LA) as indicated.
D. Electrospray mass spectra of GcvH after LplJ treatment with either octanoate or lipoate as acyl donors.
E. Assay of purified proteins for octanoyl ligation using GcvH as the substrate acceptor domain. NE denotes the control reaction that lacked enzyme.

the absence of a functional copy of *lipL* was the cause of the growth phenotype of strain NM51.

The growth phenotype of the Δ*lipL* strain indicated that it should have decreased levels of protein lipoylation and any residual modification should be abolished upon introduction of a Δ*lplJ* lesion. This was the case (Fig. 4). 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 threefold less than those seen in extracts of the wild-type strain (Fig. 4). Strain NM51 required both acetate and BCFA precursors for growth indicating that the BKDH and PDH E2 subunits were non-functional in absence of LipL. Thus, the band observed seems likely to be the non-essential 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* Δ*lplJ* strain, NM67 (Fig. 4), 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 twofold stronger than that of extracts from the wild-type strain (Fig. 4). This observation 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

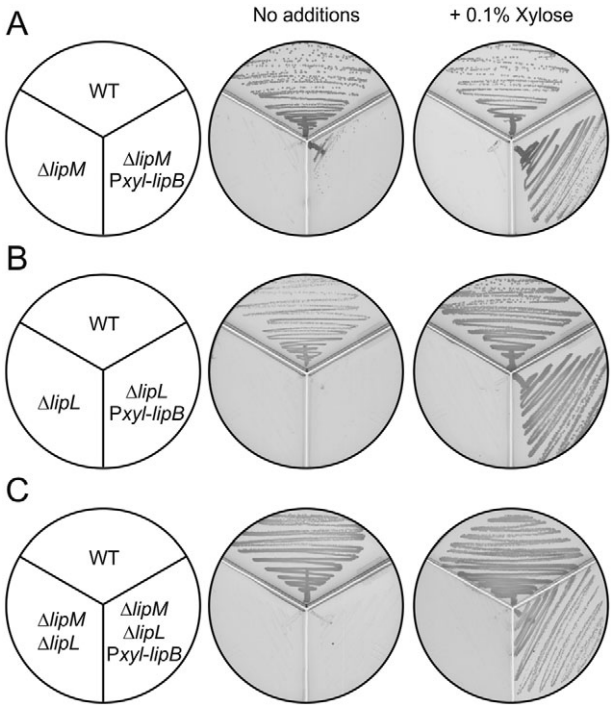

Fig. 7. Complementation of *B. subtilis* mutants deficient in lipoate synthesis by expression of *E. coli* LipB. *B. subtilis* strains JH642 (wild type), NM57 (Δ*lipM*), NM11 (Δ*lipM amyE*::P*xyl-lipB*), NM51 (Δ*lipL*), NM12 (Δ*lipL amyE*::P*xyl-lipB*), CM28 (Δ*lipL* Δ*lipM*) and NM14 (Δ*lipL* Δ*lipM 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.

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 and Cronan, 2009). However, this does not seem to be the case in *B. subtilis* since no lipoylated proteins were detected in a *ΔlipM* strain (Fig. 4).

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* Δ*lipL* double mutant strain CM28 (Fig. 7). However, when expressed in *E. coli* mutant strains, LipL had no detectable ability to replace either *E. coli* LplA or LipB (Fig. 5). 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* (Christensen and Cronan, 2010) 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 (Christensen *et al*., 2011). 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 (Fig. 5D).

Although LipL expression failed to complement 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 catalyse 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* (Christensen and Cronan, 2010). Moreover, crude extracts of *B. subtilis ΔlipL* mutants readily catalysed the LipM reaction; transfer of octanoate from octanoyl-ACP to GcvH (Fig. 8).

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 analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described in *Experimental procedures*. The PDH complexes were purified from extracts of strains NM60 (Δ*lplJ*), NM65 (Δ*lipM* Δ*lplJ*), NM67 (Δ*lipL* Δ*lplJ*) and

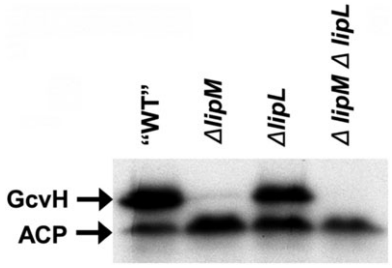

Fig. 8. LipM acts before LipL. Autoradiogram of an SDS-PAGE gel of the products of octanoyltransfer assays from octanoyl-ACP (synthesized with AasS) to GcvH. The enzyme source was crude extracts of the indicated *B. subtilis* strains. All strains, including the 'WT', carried an Δ*lplJ* lesion to prevent possible complications by ligation of octanoate.

A

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

B

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

Fig. 9. Liquid chromatography-tandem mass spectrometric analysis of PDH lipoyl domain tryptic peptides.
A. The modification states of PDH residue K43 detected.
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.

NM68 (Δ*lipM* Δ*lipL* Δ*lplJ*) 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 (Fig. 9) and modification was determined using an error tolerant search as described in *Experimental procedures* to determine the presence or absence of post-translational 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 (Fig. 9). These results indicate that PDH was not modified in Δ*lplJ* strains when either LipM or LipL was non-functional and that LipL is required for E2 lipoyl domain modification rather than for sulphur 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 Δ*lipL* Δ*lplJ* strain (Fig. 4) 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 article 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 behaviour. Its expression in *E. coli* fails to complement a Δ*lipB* mutation whereas expression of LipB complements a *B. subtilis* Δ*lipL* 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* (Christensen and Cronan, 2010) and the data in this and the companion article indicate that LipM and LipL catalyse 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. 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 *Staphylococcus 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 companion article (Christensen *et al.*, 2011) 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.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains used in the present study are listed in Table 1. *E. coli* and *B. subtilis* strains were routinely grown in Luria–Bertani (LB) broth (Sambrook *et al.*, 1989). 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-methylbutyric 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 sulphate (Km), 5; and spectinomycin sulphate (Sp), 50.

Genetic techniques

Escherichia coli competent cells were transformed with supercoiled plasmid DNA using the calcium chloride procedure (Sambrook and Russell, 2001). Transformation of *B. subtilis* was carried out by the method of Dubnau and Davidoff-Abelson (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

In all cases DNA fragments were obtained by PCR using the oligonucleotides described in Table 2. Chromosomal DNA from wild-type *B. subtilis* was used as a template. Sequencing corroborated the identity and correct sequence of all the cloned fragments.

A strain with a deletion of the *yqhM* (*lipM*) gene was constructed by gene replacement with a kanamycin resistance determinant, through a double-cross-over 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 (Table 2) 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 (Table 2) 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.

A *yhfJ* (*lplJ*) knockout 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 (Table 2) and cloned into the XbaI site of vector pJM134 (M. Perego, unpublished), and 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 (Table 2) 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 (Table 2) and inserted between the SacI and SmaI sites of plasmid pJM134 (M. Perego, unpublished). 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 (Table 2) 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 ScaI 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-

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
B. subtilis		
JH642	*trpC2 pheA1*	Laboratory stock
NM57	JH642 *lipM*::Km^r	This study
NM08	NM57 *amyE*::P*xyl-lipM*	This study
NM11	NM57 *amyE*::P*xyl-lipB*	This study
NM51	JH642 *lipL*::Sp^r	This study
NM09	JH642 *amyE*::P*xyl-lipB*	This study
NM10	JH642 *amyE*::P*xyl-lipL*	This study
NM12	NM51 *amyE*::P*xyl-lipB*	This study
NM13	NM51 *amyE*::P*xyl-lipL*	This study
CM28	NM57 *lipL*::Sp^r	This study
NM14	CM28 *amyE*::P*xyl-lipB*	This study
NM60	JH642 *lplJ*::Sp^r	This study
NM65	NM60 *lipM*::Km^r	This study
NM67	NM60 *lipL*::Sp^r	This study
NM68	NM65 *lipL*::Cm^r	This study
E. coli		
DH5α	*supE44 thi-1* Δ*lacU169(f80lacZ*Δ*M15) 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 and Cronan (1992)
QC031	*rpsL8 lipB::*Tn*1000 lplA*::Tn*10*/pQC003	This study
QC032	*rpsL8 lipB::*Tn*1000 lplA*::Tn*10*/pQC004	Christensen and Cronan (2010)
QC035	*rpsL8*/pBAD322G	Christensen and Cronan (2009)
QC038	*rpsL8 lipA::*Tn*1000 lplA*::Tn*10*/pBAD322G	Christensen and Cronan (2009)
QC057	*rpsL8 lipB::*Tn*1000 lplA*::Tn*10*/pBAD322G	Christensen and Cronan (2010)
QC080	*rpsL8 lipB::*Tn*1000 lplA*::Tn*10*/pQC032	This study
QC081	*rpsL8 lipA::*Tn*1000 lplA*::Tn*10*/pQC032	This study
QC086	*rpsL8 lipB::*Tn*1000 lplA*::Tn*10*/pQC003	This study
QC087	*rpsL8 lipB::*Tn*1000 lplA*::Tn*10*/pQC004	Christensen and Cronan (2010)
QC134	*rph-1* IN(*rrnD-rrnE*)1 φ(*fabA–lacZ*)1(Hyb)*cat*	Christensen and Cronan (2010)
QC142	*rph-1* IN(*rrnD-rrnE*)1 φ(*fabA–lacZ*)1(Hyb)*cat*/pCY598, pQC015	Christensen and Cronan (2010)
QC143	*rph-1* IN(*rrnD-rrnE)*1 φ(*fabA–lacZ*)1(Hyb)*cat*/pCY598, pQC033	This study
QC161	*rph-1* Δ*lplA*::FRT Δ*lipB*::FRT/pQC057, pTARA	Christensen and Cronan (2010)
QC168	*rpsL lipB*::Kn *lplA*::Tc φ(*fabA- lacZ*)1(Hyb)*cat*	This study
QC175	*rpsL lipB*::Kn *lplA*::Tc φ(*fabA- lacZ*)1(Hyb)*cat*/pBAD322G	This study
QC176	*rpsL lipB*::Kn *lplA*::Tc φ(*fabA- lacZ*)1(Hyb)*cat*/pQC003	This study
QC177	*rpsL lipB*::Kn *lplA*::Tc φ(*fabA- lacZ*)1(Hyb)*cat*/pQC004	This study
QC178	*rpsL* (SmR) *lipB*::Kn *lplA*::Tc φ(*fabA–lacZ*)1(Hyb)*cat*/pQC032	This study
Plasmids		
pGES40	pBluescript (Stratagene) containing the *xylR* gene and P*xylA* promoter	G. Schujman (unpublished)
pJM116	Integrative vector to construct transcriptional fusions to *lacZ*; integrates at the *amyE* locus of *B. subtilis*; Cm^r	Dartois (1996, #3}
pJM105A	Integrational vector; Cm^r	Perego (1993)
pJM114	Integrational vector; Km^r	Perego (1993)
pJM134	Integrational vector; Sp^r	M. Perego (unpublished)
pBAD322G	Arabinose inducible expression vector	Cronan (2006)
pTARA	pACYC origin, arabinose-inducible T7 polymerase	Wycuff and Matthews (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 spectinomycin cassette	This study
pNM07	pJM114 containing *lipM* interrupted with a kanamycin cassette	This study
pNM48	pJM134 containing *lplJ* interrupted with a spectinomycin cassette	This study
pNM47	pJM114 containing *lipL* interrupted with a kanamycin cassette	This study
pNM53	pJM105A containing *lipL* interrupted with a chloramphenicol cassette	This study
pNM57	Contains *xylR* P*xylA-lipM* into BamHI site of pJM116	This study
pNM58	Contains *xylR* P*xylA-lipL* into BamHI site of pJM116	This study
pNM59	Contains *xylR* P*xylA-lipB* into BamHI site of pJM116	This study
pSJ120	*E. coli* LipB expression vector	Jordan and Cronan (2003)
pQC003	LplJ expression vector	This study
pQC004	LipM expression vector	Christensen and Cronan (2010)
pQC015	N-terminal hexahistidine LipM expression vector	Christensen and Cronan (2010)
pQC032	LipL expression vector	This study
pQC033	N-terminal hexahistidine lipL expression vector	This study

a. Cm^r, Gm^r Km^r, Sp^r, Amp^r denote resistance to chloramphenicol, gentamicin, kanamycin, spectinomycin and ampicillin respectively.
CGSC, Coli Genetic Stock Center.

Table 2. Oligonucleotide primers.

Primer name	Sequence (5′–3′)[a]
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

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

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, an 882 bp fragment containing *lipM* with its ribosome binding site was PCR-amplified with oligonucleotides XVII and XVIII (Table 2) and the product inserted between the SalI and KpnI sites of pGES40 (G. 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 (Table 2) and cloned into SalI and KpnI sites of pGES40 (G. Schujman, unpublished). 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 (Table 2) and inserted between the SalI and KpnI sites of pGES40 (G. Schujman, unpublished). 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 of the genes, they 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 and Moffatt, 1986).

Growth curves of B. subtilis *cultures*

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 hour.

Complementation of E. coli *lipoate auxotrophs*

Bacillus subtilis genes were expressed in *E. coli* from plasmids with an arabinose-inducible promoter as described (Christensen and Cronan, 2010). Complementation of the *lipB lplA* strain TM136 was tested in M9 minimal medium containing 0.2% arabinose, 0.1% Vitamin-Assay Casamino Acids and gentamicin. Complementation of the *lipA lplA* strain TM131 was tested using the same medium containing 5 µg ml^{-1} sodium lipoate. Growth was measured by absorbance (OD_{600}) in a Beckman DU600 spectrophotometer.

The activities of the lipoate-dependent dehydrogenases were assayed using the continuous spectrophotometric assay previously described (Christensen and Cronan, 2009). Briefly, derivatives of TM136 carrying various plasmids were subcultured to an OD_{600} of 0.1 in LB with 0.2% arabinose, 5 mM acetate and 5 mM 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 acetylpyridine 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 acetate, 5 mM 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.

Protein purifications

Purification of GcvH. Hexahistidine-tagged *B. subtilis* GcvH was heterologously expressed in *E. coli* and purified by anion exchange on a 1.8 ml POROS HQ 20 column using an AKTA Purifier 10 (GE Healthcare) at 5 ml/min in 25 mM sodium MES (pH 6.1). The proteins were eluted with a 0–1 M gradient of LiCl in the same buffer. GcvH was verified to be in the unmodified form lacking the N-terminal methionine residue by electrospray mass spectrometry as previously described (Christensen and Cronan, 2010) 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 (Christensen and Cronan, 2010).

Purification of lipoate metabolism enzymes. LipB was purified by nickel affinity and anion exchange as described (Jordan and Cronan, 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 *Thermoplasma acidophilum* LplA (Christensen and Cronan, 2009). 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 analysed by MALDI MS as previously described (Christensen and Cronan, 2010). 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 (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 4 h at 100 V. The proteins were visualized by staining with Coomassie Blue R-250 (Sambrook and Russell, 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 *et al.*, 1987).

Acyl-adenylate intermediate formation was assayed by thin-layer chromatography and autoradiography as described for BirA (Xu and Beckett, 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. Extract 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 analysed using a modification of the method of Laskey and Mills (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 pre-flashed Biomax XAR film (Kodak) at −70°C for 24 h.

Immunoblotting analyses

Bacillus 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 h 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^{-1}) 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 sulphate-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*

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^{-1}) (Sigma) and DNase I (5 μg ml^{-1}) (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 *g* for 30 min. The cleared lysate was treated with 32 μg ml^{-1} ribonuclease and incubated at 15°C for 70 min to degrade ribosomes. The samples were centrifuged for 30 min at 44 000 *g*. The solution of approximately 50 mg of protein ml^{-1} was layered on top of a sucrose step gradient (12.5% w/v and 70% w/w). Centrifugation was performed for 3 h at 150 000 *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 dialysed 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.

Preparation of the lipoyl domain of B. subtilis *PDH complex*

Bacillus subtilis PDH complex (1 ml; 5–20 mg ml^{-1}) in 50 mM sodium phosphate buffer (pH 7.0) was digested at 30°C with *S. 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 r.p.m. in a bench top centrifuge and supernatants analysed 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.

LC-MS/MS analysis

Trypsin digestion and LC-MS/MS analysis were performed by the University of Illinois Biotechnology Center Protein Sciences Facility as follows. Gel slices were crushed and rinsed with water. Twenty-five microlitres of trypsin (12.5 μg ml^{-1}) in 25 mM ammonium bicarbonate was added and the samples were digested in a CEM Discover microwave reactor for 15 min at 55°C and 50 watts. The gel pieces were extracted with 100 μl of 50% acetonitrile and 5% formic acid for 10 min 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 × 150 mm 3 μm Atlantis dC18) and Atlantis dC18 5 μm Nanoease trap columns. A 60 min linear gradient of 1–60% 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.

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