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Unravelling the lipoyl-relay of exogenous lipoate utilization in *Bacillus subtilis*

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SUMMARY

Lipoate is an essential cofactor for key enzymes of oxidative and one-carbon metabolism. It is covalently attached to E2 subunits of dehydrogenase complexes and GcvH, the H subunit of the glycine cleavage system. *Bacillus subtilis* possess two protein lipoylation pathways: biosynthesis and scavenging. The former requires octanoylation of GcvH, insertion of sulfur atoms and amidotransfer of the lipoate to E2s, catalyzed by LipL. Lipoate scavenging is mediated by a lipoyl protein ligase (LplJ) that catalyzes a classical two-step ATP-dependent reaction. Although these pathways were thought to be redundant, a Δ*lipL* mutant, in which the endogenous lipoylation pathway of E2 subunits is blocked, showed growth defects in minimal media even when supplemented with lipoate, and despite the presence of a functional LplJ. In this study we demonstrate that LipL is essential to modify E2 subunits of branched chain ketoacid and pyruvate dehydrogenases during lipoate scavenging. The crucial role of LipL during lipoate utilization relies on the strict substrate specificity of LplJ, determined by charge complementarity between the ligase and the lipoylable subunits. This new lipoyl-relay required for lipoate scavenging highlights the relevance of the amidotransferase as a valid target for the design of new antimicrobial agents among Gram-positive pathogens.

Introduction

Lipoic acid (LA) is an organosulfur compound distributed in all domains of life. Five lipoate-dependent multienzyme complexes, which are involved in oxidative and one-carbon metabolism, have been characterized (Cronan, 2016). Pyruvate dehydrogenase (PDH) converts pyruvate into Acetyl-CoA; oxoglutarate dehydrogenase (ODH), a tricarboxylic citric acid cycle enzyme, converts oxoglutarate into succinyl-CoA; branched-chain 2-oxoacid dehydrogenase (BKDH) is an enzyme involved in branched chain fatty acids (BCFA) synthesis; acetoin dehydrogenase (ADH) acts in stationary phase of growth and converts acetoin into Acetyl-CoA. These lipoate-requiring complexes share the same architecture: they are composed of many copies of three catalytic subunits, known as E1, E2, and E3. The fifth complex, the glycine cleavage system (GCS), catalyzes the oxidative decarboxylation of glycine and is composed of four proteins, called P, H (GcvH), T and L proteins. LA is linked through an amide bond to a specific and conserved lysine residue of the lipoylable domains (LD) present in E2 and GcvH proteins, where it acts as a swinging arm transferring reaction intermediates among the multiple active sites of the enzyme complexes (Perham, 2000).

LA metabolism has been thoroughly characterized in the Gram-negative bacterium *Escherichia coli*. This organism has two redundant pathways for protein lipoylation: an endogenous, or *de novo* synthesis, and a scavenging pathway of the cofactor from the environment (Table S1). In the first step of LA synthesis an octanoyltransferase (LipB) catalyzes the attachment of octanoate derived from fatty acid synthesis to LD in the E2 subunits, PDH-E2 (E2p, dihydrolipoamide acetyltransferase), ODH-E2 (E2o, dihydrolipoamide transsuccinylase), and GcvH. Then, the LA synthase (LipA) catalyzes the conversion of the octanoyl side chain to lipoyl, by introduction of a pair of sulfur atoms (Reed and Cronan, 1993). The scavenging pathway is directly carried out by

lipoyl protein ligase A (LplA) which attaches exogenous LA to the apoproteins by a two-step ATP-dependent reaction: a) the activation of LA to lipoyl-AMP and b) the transfer of this activated lipoyl species to E2 subunits and GcvH, with the concomitant liberation of AMP (Cronan, 2016; Morris, Reed and Cronan, 1995; Morris, Reed and Cronan, 1994).

LA synthesis in the model Gram-positive *Bacillus subtilis* involves more proteins than the corresponding *E. coli* pathway: it requires four protein activities to lipoylate its apoproteins *de novo*, instead of the two enzymes necessary in the Gram-negative bacterium (Fig 1A). First, the octanoyl-acyl carrier protein (ACP):protein-N-octanoyltransferase, LipM, transfers the octanoyl moieties to GcvH (Christensen and Cronan, 2010). Then, LipA inserts sulfur atoms into C6 and C8 of the octanoyl moieties (Martin, Lombardía, Altabe, de Mendoza and Mansilla, 2009). Finally, the amidotransferase, LipL, transfers the lipoyl side chain from GcvH to the E2 subunits (Christensen, Martin, Mansilla, de Mendoza and Cronan, 2011b). This lipoyl-relay pathway was found not only in Gram-positive bacteria, but also in yeast and humans (Cao, Hong, Zhu, Hu and Cronan, 2017; Cao , Zhu , Song , Hu and Cronan, 2018; Schonauer, Kastaniotis, Kursu, Hiltunen and Dieckmann, 2009; Zorzoli, Grayczyk and Alonzo, 2016). *B. subtilis* has a sole lipoate ligase, LplJ, which catalyzes the same ATP-dependent reaction as LplA, as demonstrated by *in vitro* modification of *E. coli* and *B. subtilis* apoproteins (Martin, Christensen, Mansilla, Cronan and de Mendoza, 2011). However, a mutant which lacks LipL, a protein that belongs to the endogenous lipoylation pathway, shows growth defects in minimal medium supplemented with this cofactor (Martin *et al.*, 2011). It is also interesting to note that lipoate supplementation fully restored growth of a Δ*gcvH* mutant, which correlated with modification of all the E2 subunits (Christensen *et al.*, 2011b). These results indicate that LipL, but not GcvH,

is involved in the scavenging pathway. We hypothesized that LipL would play a role in lipoate scavenging by regulating LplJ activity or modulating global changes in gene expression of the target proteins, making lipoyl scavenging insufficient in its absence. In this paper, we establish the essential role of LipL in both pathways of lipoate post-translational modification and report an unexpected donor of lipoyl moieties for the amidotransfer reaction. Whereas LipL does not share sequence homology with eukaryotic enzymes, there are Gram-positive pathogenic bacteria that contain homologous proteins to LipL, underscoring its relevance as a valid target for the design of new antimicrobial agents against these bacteria.

Results

B. subtilis lipoyl protein ligase LplJ requires the amidotransferase LipL to modify all E2 subunits

It was previously described that LplJ, the lipoyl protein ligase, is the sole enzyme that links lipoate to the apoproteins of *B. subtilis* (Martin *et al.*, 2011). Expression of LplJ in *E. coli* Δ*lipA* Δ*lplA* cells, that are unable to synthesize and ligate LA, restored their ability to ligate LA to all *E. coli* apoproteins (Martin *et al.*, 2011). However, modification of *B. subtilis* E2p by LplJ has not been detected *in vitro* (Martin *et al.*, 2011). In addition, it was observed that *B. subtilis* Δ*lipL* mutants were unable to grow in Spizizen minimal medium (SMM) (Spizizen, 1958) even though it was supplemented with LA, and although a functional LplJ was present (Martin *et al.*, 2011; Fig 1B). These observations suggest that LipL is also involved in the LA salvage process.

To determine the role of LipL in lipoate ligation to the apoproteins we performed Western blot analysis on cell extracts of *B. subtilis* mutants defective in the synthesis or scavenging pathways, grown in the presence or absence of exogenously provided lipoate. Anti-LA immunoblot of extracts from the Δ*lplJ* strain showed a wild-type pattern of lipoylated proteins: two major bands, with apparent masses of 60 and 52 kDa, were detected both in the absence and presence of LA (Fig 1C, lanes 1 and 2). The higher molecular weight band corresponds to the E2p and E2o subunits, which run with the same apparent molecular weight in SDS-PAGE, whereas the lower molecular weight band corresponds to the E2b subunit (BKDH-E2, lipoamide acyltransferase). These results were expected since in the Δ*lplJ* strain the LA biosynthetic pathway is still functional. By contrast, immunoblot analysis of crude extracts of strain Δ*lipL* grown in the presence of LA showed only the higher molecular weight band, (Fig 1C, lane 4). This result denotes that LipL is required for the ligation of exogenously provided LA to,

at least, the E2b subunit and explains the observed growth defect of the Δ*lipL* mutant in SMM supplemented with LA (Fig 1B). Surprisingly, this band was also detected when Δ*lipL* cells were grown in SMM without LA (Fig 1C, lane 3), meaning that the biosynthesis pathway is involved in this modification. It was previously described by our group that the lipoyl moiety from lipoyl-GcvH is not transferred to any E2 subunit in the absence of both the amidotransferase and the lipoyl ligase, resulting in its accumulation (Martin *et al.*, 2011). Besides, a Δ*lipL* Δ*lipM* double mutant, unable to transfer the octanoyl residue to GcvH and in consequence to synthesize lipoyl-GcvH (Martin et al., 2011), does not lipoylate its E2 subunits in the absence of the exogenously provided cofactor (Fig 1C, lane 5). Then we reasoned that, in the absence of the amidotransferase, LplJ is transferring the endogenously synthesized LA from lipoyl-GcvH to at least one of the E2 subunits of higher apparent molecular weight (see below).

As observed using extracts of Δ*lipL* cells, when a Δ*lipL* Δ*lipM* double mutant is grown in SMM supplemented with LA, the higher molecular weight band can be detected, due to the ligation of the exogenous cofactor by LplJ (Fig 1C, lane 6). On the contrary, in extracts of Δ*lipM* Δ*lplJ* cells there aren´t any lipoylated proteins even when LA is present in the medium (Fig 1C, lane 7), an expected result as these cells are defective both in the biosynthetic and salvage pathways.

As the E2o and E2p subunits have the same apparent molecular weight in SDS-PAGE, media supplementation analysis was performed in order to determine if both proteins were functional in the Δ*lipL* mutant. To this end, the Δ*lipL* strain was grown in SMM supplemented with BCFA precursors (BCFAP) or both sodium acetate and BCFAP, as it is already known that exogenous succinate is not a requirement for *B. subtilis* growth in SMM (Martin *et al.*, 2009). As shown in Fig 1B, this strain is only able to grow if

both sodium acetate and BCFAP are added to SMM, indicating that the PDH complex is not functional in a Δ*lipL* strain. These results also suggest that the 60 kDa lipoylated band observed in the immunoblotting analysis of this mutant (Fig 1C, lane 3 and 4) corresponds to the E2o subunit.

Together, these results indicate that the lipoyl protein ligase enzyme LplJ is essential to transfer exogenous LA to GcvH and E2o, but also requires LipL to modify E2p and E2b subunits. This path differs from other lipoyl ligase enzymes, such as *E. coli* LplA, which can transfer exogenous lipoate to all E2 subunits without the requirement of an additional protein. Expression of *E. coli* LplA under the control of the IPTG-inducible promoter P*spac* in a *B. subtilis* Δ*lipL* Δ*lplJ* mutant restores growth of this strain in SMM supplemented with lipoate (Fig 1D). Since the Δ*lipL* Δ*lplJ* double mutant is impaired in both LA biosynthesis and utilization, this result indicates that LplA can functionally bypass both pathways in *B. subtilis* without the aid of an auxiliary protein. As shown for LA synthesis, where a four-protein pathway is required in *B. subtilis* (Christensen *et al.*, 2011b; Christensen and Cronan, 2010; Martin *et al.*, 2011) instead of the two-protein lipoylation mechanism utilized by *E. coli* (Jordan and Cronan, 2003; Zhao, Miller, Jiang, Marletta and Cronan, 2003), the ligation of exogenous lipoate in this Gram-positive model bacterium also follows a more complex pathway than in the Gram-negative model (Morris *et al.*, 1995; Morris *et al.*, 1994).

LipL is also required for octanoic acid scavenging

In *E. coli*, the lipoyl ligase LplA is able to transfer both lipoate as well as octanoate to the apoproteins, albeit less efficiently (Zhao *et al.*, 2003). A similar behavior was observed in a *Staphylococcus aureus* Δ*lipM* mutant (Zorzoli *et al.*, 2016). To determine whether *B. subtilis* LplJ is able to ligate exogenously provided octanoic acid and if LipL is also involved in this process, a Δ*lipM* strain was grown in SMM supplemented with

octanoic acid or the combination of sodium acetate and BCFAP. Whereas octanoic acid supplementation allowed growth of the Δ*lipM* mutant to levels comparable to the wild type strain, the Δ*lipM* Δ*lplJ* double mutant was unable to grow in the same conditions (Fig 2A). These results indicate that LplJ is required for the transfer of exogenous octanoate to the apoproteins while LipM activity is not essential in this process. As shown in Fig 2B, a Δ*lipL* mutant strain showed the same growth defect in SMM supplemented with octanoic acid as the observed for a Δ*lipM* Δ*lplJ* strain, indicating that in *B. subtilis* both LplJ and LipL are required not only for lipoate attachment, but also for octanoic acid scavenging. Besides, since octanoate supplementation fully restored growth of a Δ*gcvH* mutant (Fig 2B), we concluded that octanoate ligation by LplJ and LipL does not require the formation of an octanoate-GcvH intermediate. This result also indicates that introduction of sulfur atoms mediated by LipA can occur either on octanoyl-GcvH or on octanoyl-E2 (at least on octanoyl-E2o, see below).

As reported for *E. coli* and *S. aureus*, higher concentrations of exogenous octanoic acid (125 µM) than LA (25 nM) are required to supplement growth of a Δ*lipM* mutant (Fig S1) (Jordan and Cronan, 2003; Zorzoli *et al.*, 2016). Thereby, even though *B. subtilis* LipL and LplJ are capable of transferring both exogenously provided lipoic and octanoic acid to the E2 subunits, lipoate transfer seems to be more efficient. Sequence alignment of LplJ and LplA shows that the *B. subtilis* lipoyl ligase contains the residues predicted to form hydrophobic interactions with the dithiolane ring and the hydrophobic tail of LA, as inferred from LplA crystal structure (Fujiwara *et al.*, 2005). Unspecific van der Waals interactions may permit LplJ to bind LA analogues and octanoic acid, but hydrophobic interaction would be stronger when the dithiolane ring of LA is present. This might explain why LplJ, like LplA, has a higher affinity for lipoate than for octanoate.

Functional LipL is required for lipoate scavenging

We demonstrated that during lipoate scavenging the *B. subtilis* E2p and E2b subunits are only lipoylated when both LplJ and LipL are present in the cell (Fig 1C, lanes 4, 6 and 7). We therefore wondered whether LplJ and LipL could be acting sequentially, or if the amidotransferase could be involved in LplJ expression or modulating its activity, or if the dual requirement for these proteins in the utilization of exogenously provided lipoate could arise from the need of LipL and LplJ to interact forming a functional complex. For example, protein-protein interaction in lipoate synthesis have been proposed to occur in yeast, among Lip3 (amidotransferase), the H protein, and perhaps Lip2 (octanoyltransferase) and Lip5 (lipoate synthase), which could be forming a lipoylation complex (Schonauer *et al.*, 2009). To discern between these possibilities, we used the bacterial adenylate cyclase two-hybrid system to test for LipL and LplJ interactions (Karimova, Ullmann and Ladant, 2000). In this system the interaction between target proteins results in the functional complementation between adenylate cyclase T18 and T25 domains, which in turn results in production of cAMP and a concomitant increase in β-galactosidase activity in *E. coli* cells. LplJ was fused to the T18 domain of the adenylate cyclase, either to the N-term and C-term, and LipL was fused to the T25 domain, also in both positions. Colonies transformed with the four possible plasmid combinations formed white colonies in LB supplemented with X-gal (Fig S2A), even though the system successfully worked when the T18 and T25 domains were fused to interacting leucine zipper proteins (Fig S2B). These results suggest that LipL and LplJ are not interacting *in vivo.*

To determine if it was indeed the amidotransferase activity of LipL required for E2p and E2b modification during lipoate scavenging or if LipL was somehow regulating LplJ expression or activity, we analyzed the growth phenotype of a Δ*lipL* mutant in which a

catalytically inactive form of LipL was expressed. It was previously described that LipL residue C150 is essential for catalysis: mutagenesis of this cysteine residue resulted in loss of enzymatic activity and the inability to form an acyl-enzyme intermediate (Christensen *et al.*, 2011b). Based on this evidence, an in-frame fusion of LipLC150A to the green fluorescent protein (GFP) was expressed under a xylose-inducible promoter in a Δ*lipL* mutant. As observed in Fig 3A, the expression of the wild type version, LipL-GFP restored the growth of the Δ*lipL* mutant. By contrast, expression of LipLC150A did not allow the growth of the Δ*lipL* mutant in the presence of LA. This result correlates with the detection of just one of the two lipoylated bands in the immunoblot corresponding to the E2p and/or E2o proteins (Fig 3B). Although the pattern of lipoylated proteins in this strain was identical to the one of a Δ*lipL* strain (Fig 1B), it was still possible that expression of the LipLC150A protein allowed at least modification of the E2p subunit. However, this strain was unable to grow in SMM supplemented only with BCFAP (Fig 3A), indicating that the PDH complex was still not functional. To rule out the possibility that the observed phenotype was the result of lack of expression of the mutant version of LipL, the fluorescence produced by the fusion protein LipLC150A-GFP was monitored by microscopy. As shown in Fig 3C, fluorescence was observed after the addition of the inductor to SMM, however, growth was restored only when the media was supplemented with both sodium acetate and BCFAP (Fig 3A). These results indicate that LipL must be functional to allow E2p and E2b modification by exogenous lipoate.

Deciphering the enigmatic role of LipL in lipoate utilization

We have demonstrated that *B. subtilis* requires the presence of a functional amidotransferase in order to lipoylate E2p and E2b with exogenously provided LA. GcvH, the only known substrate of LipL, and essential during LA *de novo* biosynthesis,

was dispensable for lipoate utilization (Christensen *et al.*, 2011b). Therefore, we hypothesized that another lipoylated protein was acting as a source for the amidotransfer reaction during lipoate utilization. In Western blot assays of protein extracts of Δ*lipL* mutants grown in the presence of LA, a band corresponding to the lipoylated E2o, was observed (Fig 1 and 3). This protein has not been previously described as a lipoate donor in the biosynthesis pathway, since LipM only transfers octanoate from octanoyl-ACP to GcvH (Christensen and Cronan, 2010; Christensen *et al.*, 2011b). However, we reasoned that lipoyl-E2o might be a good source of the cofactor for LipL amidotransfer reaction during the lipoate scavenging pathway, in the absence of lipoyl-GcvH. Since it was demonstrated that *L. monocytogenes* LipL catalyzes a reversible reaction (Christensen, Hagar, O'Riordan and Cronan, 2011a), it is possible that the *B. subtilis* lipoylation relay uses E2o as a LA donor to transfer the cofactor to E2b and E2p. To test this hypothesis, we constructed a Δ*gcvH* Δ*odhB* strain (being *odhB* the gene encoding E2o). This double mutant was unable to grow in SMM even when supplemented with LA (Fig 4A), and its E2s are not lipoylated in these growth conditions (Fig 4B). These results indicate that the Δ*gcvH* Δ*odhB* double mutant lost the ability to utilize exogenous lipoate, even when wild type LplJ, LipL, and the essential lipoyl-dependent E2p and E2b are present in the cell. As expected, a Δ*odhB* strain is able to synthesize LA, and thus, grows and lipoylates its apoproteins in SMM (Fig 4). All these results allowed us to propose a model of LA biosynthesis and utilization in *B. subtilis*, where the amidotransferase LipL plays a central role in both pathways, transferring lipoyl moieties from GcvH and E2o to the rest of the E2 subunits (Fig 5).

LplJ-mediated lipoyl linking requires a specific glutamate residue in the target apoprotein

We demonstrated that LplJ could only ligate LA to GcvH and to E2o, while this enzyme does not modify E2p and E2b. We hypothesized that LplJ substrate specificity could be due to the orientation of the lipoylable lysines in GcvH and E2o, which allows a convenient interaction between the ligase and these subunits, while the orientation of the corresponding lysines on E2p and E2b apoproteins would not favor LplJ interaction. To corroborate our hypothesis, we aligned E2o-LD I-Tasser generated models with E2p-LD, E2b-LD and GcvH models. As shown in Fig S3, E2o and E2p lysine residues have different orientations, which would account for their differences in lipoylation. However, even though the E2b conserved lysine has the same orientation than the corresponding E2o residue, LplJ is not able to ligate lipoate to E2b. Besides, conserved lysines from E2o and GcvH, both lipoylated by LplJ, have different orientations. We conclude that orientation of the lipoylable lysine residue is not a determinant during lipoate ligation by LplJ.

To try to identify amino acid residues that would be involved in LplJ substrate specificity, we aligned E2 LDs and GcvH sequences from *B. subtilis* and *E. coli*. As shown in Fig 6A, the E2 subunits that can act as substrates for LplJ have a conserved glutamate (a negatively charged residue), located three residues to the N-terminal side from the lipoylable Lys. By contrast, E2p and E2b have a glutamine or a methionine residue instead (uncharged residues). Thus, the substrate specificity of the ligase for the lipoate acceptor protein could be determined by charge complementarity between the ligase and the lipoylable subunits, as predicted by modelling of complexes between an archeal LplA and LD of Gram-negative bacteria (Kim *et al.*, 2005). To determine if the negative charge of E2o Glu^{39} is essential during lipoylation by LplJ, we generated a mutant subunit where this glutamate residue was replaced by Gln (E2o-E39Q). As previously shown, the double mutant strain Δ*gcvH* Δ*odhB* is unable to grow in SMM

supplemented with LA (Fig 4), due to the lack of an appropriate protein recipient for LplJ ligation. When this strain was transformed with a plasmid that allows expression of the E2o wild type copy, it recovered its ability to attach LA and hence to grow in SMM in the presence of the cofactor (Fig 6B). However, when the mutant copy E2o-E39Q was expressed, the bacterial strain was unable to ligate exogenous LA, demonstrating that Glu^{39} is indeed essential for the ligation reaction carried out by LplJ (Fig 6B).

Taking this into account, we wondered if a mutated version of E2p, containing a glutamate residue instead of the conserved glutamine in position 40, would be recognized by LplJ. We therefore transformed a *B. subtilis* Δ*gcvH* Δ*odhB* mutant with an integrative plasmid containing the coding sequence for the mutated version of E2p (E2p-Q40E) or the wild type version cloned under an IPTG inducible promoter. As expected, these strains could not grow in SMM, but the addition of LA to the medium allowed the growth of the strain expressing E2p-Q40E (Fig 6C). This indicates that PDH is functional, and thus that LplJ had gained specificity for the mutated version of E2p. It is interesting to note that BCFAP addition to the medium was not necessary, which means that BKDH is also functional. As this strain contains a wild type LipL, we conclude that LipL would also use lipoyl-E2p-Q40E as substrate for amidotransfer to E2b. Confirming this hypothesis, a Δ*lipL* mutant expressing E2p-Q40E, requires BCFAP addition to grow on LA-containing SMM (Fig S4.A).

We have demonstrated that LplJ is able to ligate lipoate to GcvH, E2o and E2pQ40E. As this enzyme also ligates octanoate to the receptor proteins, we wonder if *B. subtilis* LipA would also accept octanoyl-E2p as substrate. Addition of octanoic acid to SMM does not restore the growth of Δ*gcvH* Δ*odhB* mutant expressing E2p-Q40E (Fig 6C), as occurred in Δ*lipM* and Δ*gcvH* mutants (Fig 2), so clearly LipA is not inserting sulfur atoms on octanoyl-E2p-Q40E.

All together these experiments put in evidence that the glutamate residue located three residues to the N-terminal side from the lipoylable lysine in the apoprotein is key for recognition as a lipoylable substrate by LplJ.

Discussion

Protein lipoylation is a post-translational modification present from bacteria to mammals. It is essential for the activity of 2-oxoacid dehydrogenase complexes and the GCS. Different organisms have evolved diverse strategies for protein lipoylation: some of them synthesize the cofactor, others utilize LA acquired from the environment, and others encode both pathways. To add to the complexity of protein lipoylation pathways, modification of apoproteins using exogenous lipoate occurs by several mechanisms. In *E. coli* LA is transferred to apoproteins in an ATP-dependent process by the lipoyl protein ligase A (LplA). In Gram-positive bacteria, the scavenging pathways are even more diverse. The pathogenic bacterium *Listeria monocytogenes* possess two lipoyl protein ligases. While LplA1 is required for intracellular growth and can use host-derived lipoyl-peptides as substrates, LplA2 utilizes only free lipoate and is dispensable for intracellular growth (Keeney, Stuckey and O'Riordan, 2007). Additionally, LplA1 has a tight substrate specificity as it only ligates lipoate to GcvH (Christensen *et al.*, 2011a). Modification of E2 LDs requires the activity of the amidotransferase, LipL, which utilizes lipoylated GcvH as substrate (Christensen *et al.*, 2011a). *Staphylococcus aureus* also has two ligases: LplA1 and LplA2. LplA1 is the primary LA salvage enzyme in broth culture, while either LplA1 or LplA2 stimulate bacterial survival within macrophages in a manner dependent on exogenous LA provision (Zorzoli *et al.*, 2016). *In vitro* studies determined that these ligases target different LD-containing proteins: LplA1 is able to modify GcvH and E2o, while LplA2 modifies all oxoacid dehydrogenase E2 subunits (Laczkovich *et al.*, 2018). As expression of LplA2 is limited

in broth culture, modification of E2b and E2p in this condition requires the transfer of the lipoyl moiety from lipoyl-GcvH to the apoproteins, mediated by LipL (Zorzoli *et al.*, 2016). By contrast, the model bacterium *B. subtilis* has a sole lipoyl protein ligase, LplJ, which catalyzes the same ATP-dependent reaction as *E. coli* LplA (Martin *et al.*, 2011), but it is not enough to attach LA to all E2 subunits, as it was demonstrated in this study.

B. subtilis relies on two pathways for protein lipoylation, but they are not completely redundant: growth and lipoylation phenotypes observed in a Δ*lipL* mutant pointed out that the amidotransferase acts in both the scavenging and the *de novo* biosynthetic pathway of the cofactor. In this paper we demonstrated that although LplJ is able to modify all the *E. coli* E2s, in *B. subtilis* it can only transfer exogenous lipoate to GcvH and E2o. For E2p and E2b lipoylation, the presence of LipL is necessary, which correlates with *in vitro* evidence of LplJ lipoylating GcvH but not E2p (Christensen *et al.*, 2011b). Until this study, the exact role of this amidotransferase during LA scavenging in *B. subtilis* remained elusive. The first considered interpretation was that LplJ modifies GcvH and then LipL catalyzes the amidotransfer reaction from GcvH to E2 subunits, as already described for *S. aureus* and *L. monocytogenes* (Christensen *et al.*, 2011a; Laczkovich *et al.*, 2018; Zorzoli *et al.*, 2016). Nevertheless, it was reported that a *B. subtilis* Δ*gcvH* mutant is able to grow in SMM supplemented with lipoate, showing a strong lipoylation of the E2 subunits (Martin *et al.*, 2011), which indicates that GcvH is not an essential intermediate during LA scavenging. An alternative hypothesis was that LipL and LplJ form a complex, as it was proposed to occur with the proteins Lip3, the H protein and probably Lip2 and Lip5, involved in LA synthesis in yeast (Schonauer *et al.*, 2009). However, we demonstrated via two-hybrid assay that LipL and LplJ are not interacting, indicating that these enzymes are probably

functioning in successive enzymatic steps. Evidence in support to this result stems from the finding that LipL must be functional during LA scavenging process.

Considering that GcvH is not required during lipoate scavenging, that LplJ can only modify E2o subunits and GcvH, and that LipL activity is necessary to lipoylate the E2p and E2b subunits, we propose that the scavenging pathway could consist of successive steps that include the dihydrolipoamide transsuccinylase (E2o). Initially, LplJ would activate LA and modify the E2o and GcvH subunits. In a subsequent reaction, LipL would catalyze the amidotransfer reaction from lipoyl-E2o and/or lipoyl-GcvH to E2b and E2p subunits. The growth and lipoylation phenotypes from a Δ*gcvH* Δ*odhB* strain support this model: this double mutant is unable to utilize exogenous lipoate, even when wild type LplJ, LipL, and the lipoyl-dependent E2p and E2b are present in the cell. The need for LipL activity during LA scavenging would be due to the inability of the lipoyl protein ligase to utilize E2p and E2b as substrates. A comparison between the aminoacids sequences surrounding the lipoylation site of GcvH and E2-LD from *B. subtilis* and *E. coli* highlighted key differences. While the proteins that can be modified by LplJ possess a Glu residue located 3 positions to the N-terminal side of the lipoylable Lys, a non-polar or uncharged residue was found in *B. subtilis* E2p and E2b. Replacement of this acidic residue by a non-polar one in E2o (E2o-E39Q) precluded its lipoylation by LplJ, and resulted in the inability to utilize exogenous lipoate in a Δ*gcvH* Δ*odhB* background (Fig 6B). On the contrary, when the non-polar Gln residue of E2p was replaced by a Glu, LplJ was able to recognize it as a substrate and lipoylation of the apoprotein indeed occur (Fig 6C). Thus, when an acidic residue is present in this position of the apoproteins LplJ is able to lipoylate their substrates, but the absence of this negative charge interferes with LplJ recognition. Based on structural analysis, similar interactions through a hydrogen bond, had been proposed between Glu residues

situated in equivalent positions of E2o and E2p from *E. coli* with Gly^{74} of LplA (Fujiwara *et al*., 2010). Also, modeling *T. acidophylum* LplA complexes with E2p from *A. vinelandii*, or GcvH from *T. thermophilus* predicted interactions between conserved acidic residues from the receiver apoproteins, located close to the lipoylable Lys, and basic residues from LplA, through hydrogen bond unions (Kim *et al*., 2005). It was suggested that these residues would participate in the recognition of the apoproteins by the ligase. In this study we have demonstrated their essentiality for the reaction to proceed *in vivo*.

A similar lack of recognition of E2 subunits by the lipoyl protein ligases was described in *S. aureus* and *L. monocytogenes* (Christensen *et al.*, 2011a; Zorzoli *et al.*, 2016). Using alignments of primary sequences of lipoate-modified proteins, we found that E2o from *S. aureus* and GcvH from both bacteria, which can be modified by LplA1 ligases, contain the conserved Glu residue located 3 positions to the N-terminal side of the lipoylable Lys (Fig S5). As expected, the E2 apoproteins that require LipL activity to get lipoylated contain uncharged or non-polar residues occupying these positions (Fig S5). It is interesting to note that LplA1 from both bacteria have higher sequence similarity to *B. subtilis* LplJ than LplA2 (*S. aureus* LplA1 and LplA2 57% and 39% identity; *L. monocytogenes* LplA1 and LplA2 65% and 51% identity, respectively) and they share the same recognition requirements (Christensen *et al.*, 2011a; Laczkovich *et al.*, 2018). The growth phenotype and lipoylation pattern of *S. aureus* Δ*lipL* mutants (Zorzoli *et al*., 2016) indicate that the amidotransferase would be performing the same role in lipoate scavenging as its *B. subtilis* orthologue.

LplJ has an additional overlooked activity: it can take the lipoyl moiety synthesized on GcvH and ligate it to apoproteins that are able to interact with the ligase. As seen in Fig1C, in a Δ*lipL* mutant E2o became lipoylated even in the absence of exogenous LA

(lane 3), but these modification does not occur if the strain is unable to synthesize octanoyl-GcvH (Δ*lipM* Δ*lipL* mutant, lane 5), or lacks the ligase (Δ*lipL* Δ*lpJ* mutant, Martin *et al*, 2011). This transfer also takes place on the E2p-Q40E version, which is able to interact with LplJ. When this modified E2p subunit was expressed in a Δ*lipL* mutant the addition of BCFAP to SMM restored its growth, indicating that PDH was active, and thus LplJ was modifying E2p-Q40E with endogenously produced LA (Fig S4.A). Indeed, growth of a Δ*lipL* Δ*lplJ* mutant expressing E2p-Q40E requires addition of both acetate and BCFAP to SMM, indicating that in the absence of the lipoyl ligase the transference of endogenous synthesized LA to this protein is not occurring (Fig S4.B).

B. subtilis E2p and E2b have a glutamine and a methionine instead of glutamate in the position equivalent to E2o-Glu[39], so as it was already explained, they cannot be modified with exogenous lipoate by LplJ and hence require LipL amidotransfer activity. This indicates that LipL is able to transfer lipoyl moieties to apoproteins that have aminoacid residues other than Glu in the mentioned position. It is interesting to note that mammalian amidotransferases exhibit a different pattern of substrate recognition. It had been demonstrated that Glu residues located in equivalent positions of bovine liver mitochondria E2 subunits are essential for the lipoate attachment reaction using the bovine amidotransferase LIPT1 (Fujiwara, Okamura-Ikeda and Motokawa, 1996). However, the assayed reaction of LIPT1 corresponds to the formerly believed lipoyltransferase activity of this protein: transference of the lipoyl moiety from lipoyl-AMP to apo-LDs, which might be a moonlighting activity of the enzyme. The role of LIPT1 as an amidotransferase has been recently confirmed using human E2p as the receptor apoprotein (Cao *et al.*, 2018). It remains to be determined if human E2b, that contains a Gln residue instead of Glu located 3 residues to the N-terminal side of the

lipoylation site, can also act as a substrate in this reaction, or if another enzyme is required for lipoamide acyltransferase modification. This is likely to be the case as LIPT1 deficiency in humans greatly alters E2p and E2o lipoylation, but E2b modification is only partly affected (Soreze *et al.*, 2013). These differences of specificity between bacterial and human amidotransferases might be a consequence of the lack of significant sequence similarity between both proteins or to differences in their mechanisms of reaction. It is worth noting that LipL is able to transfer the octanoyl moiety from [1-^{14}C]octanoyl-GcvH to PDH LDs of both *E. coli* and *B. subtilis* (Christensen *et al.*, 2011). However, we observed that LipA is not able to insert sulfur atoms on octanoyl-E2p (Fig 6C) as it does on octanoyl-E2o (Fig 2) or, as already reported, on octanoyl-GcvH (Martin *et al.*, 2009). It therefore seems that although LipL can transfer octanoate *in vitro*, this is just a secondary function. Moreover, the human amidotransferase LIPT1 is not able to use octanoate *in vitro* (Cao *et al.*, 2018), agreeing with the fact that LipL main physiological reaction would be the transfer of lipoate moieties.

It was previously postulated that GcvH provides an environment that facilitates the LipL reaction and that the E2-LD lack this property (Cao *et al.*, 2017). However, in this work we demonstrate that lipoyl-E2o is a good substrate for LipL, and that indeed lipoyl-E2p-Q40E can function as a donor in the amidotransferase reaction. Therefore, LipM ability to transfer octanoate only to GcvH, and not to any E2, would be the cause of the lipoate relay during LA synthesis in *B. subtilis*. This study demonstrated that LipL is more flexible in substrate recognition than LipM and LplJ. LipM is able to modify all *E. coli* E2 subunits (Christensen and Cronan, 2010), but is unable to modify any *B. subtilis* E2, *Homo sapiens* E2p or *Aquifex aeolicus* GcvH2, GcvH3 and GcvH5, even when most of these proteins share the pattern of recognition of the lipoyl protein

ligases (Cao *et al.*, 2017; Martin *et al.*, 2011). Further work would be required to define the determinants of LipM substrate specificity.

Based on our results, we propose a model for lipoate biosynthesis and utilization in *B. subtilis*, where LipL plays an essential role in both pathways, transferring LA to the essential E2p and E2b, using either GcvH or E2o as donors (Fig 5). The lipoyl-relay required for biosynthesis and scavenging of lipoate would reflect the age of the reactions involved. By phylometabolic analysis, it was proposed that the *B. subtilis* variant might be the ancestral pathway to lipoate synthesis (Braakman and Smith, 2014). We postulate that the route of LA salvage in this Gram-positive model would have appear to satisfy only the GCS requirement, and afterwards the use of the oxidative Krebs cycle or the degradation of branched chain amino acids demanded the amidotransferase activity. The ability of E2o to become substrate of LplJ would be a gain of function that E2p and E2b have not still achieved along evolution. The pathways of protein lipoylation found in proteobacteria, with a single all-purpose octanoyltranferase (LipB) and a unique ligase (LplA) to modify all apoproteins would reflect metabolic innovations of more recent emergence.

The similarities between protein lipoylation requirements in *B. subtilis*, *S. aureus* and *L. monocytogenes* suggest that this lipoyl-relay during lipoate utilization is conserved among Gram-positive bacteria (Christensen *et al.*, 2011a; Martin *et al.*, 2011; Zorzoli *et al.*, 2016). Due to the involvement of LA metabolic proteins in pathogenesis, multidrug resistance and intracellular growth of pathogens (Grayczyk, Harvey, Laczkovich and Alonzo, 2017; Ma *et al.*, 2006; Rachman *et al.*, 2006; O`Riordan, Moors and Portnoy, 2003), the finding of essential proteins implicated in LA metabolism would provide new targets for antimicrobials. Besides, as LipL has no significant primary sequence homology with human proteins, we propose that this

enzyme would be a good target for the design of new antimicrobial agents.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains used in this work are listed in Table 1. *B. subtilis* strains are derivatives of JH642. *E. coli* and *B. subtilis* strains were routinely grown in Luria Bertani (LB) broth (Sambrook, Fritsch and Maniatis, 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 (SMM) for *B. subtilis*. SMM was supplemented with 50 nM or 0.5 mM DL-α-LA, 10 mM sodium acetate and 0.1 mM each BCFA precursor (BCFAP, isobutyric acid, isovaleric acid and 2-methylbutyric acid), as indicated. Xylose was added to 0.1% and isopropyl β-D-thiogalactopyranoside (IPTG) was added to 1 mM as required. Glycerol (0.5%) was used as a carbon source instead of glucose for the experiments involving gene expression under the control of the xylose-inducible promoter (P*xylA*). Antibiotics were added at the following concentrations: sodium ampicillin (Amp), 100 μg ml-1; chloramphenicol (Cm), 5 μg ml-1; kanamycin sulfate (Km), 5 μg ml-1 for *B. subtilis* or 50 μg ml-1 for *E. coli*; streptomycin (Str), 100 μg ml-1; erytromycin (Em), 0.5 μg ml-1; lincomycin (Lm), 12.5 μg ml-1 and spectinomycin sulfate (Sp), 50 μg ml-1.

Genetic techniques

E. coli competent cells were transformed with supercoiled plasmid DNA using the calcium chloride procedure (Ausubel *et al.*, 1987). Transformation of *B. subtilis* was carried out by the method of Dubnau and Davidoff-Abelson (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, Takada and Okada, 1975). Under these conditions, *amy*$^+$ colonies produced a clear halo, whereas *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 *B. subtilis* JH642 was used as a template. Sanger sequencing was used to corroborate the identity and correct sequence of all the cloned fragments. Plasmids used in this study are listed in Table 3.

A strain with a deletion of the *lipL* gene was constructed by gene replacement with a kanamycin resistance determinant, through a double crossover event. For this purpose plasmid pNM47 (Martin *et al*., 2011) was linearized with *Sca*I and used to transform strain JH642, yielding strain NM28.

For Δ*lipL* complementation and expression analyses, a plasmid expressing LipLC150A was constructed as follows: a 952 bp fragment containing *lipL* with its ribosome binding site (RBS) was PCR-amplified with oligonucleotides LFwSal and LRevBK (Martin *et al*., 2011). This fragment was digested with *Hind*III and *Cla*I to obtain a fragment containing the first 410 bp of the *lipL* gene, which was cloned into pBluescriptKS (Stratagene), yielding plasmid pNM82. Plasmid pQC079 (Christensen *et al*., 2011) was digested with *Cla*I to obtain a fragment of *lipL* gene containing a point mutation that replaces the cysteine 150 for alanine. This fragment was cloned into pNM82 to obtain plasmid pNR002. To construct a translational fusion of the *lipLC150A* gene contained in pNR002 to the green fluorescent protein (GFP), an 860 bp fragment containing *lipLC150A* allele was PCR amplified using oligonucleotides lipL_Kpn_FOR and lipL_Hind_REV. This fragment, cloned in pJET 1.2/blunt (pJET-*lipLC150A*) was

554 digested with *Hind*III and *Kpn*I and inserted into pSG1154 (Lewis and Marston, 1999),
555 rendering plasmid pNR005. This plasmid was used to transform strain NM28. The
556 double crossover event into the *amy* locus was assessed by the ability to metabolize
557 starch. The resulting strain was named NR008 (*lipL::*Km^r *amyE::lipLC150A*). A similar
558 strategy, using pGES40-lipL (Martin *et al*., 2011) as template, was performed to
559 construct a wild-type LipL-GFP fusion, rendering strain AL107.
560 To study complementation of Δ*lipL* Δ*lplJ* strain with *E. coli* LplA, strain NR001
561 (*lipL::*Km^r, *lplJ::*Sp^r *sacA::*P*spac-lplA*) was constructed. Briefly, wild-type copy of the
562 *E. coli lplA* gene (1055 bp fragment) was PCR-amplified from genomic DNA of strain
563 W3110 with oligonucleotides lplAHFw and lplABRv and the product inserted between
564 the *Hind*III and *BamH*I sites of pGES485 (G. Schujman, unpublished). This plasmid
565 was digested with *Eco*RI and *Bam*HI to obtain a fragment containing *lacI Pspac-lplA*,
566 which was cloned into the *sacA* locus of pSac-Cm (Middleton and Hofmeister, 2004)
567 previously digested with the same enzymes, yielding plasmid pNM85. Plasmid pNM85
568 was linearized with *Sca*I and used to transformed strain NM60 (Martin *et al*., 2011),
569 yielding strain NM107. This strain was transformed with plasmid pNM47 (Martin *et al*.,
570 2011) linearized with *Sca*I, yielding strain NR001. Transformants were screened for
571 *sacA* phenotype, as previously described (Middleton and Hofmeister, 2004).
572 For two-hybrid analyses, four plasmids were constructed: pKT25-*lipL*, pUT18-*lplJ*,
573 pKNT25-*lipL* and pUT18C-*lplJ*. These plasmids contain the *lipL* gene fused in frame to
574 the T25 fragment of *Bordetella pertussis* adenylate cyclase and the *lplJ* gene fused in
575 frame to its T18 fragment. Plasmids pKT25-*lipL* and pKNT25-*lipL* were constructed as
576 follows: an 856 bp fragment containing *lipL* gene was PCR-amplified using
577 oligonucleotides lipL Up and lipL DW and ligated into pJET 1.2/blunt to obtain plasmid
578 pJET-*lipL*. This plasmid was digested with *Xba*I and *BamH*I and the resulting fragment

was inserted into plasmids pKT25 and pKNT25 (Karimova *et al*., 2000). Plasmids pUT18-*lplJ* and pUT18C-*lplJ* were constructed as follows: a 1007 bp fragment containing *lplJ* gene was PCR-amplified using oligonucleotides lplJ Up and lplJ DW. This fragment was ligated to pJET 1.2/blunt to obtain plasmid pJET-*lplJ*. This plasmid was digested with *Xba*I and *Sac*I and the resulting fragment was inserted into the plasmids pUT18 and pUT18C (Karimova *et al*., 2000).

The Δ*gcvH* Δ*odhB* deletion mutant strain CM56, was obtained by transformation of strain NM20 with plasmid pCM1104. This plasmid was constructed as follows: a 2050 bp fragment from the 5′ upstream to the 3´ downstream region of the *odhB* gene was PCR-amplified with oligonucleotides ODHup and ODHdw and cloned in pJET1.2/blunt, yielding plasmid pCM1103. The spectinomycin-resistance cassette from plasmid pJM134 (M. Perego, unpublished) was inserted between the *Hinc*II and *Kpn*I sites of the previously generated plasmid to render plasmid pCM1104.

To obtain a Δ*odhB* deletion mutant strain, chromosomal DNA of strain CM56 was used to transform JH642. Upon selection for spectinomycin-resistance, the colonies that remain sensitive to kanamycin were selected, and the presence of wild type *gcvH* gene was confirmed by PCR (Martin *et al*., 2011). This strain was named CM57.

A wild type copy of *odhB* gene was PCR amplified with oligonucleotides ODH_Xho_FOR and ODH_Cla_REV, and inserted into *XhoI* and *ClaI* sites of plasmid pSG1154 (Fujiwara *et al*., 2005), rendering pAL35. The gene coding for the mutant copy of ODHB in which Glu^{39} is replaced by Gln was obtained as follows: oligonucleotides ODHup and ODHExQ_RV were used to amplify the 5´fragment of *odhB* gene while oligonucleotides ODHExQ_FOR and ODH_Cla_REV, to amplify the 3´end. Both fragments were used as template for an overlap extension PCR in which after 10 cycles of extension, oligonucleotides ODH_Xho_FOR and ODH_Cla_REV

were added. The product obtained, *odhBE39Q*, was inserted into *Xho*I and *Cla*I sites of vector pSG1154 (Lewis and Marston, 1999) resulting in plasmid pAL34. Plasmids pAL34 and pAL35 were then digested with *Sal*I and *Cla*I and ligated in vector pHPKS (Johansson and Hederstedt, 1999) rendering plasmids pAL36 and pAL41, respectively. Both plasmids were used to transform strain CM56.

A wild type copy of *pdhC* gene was amplified using oligonucleotides pdhC_Sph_FOR and pdhC_Bdw. The resulting fragment of 1423 bp was inserted into *Sph*I and *BamH*I sites of vector pGES485, rendering plasmid pAL39. To create the mutant version E2p-Q40E, oligonucleotides pairs pdhC_Kup and pdhQxEDW, and pdhQxEup and pdhC_Bdw were used to amplify 5´and 3´fragments of *pdhC* gene, which encodes E2p, respectively. Both fragments were used for an overlap extension PCR in which after 10 cycles of extension, oligonucleotides pdhC_Sph_FOR and pdhC_Bdw were added. The amplified gene, *pdhCQ40E*, was inserted into *Sph*I and *BamH*I sites of vector pGES485, rendering plasmid pAL40. The resulting integrative plasmids pAL39 and pAL40 were used to transform NM28 strain, rendering strain AL119 and AL110, respectively. The double crossover event into the *amy* locus was checked by the inability of cells to metabolize starch and their sensibility to macrolides.

The *ΔlipL* Δ*lplJ amyE::* P*spac-pdhCQ40E* deletion mutant strain AL113, was obtained by transformation of strain AL110 with plasmid pMN84. This plasmid was constructed as follows: the 5´ fragment of gene *lplJ* was amplified using oligonucleotides JSac5up and JXba5dw, and cloned in pCR-Blunt II-TOPO. The 521 bp product obtained by *Xba*I digestion was ligated into pJM105. The 3´fragment of gene *lplJ* was amplified using pair of oligonucleotides JSal3up and JXo3dw and ligated into *Sal*I and *Xho*I sites of pJM105 already containing 5´*lplJ*.

Strain AL117 was obtained by transformation of *B.* subtilis NM20 with plasmid pAL42. Plasmid pAL42 was constructed as follows: plasmid pJM105 was digested with *Kpn*I and *Sma*I and the chloramphenicol cassette obtained was inserted between sites *Kpn*I and *Hinc*II of plasmid pCM1103, rendering plasmid pAL42. AL117 was then transformed with plasmids pAL39 and pAL40 resulting in strains AL120 and AL118, respectively. The double crossover event into the *amy* locus was checked by the inability of cells to metabolize starch and their sensibility to macrolides.

Immunoblotting analyses

B. subtilis wild type and mutant strains were grown overnight in SMM supplemented with sodium acetate and BCFAP at 37°C. Cells were used to inoculate fresh media of the same composition with or without LA, and cultured at 37°C. After 22 hours of growth, 1 ml aliquot of each sample was 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 1 mM) per OD_{600} unit. Then, 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 sulfate-gel electrophoresis in a 12% acrylamide gel. Proteins were transferred to a nitrocellulose membrane and detected using rabbit anti-lipoate antibody (Calbiochem) and anti-rabbit immunoglobulin G conjugated to peroxidase (Bio-Rad). The bands were visualized using the ECL Plus Western Blotting Detection System (GE).

Adenylate cyclase two-hybrid assay

The method used for the adenylate cyclase two-hybrid assay was essentially that of Euromedex (Karimova *et al.*, 2000). BTH101 host cells were co-transformed with the following combinations of plasmids: pKT25-*lipL*/pUT18-*lplJ*, pKNT25-*lipL*/pUT18-*lplJ*, pKT25-*lipL*/pUT18C-*lplJ* and pKNT25-*lipL*/pUT18C-*lplJ*. Transformed colonies were grown on LB plates containing 5-Bromo-4-Chloro-3-indolyl-β-D-galactopyranoside (X-gal; 40 μg ml-1) and isopropyl β-D-1-thiogalactopyranoside (0.5 mM IPTG) at 30°C for 24 h.

Fluorescence microscopy

B. subtilis NR008 strain was grown overnight in SMM supplemented with sodium acetate and BCFAP at 37°C. Cells were used to inoculate fresh media of the same composition with or without xylose, and cultured at 37°C until they reached exponential growth phase. An aliquot of these cultures was used for microscopy. Microphotographs were taken with a Nikon Eclipse 800 microscope and an Andorclara camera. Exposure time was 30 ms for bright-field microscopy and 5 s for fluorescence microscopy. Images were processed and analyzed with Nis Elements and ImageJ.

Bioinformatics

Protein sequences were analyzed with the program BLASTP (Altschul and Lipman, 1990). Sequence alignments were performed using T-Coffee (Notredame, Higgins and Heringa, 2000) and drawn using Boxshade (http://sourceforge.net/projects/boxshade/). The computer program I-Tasser (Zhang, 2008) was used to construct a model of the ODH, PDH, BKDH and GcvH lipoylable domains. The models were aligned and visualized in PyMOL (DeLano, 2002).

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AUTHOR CONTRIBUTIONS

NBR, AL and NM performed the experiments. MCM designed the study and conceived the experiments. All authors analyzed the data. NBR, AL and MCM wrote the manuscript, with input from NM. All authors have read and approved the final version.

GRAPHICAL ABSTRACT

ABBREVIATED SUMMARY

The amidotransferase LipL, previously believed to be involved only in lipoate biosynthesis, plays a crucial role during scavenging of exogenously provided cofactor. Due to the substrate specificity of the ligase LplJ, determined by charge complementarity with the lipoylable subunits, only oxoglutarate dehydrogenase complex E2 and the H protein of the glycine cleavage system are modified. Both

lipoylated proteins participate in the lipoyl-relay pathway of lipoate scavenging, that enables modification of the remaining E2 subunits.

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838 **Table 1.** Bacterial strains used in this study.

Strain	Relevant characteristics†	Source or reference
B. subtilis		
AL107	*lipL*::Km^r *amyE*::P*xyl-lipL-gfp*	This study
AL110	*lipL*::Km^r *amyE*::P*spac-pdhCQ40E*	This study
AL113	*lipL*::Km^r *lplJ*::Cm^r *amyE*::P*spac-pdhCQ40E*	This study
AL117	*gcvH*::Km^r *odhB*::Cm^r	This study
AL118	*gcvH*::Km^r *odhB*::Cm^r *amyE*::P*spac-pdhCQ40E*	This study
AL119	*lipL*::Km^r *amyE*::P*spac-pdhC*	This study
AL120	*gcvH*::Km^r *odhB*::Cm^r *amyE*::P*spac-pdhC*	This study
CM28	*lipM*::Km^r *lipL*::Sp^r	(Martin *et al.*, 2011)
CM56	*gcvH*::Km^r *odhB*::Sp^r	This study
CM57	*odhB*::Sp^r	This study
JH642	*trpC2 pheA1*	Laboratory stock
NM107	*lplJ*::Sp^r *sacA*::P*spac-lplA*	This study
NM20	*gcvH*::Km^r	(Christensen *et al.*, 2011)
NM28	*lipL*::Km^r	This study
NM51	*lipL*::Sp^r	(Martin *et al.*, 2011)
NM57	*lipM*::Km^r	(Martin *et al.*, 2011)
NM60	*lplJ*::Sp^r	(Martin *et al.*, 2011)
NM65	*lplJ*::Sp^r *lipM*::Km^r	(Martin *et al.*, 2011)

NM67	*lplJ*::Sp^r *lipL*::Km^r	(Martin *et al.*, 2011)
NR001	*lplJ*::Sp^r *lipL*::Km^r *sacA::*P*spac-lplA*	This study
NR008	*lipL*::Km^r *amyE::*P*xyl-lipLC150A-gfp*	This study
E. coli		
BTH101	F-, *cya-99, araD139, galE15, galK16, rpsL1 (Str^r), hsdR2, mcrA1, mcrB1.*	(Karimova *et al.*, 2000)
DH5α	*supE44 thi-1* Δ*lacU169(Φ80lacZ*ΔM15*)endA1 recA1 hsdR17 gyrA96 relA1 trp6 cysT329::lacinm$^{\lambda\ p1(209)}$*	Laboratory stock
W3110	F$^-$ lambda$^-$ IN(rrnD-rrnE)1 rph-1	(Bachmann, 1996)

† Amp, Cm, Km, MCL and Sp denote ampicillin, chloramphenicol, kanamycin, macrolides and spectinomycin resistance cassettes, respectively.

843 **Table 2.** Oligonucleotides used in this study.

Name	**Sequence**[†]
JSac5up	5'-GGAGCTCGATGCTGATATAGAACAGTTTCA -3'
JXba5dw	5'-ATTCTAGAGCGGATCATTGATATTTTGATTG-3'
JSal3up	5'-TGGGGTCGACACGAAAGAGGATTTC-3'
JXo3dw	5'-CAGCCTCGAGCTTGGCCACATAATA -3'
LFwSal	5´- AGTTGTCGACCAATAAGCCTAACATGAAAGGG -3´
lipL DW	5'-ACAGGATCCACCTTTGCATTCCGC-3'
lipL UP	5'-TATTCTAGAGATGGCAAACCAACCG-3'
lipL_Hind_REV	5'-CAAAGCTTCCCAAATACCTTTGC-3'
lipL_Kpn_FOR	5'-AAGGTACCATGGCAAACCAACC-3'
lplABRv	5'-TGGGATCCTGGGCGGGTAACTACCTTAC-3'
lplAHFw	5'-TAAAGCTTGGAGGATCGTTATGTCCACATTACG-3'
lplJ DW	5'-TCGAGCTCATCAGATCAAGGAAATCC-3'
lplJ UP	5'-ACTCTAGACATGTTATTTATAGACAATC-3'
LRevBK	5´- AGGGTACCGGATCCTTGAGATAAAAAATGCATG -3´
ODHdw	5´-TTCTCGAGGTTTCTTTGTGCAAAGC-3´
ODHExQ_FOR	5´- TTGAACTACAAACGGATAAAGTG-3´
ODHExQ_RV	5´- TCACTTTATCCGTTTGTAGTTCAAGC-3´
ODHup	5´-ATCTAGACGCCGAAGAGCCTTCTTC-3´
ODH_Cla_REV	5´- TAATCGATTTATTATCCTTCTAATAAAAGC-3´
ODH_Xho_FOR	5´- TACTCGAGACGTATTGTATCTGATAGC-3´
pdhC_Kup	5´- TATGGTACCGCGATTGTCGTTCAAG-3´
pdhC_Sph_FOR	5´- TAGCATGCCTGTTTTCAATGCTTACGATG-3´
pdhQxEup	5´- GGCTGAAGTCGAAAATGATAAAGC-3´

pdhQxEdw	5´- GCTTTATCATTTTCGACTTCAGC-3´
pdhC_Bdw	5´- CACGGATCCTACTACCATAACATTACGC-3´

844 †*Restriction sites are underlined*

846 **Table 3.** Plasmids used in this study.

Plasmid	**Relevant characteristics**‡	**Source or reference**
pAL26	pSG1154 containing *lipL* gene between *Kpn*I-*Hind*III	This study
pAL34	pSG1154 containing *odhBE39Q* alele between *Xho*I and *Cla*I sites	This study
pAL35	pSG1154 containing *odhB* gene between *Xho*I and *Cla*I sites	This study
pAL36	pHPKS containing *odhBE39Q* gene under a xylose inducible promoter	This study
pAL39	pGES485 containing *pdhC* gene under an IPTG inducible promoter	This study
pAL40	pGES485 containing *pdhCQ40E* gene under an IPTG inducible promoter	This study
pAL41	pHPKS containing *odhB* gene under a xylose inducible promoter	This study
pAL42	pJET1.2/blunt containing *odhB* interrupted with a Cm^r cassette	This study
Bluescript KS	*E. coli* cloning vector, Amp^r	Stratagene
pCM1103	pJET1.2/blunt containing *odhB*	This study
pCM1104	pJET1.2/blunt containing *odhB* interrupted with a Sp^r cassette	This study
pGES40-LipL	pGES40 containing *lipL* with its ribosome binding site cloned into *Sal*I and *Kpn*I sites	(Martin *et al.*, 2011)

pGES485	Integrative vector, Sp^r	G.E. Schjuman (unpublished)
pHPKS	*B. subtilis* low copy number replicative vector, MCL^r	(Johansson and Hederstedt, 1999)
pJET 1.2/blunt	*E. coli* cloning vector, Amp^r	Thermo Scientific
pJET-*lipL*	*lipL* PCR amplified with oligonucleotides lipL UP and lipL DW cloned in pJET 1.2/blunt	This study
pJET-*lipLC150A*	*lipL*C150A PCR amplified with oligonucleotides lipL_Kpn_FOR and lipL_Hind_REV cloned in pJET 1.2/blunt	This study
pJET-*lplJ*	*lplJ* PCR amplified with oligonucleotides lplJ UP and lplJ DW cloned in pJET 1.2/blunt	This study
pJM105	Integrative vector, Cm^r	(Perego, 1993)
pJM134	Integrative vector, Sp^r	M. Perego (unpublished)
pKNT25	Derived from plasmid pSU40, it allows to create in-frame fusions to the N-terminal end of the T25 fragment of *B. pertussis* adenylate cyclase, Km^r	(Karimova *et al*., 2000)
pKNT25-*lipL*	pKNT25 containing *lipL* between *Xba*I-*BamH*I	This study
pKNT25-zip	Derivative of pKT25 in which a leucine zipper is genetically fused in frame to the T25 fragment	(Karimova *et al*., 2000)

pKT25	Derived from plasmid pSU40, it allows to create in-frame fusions to the C-terminal end of T25 fragment, Km^r	(Karimova *et al*., 2000)
pKT25-*lipL*	pKT25 containing *lipL* between *Xba*I-*BamH*I	This study
pNM47	pJM114 containing *lipL* interrupted with a kanamycin cassette	(Martin *et al*., 2011)
pNM82	pBluescript containing a 410 bp fragment of *lipL* gene between *Hind*III-*Cla*I	This study
pMN84	pJM105 containing *lplJ* interrupted by a chloramphenicol resistance cassette	This study
pNM85	pSac-Cm containing *lacI Pspac-lplA* cloned into the *sacA* locus	This study
pNR002	pNM82 containing a 846 bp fragment of *lipLC150A* gene in *Cla*I	This study
pNR005	pSG1154 containing *lipLC150A* gene between *Kpn*I-*Hind*III	This study
pQC079	Vector which expresses LipLC150A with a 6-His tag in N-terminus	(Christensen *et al*., 2011)
pSac-Cm	Integrative vector for *SacA* locus, Cm^r	(Middleton and Hofmeister, 2004)
pSG1154	Vector used to fuse GFP onto the C-terminus of any protein under the control of a xylose inducible promoter. The fusion is integrated into *B. subtilis amyE* locus	(Lewis and Marston, 1999)

pUT18	Derivative of the plasmid pUC19. It allows to create in-frame fusions to the N-terminal end of the T18 fragment of *B. pertussis* adenylate cyclase, Amp^r	(Karimova *et al*., 2000)
pUT18C	Derivative of the plasmid pUC19. It allows to create in-frame fusions to the C-terminal end of T18 fragment, Amp^r	(Karimova *et al*., 2000)
pUT18c-lplJ	pUT18C containing *lplJ* gene between *Xba*I-*Sac*I	This study
pUT18C-zip	Derivative of pUT18C in which a leucine zipper is genetically fused in frame to the T18 fragment, Amp^r	(Karimova *et al*., 2000)
pUT18-lplJ	pUT18 containing *lplJ* gene between *Xba*I-*Sac*I	This study

‡ Amp, Cm, Km, MCL and Sp denote ampicillin, chloramphenicol, kanamycin, macrolides and spectinomycin resistance cassettes, respectively.

FIGURE LEGENDS

Figure 1. A. Current model for lipoic acid synthesis and scavenging in *B. subtilis.* During lipoic acid (LA) synthesis LipM transfers octanoic acid ligated to the acyl carrier protein (ACP), from the fatty acid biosynthesis, to the H protein of the Glycine cleavage system (GcvH). Then, LipA generates lipoyl-GcvH and LipL transfers the lipoyl group from GcvH to the lipoylable domain (LD) of the E2 subunits. Exogenous lipoate is transferred to the LDs and the GcvH subunit by LplJ in an ATP-dependent two steps reaction. **B. Growth phenotype of a *B. subtilis* mutant deficient in amidotransferase.** Δ*lipL* strain (NM51) was grown overnight in Spizizen minimal medium (SMM) supplemented with acetate and branched chain fatty acid precursors (BCFAP). The cultures were centrifuged and the cells resuspended in SMM or with the addition of the indicated supplements. Wild type strain (JH642) was included as a positive growth control. Growth was determined by measuring the OD_{600} of the cultures at 22 h of incubation at 37°C. Values reported are the means ± S.D. (n=2). **C. Immunoblotting analysis of mutant strains with an anti-LA antibody.** Strains were grown overnight in SMM supplemented with acetate and BCFAP. The cells were diluted in fresh medium of the same composition with or without the addition of LA, as indicated, and grown for 22 h before analysis. **D. Effect of complementation with the *E. coli* lipoate ligase.** Strains Δ*lipL* Δ*lplJ* (NM67) and Δ*lipL* Δ*lplJ* P*spac-lplA* (NR001) were grown overnight in SMM supplemented with acetate and BCFAP. Cells were collected and resuspended in SMM, or with the addition of the indicated supplements. Wild type strain (JH642) was included as a positive growth control. OD_{600} values of the cultures were measured after 22 h of growth at 37°C. Values reported are the means ± S.D. (n=2).

875 **Figure 2. Growth of lipoic acid auxotrophs upon octanoic acid supplementation. A.**
876 Growth of wild type (JH642), Δ*lipM* (NM57) and Δ*lipM* Δ*lplJ* (NM65) bacterial strains.
877 **B.** Growth of Δ*lipM* (NM57), Δ*lipL* (NM51) and Δ*gcvH* (NM20). The strains were
878 streaked onto minimal medium plates containing the supplements indicated above and
879 incubated for 48 h at 37°C.
880 **Figure 3. A. Growth phenotype of Δ*lipL* strain expressing LipLC150A.** Strains
881 Δ*lipL amyE::*P*xyl-lipLC150A-gfp* (NR008) and Δ*lipL amyE::*P*xyl-lipL-gfp* (AL107)
882 were grown overnight in SMM supplemented with acetate and branched chain fatty acid
883 precursors (BCFAP). Cultures were centrifuged and cells resuspended in Spizizen
884 minimal medium (SMM) in the presence of the inductor, with the addition of
885 supplements, as indicated. The OD_{600} values of the cultures were measured after 22 h of
886 growth at 37°C. Values reported are the means ± S.D. (n=2). **B. Lipoylated proteins of**
887 **Δ*lipL* strain expressing LipLC150A.** Strain Δ*lipL amyE::*P*xyl-lipLC150A-gfp*
888 (NR008) was grown overnight in SMM supplemented with acetate and BCFAP. Cells
889 were diluted in fresh medium of the same composition with the addition of lipoate and
890 with or without xylose, as indicated, and grown for 22 h before analysis. **C. Expression**
891 **of LipLC150A-GFP.** Strain Δ*lipL amyE::*P*xyl-lipLC150A-gfp* (NR008) was grown in
892 SMM supplemented with sodium acetate and BCFAP (panels A and B). Xylose was
893 added (0.1%) to induce the expression of LipLC150A-GFP (panels D and E). Panels C
894 and F show the merge between fluorescence microscopy and light field microscopy in
895 each condition. Scale bars represent 5 μm.
896 **Figure 4. Role of oxoglutarate dehydrogenase in lipoic acid scavenging. A.** Growth
897 of bacterial strains Δ*odhB* (CM57), Δ*gcvH* (NM20) and Δ*gcvH* Δ*odhB* (CM56). Strains
898 were streaked onto Spizizen minimal medium (SMM) plates containing the supplements
899 indicated above and incubated for 48 h at 37°C. **B.** Immunoblotting analysis of mutant

strains with an anti-lipoic acid antibody. The strains were grown overnight in SMM supplemented with acetate and branched chain fatty acid precursors (BCFAP). Cells were diluted in fresh medium of the same composition with or without the addition of lipoic acid (LA), as indicated, and grown for 22 h before analysis.

Figure 5. The role of LipL in lipoate and octanoate utilization. Open arrows: lipoic acid biosynthesis. Thin solid arrows: lipoic and octanoic acid salvage. Thick solid arrows: common steps. In the absence of lipoate biosynthesis the amidotransferase can transfer the lipoyl moiety from the oxoglutarate dehydrogenase (ODH) E2 to the others E2 subunits (dashed arrows). If LipL is absent, pyruvate dehydrogenase (PDH) and branched-chain ketoacid dehydrogenase (BKDH) E2 cannot be modified, neither by the endogenous nor by the exogenous lipoylation pathways.

Figure 6. LplJ substrate specificity for the lipoate ligation reaction. A) Sequence alignment of *B. subtilis* and *E. coli* E2-LDs and GcvH subunits. Identical residues are shown highlighted in black and similar residues are highlighted in grey. The conserved lipoylable lysine residues are indicated by an arrow. (*) Glutamate residues predicted to determine LplJ specificity. (#) Other conserved negatively charged residues that would stabilize the interaction with LplJ. Bs: *B. subtilis*; Ec: *E. coli.* **B)** Growth of bacterial strain Δ*gcvH* Δ*odhB* (CM56) transformed with plasmids that allow expression of either wild type (*odhB*) or mutated E2o copy (*odhBE39Q*). Strain Δ*odhB* (CM57) was used as a positive growth control. Strains were streaked onto minimal medium plates (SMM) containing 0.8% xylose and the supplements indicated above and incubated for 48 h at 37°C. **C)** Growth of bacterial strains Δ*gcvH* Δ*odhB amyE::*P*spac-pdhC* (AL120) and Δ*gcvH* Δ*odhB amyE::*P*spac-pdhCQ40E* (AL117), which express the wild type or mutated copy of E2p, respectively. Wild type strain (JH642) was used as a growth

924 control. Strains were streaked onto SMM plates containing 1 mM IPTG and the
925 indicated supplements. Plates were incubated for 72 h at 37ºC.

A

Fatty acid Biosynthesis

Octanoyl-ACP

LipM

Octanoyl-GcvH

LipA

Lipoyl-GcvH

LipL

Lipoylated E2 Domain

LplJ

AMP

GcvH

LD

Lipoyl-AMP

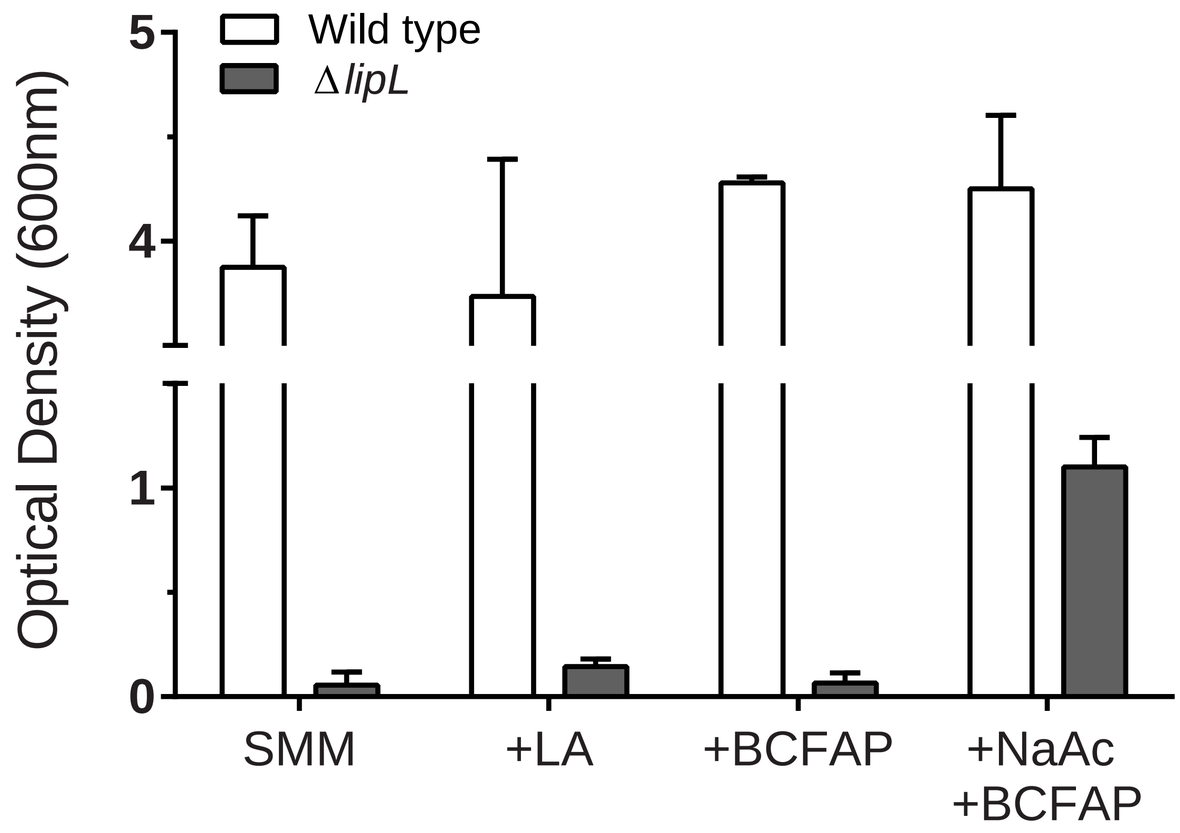
LplJ

PPi

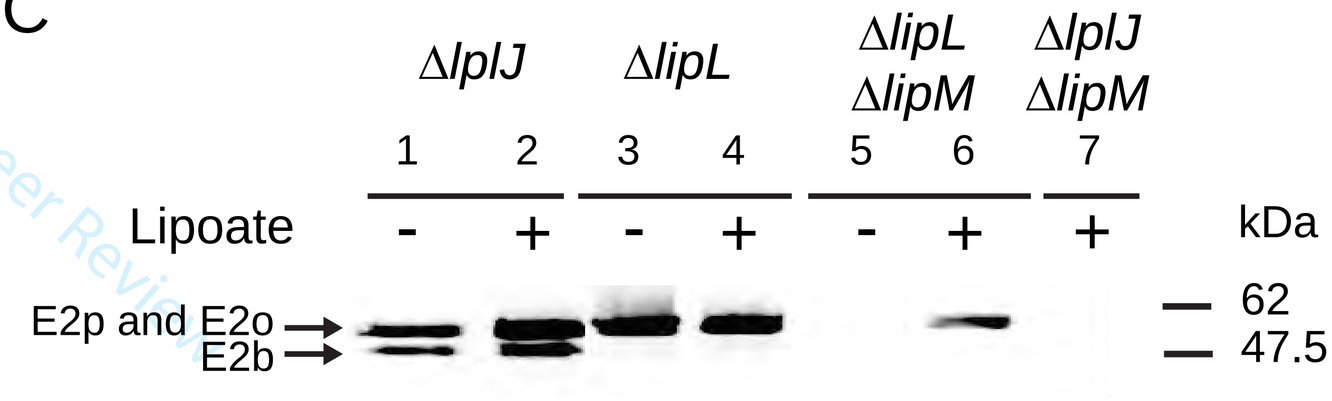
ATP

Lipoic Acid

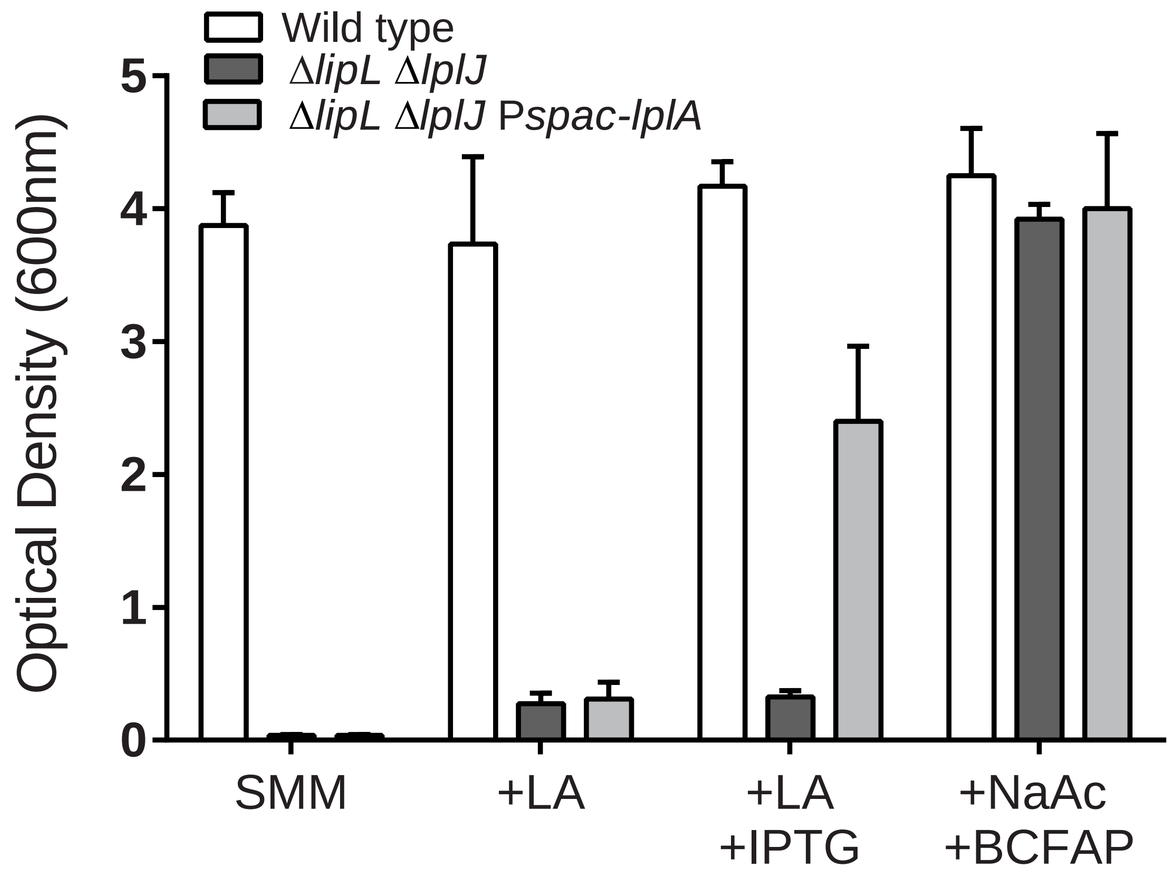
B



C

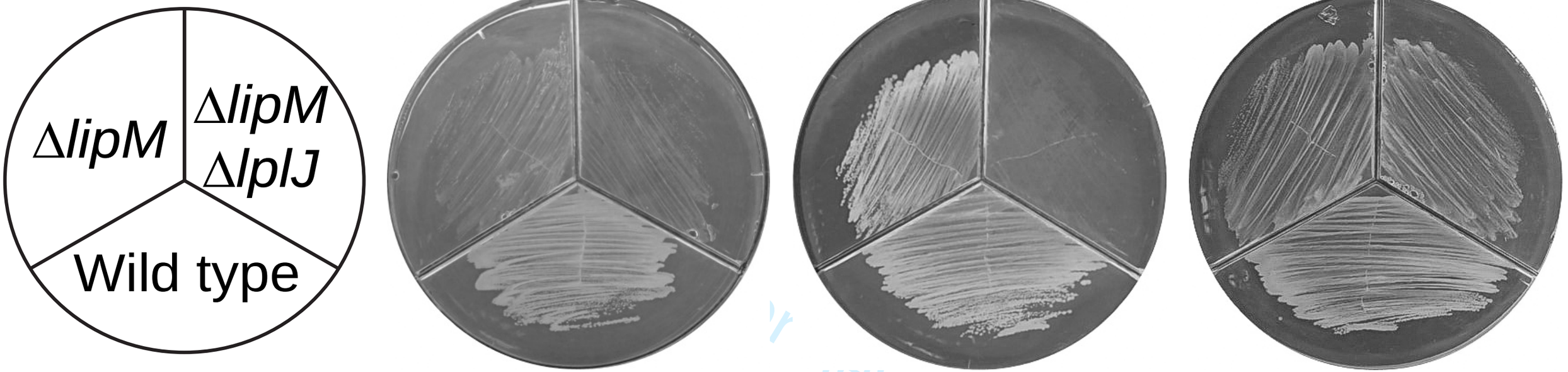


D



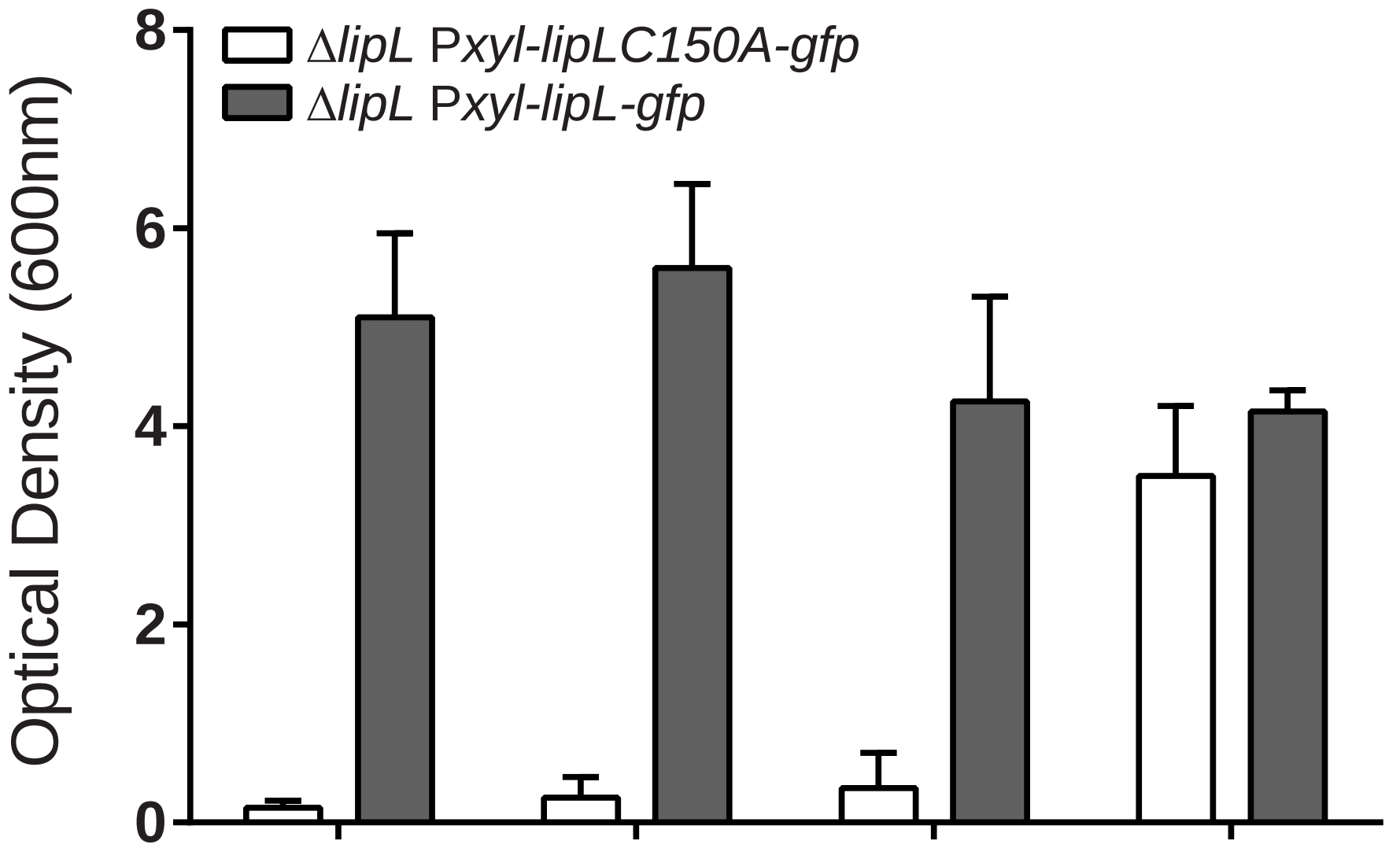
A

SMM + Octanoic acid + NaAc + BCFAP

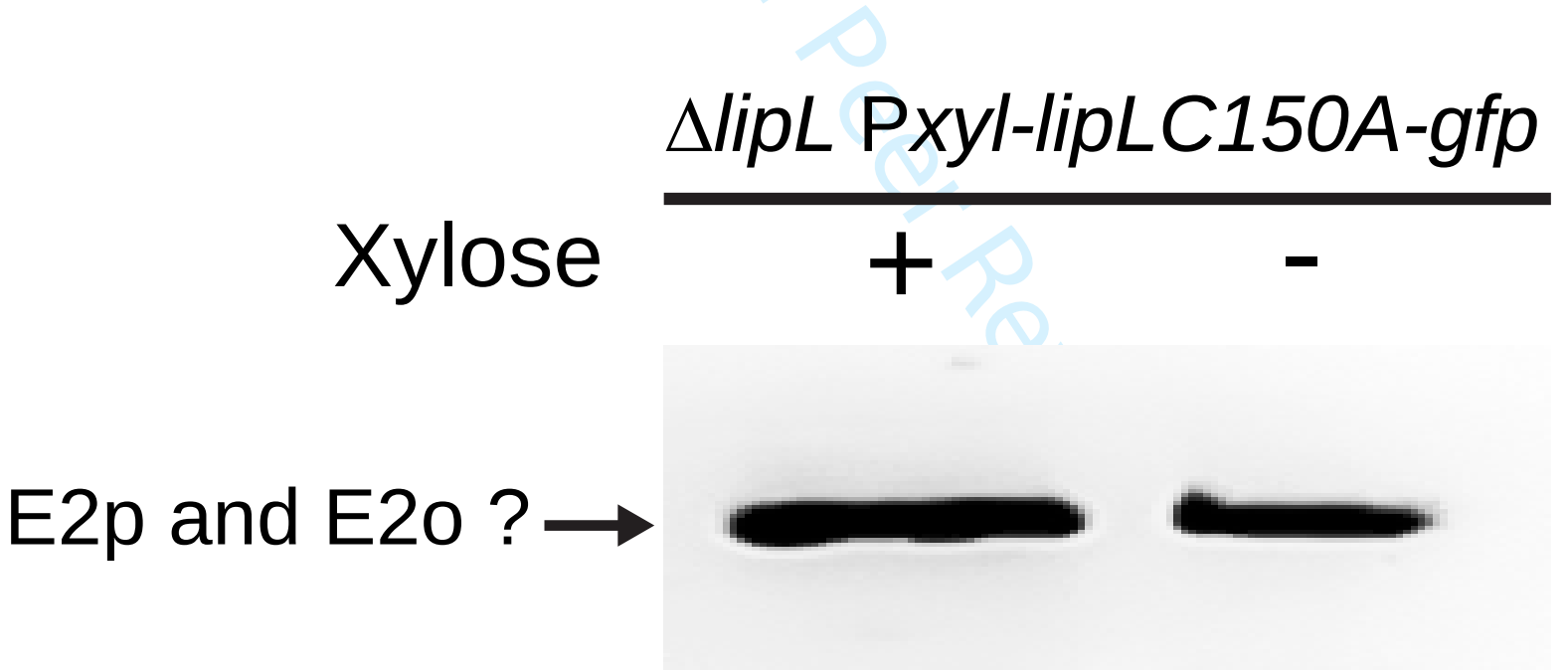


B

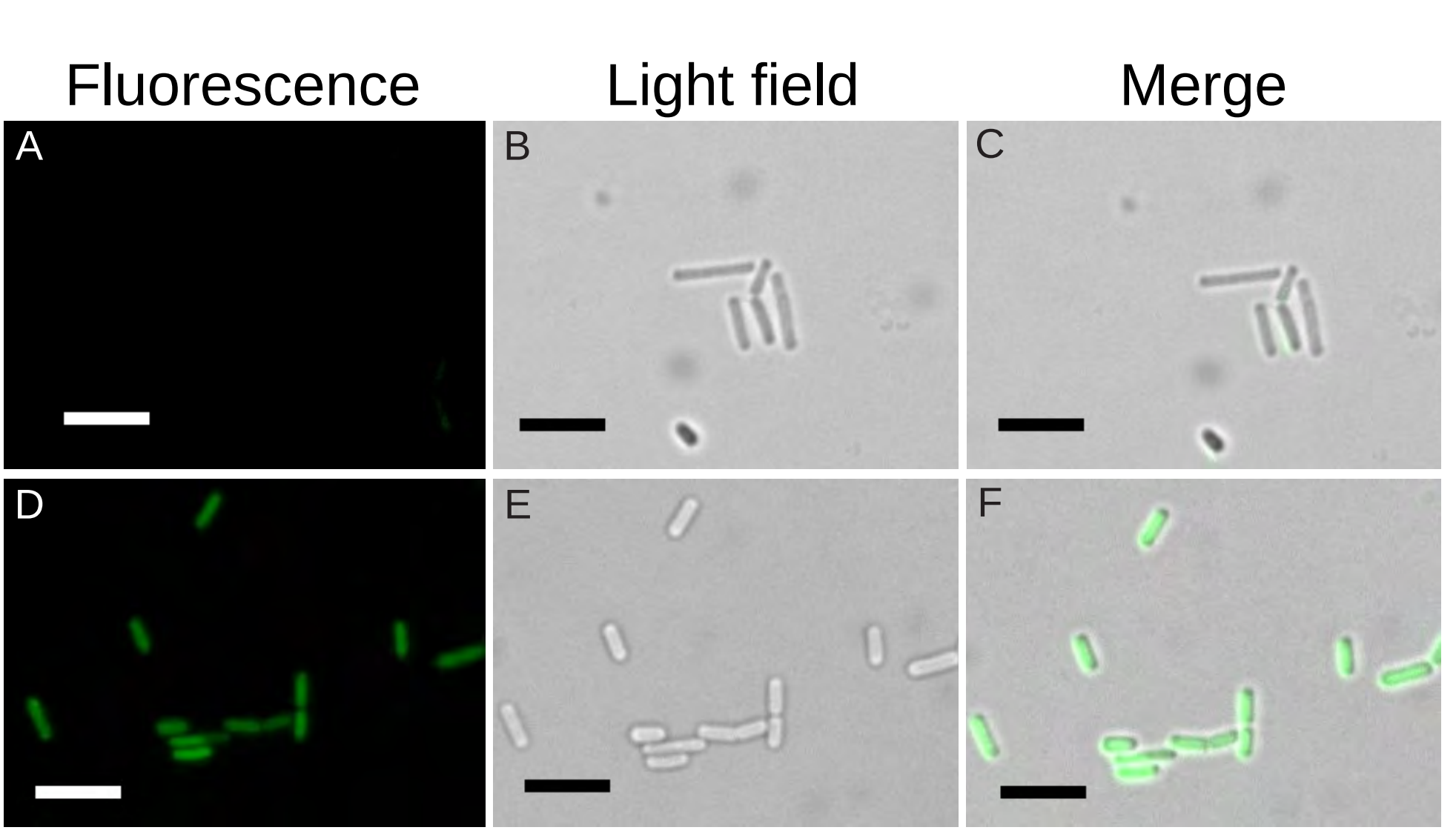
A


ΔlipL Pxyl-lipLC150A-gfp
ΔlipL Pxyl-lipL-gfp
Optical Density (600nm)
0
2
4
6
8
SMM
+ LA
+ BCFAP
+ NaAc
+ BCFAP

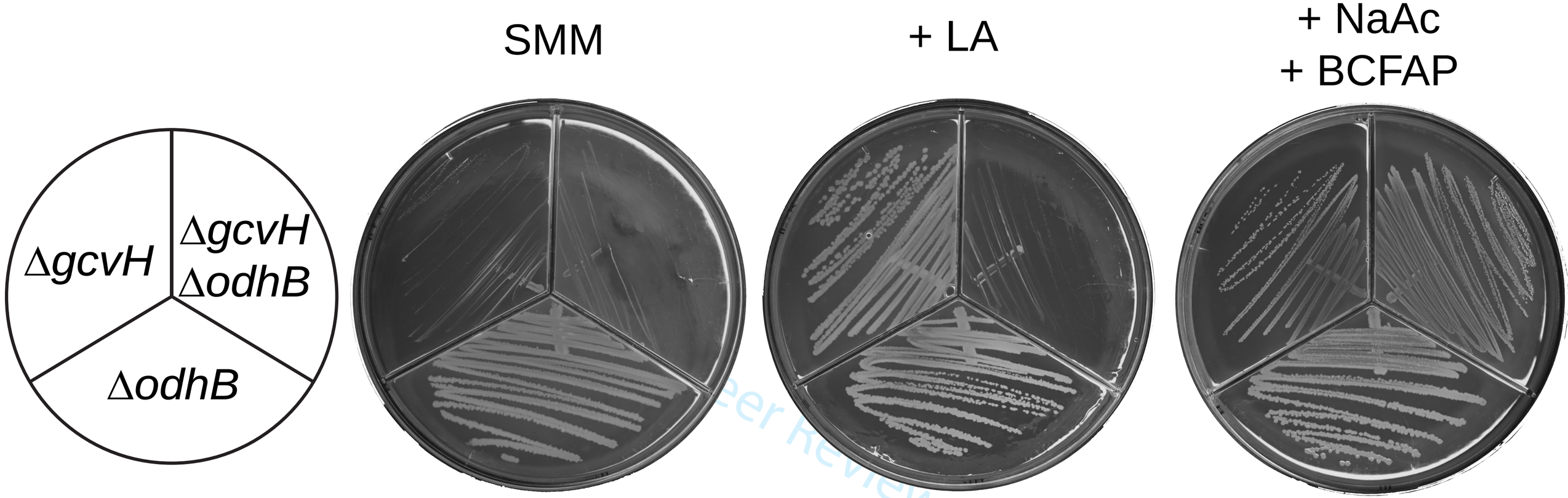
B


ΔlipL Pxyl-lipLC150A-gfp
Xylose
+
-
E2p and E2o ?

C


Fluorescence
Light field
Merge
Xylose -
Xylose +
A
B
C
D
E
F

A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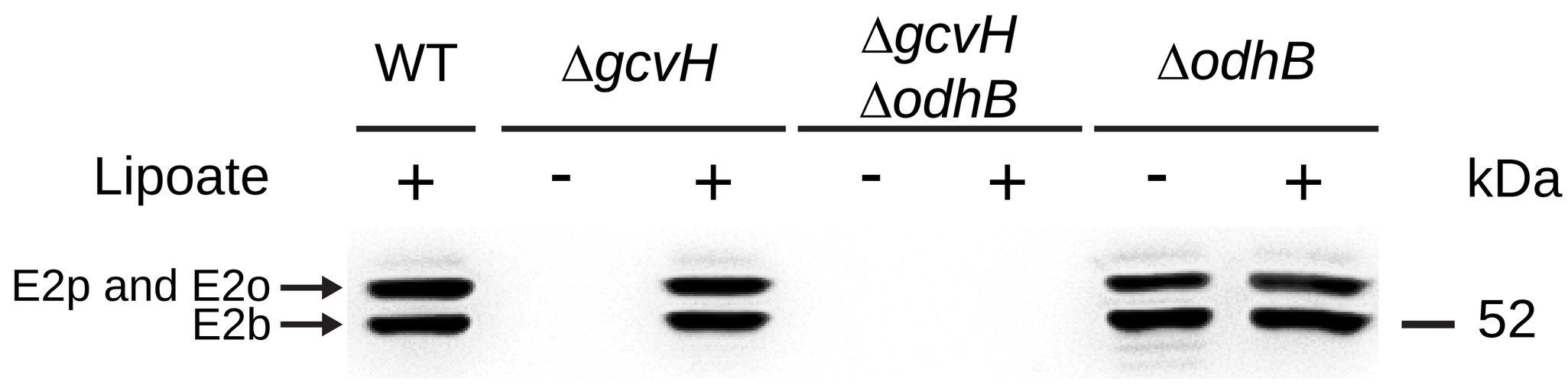
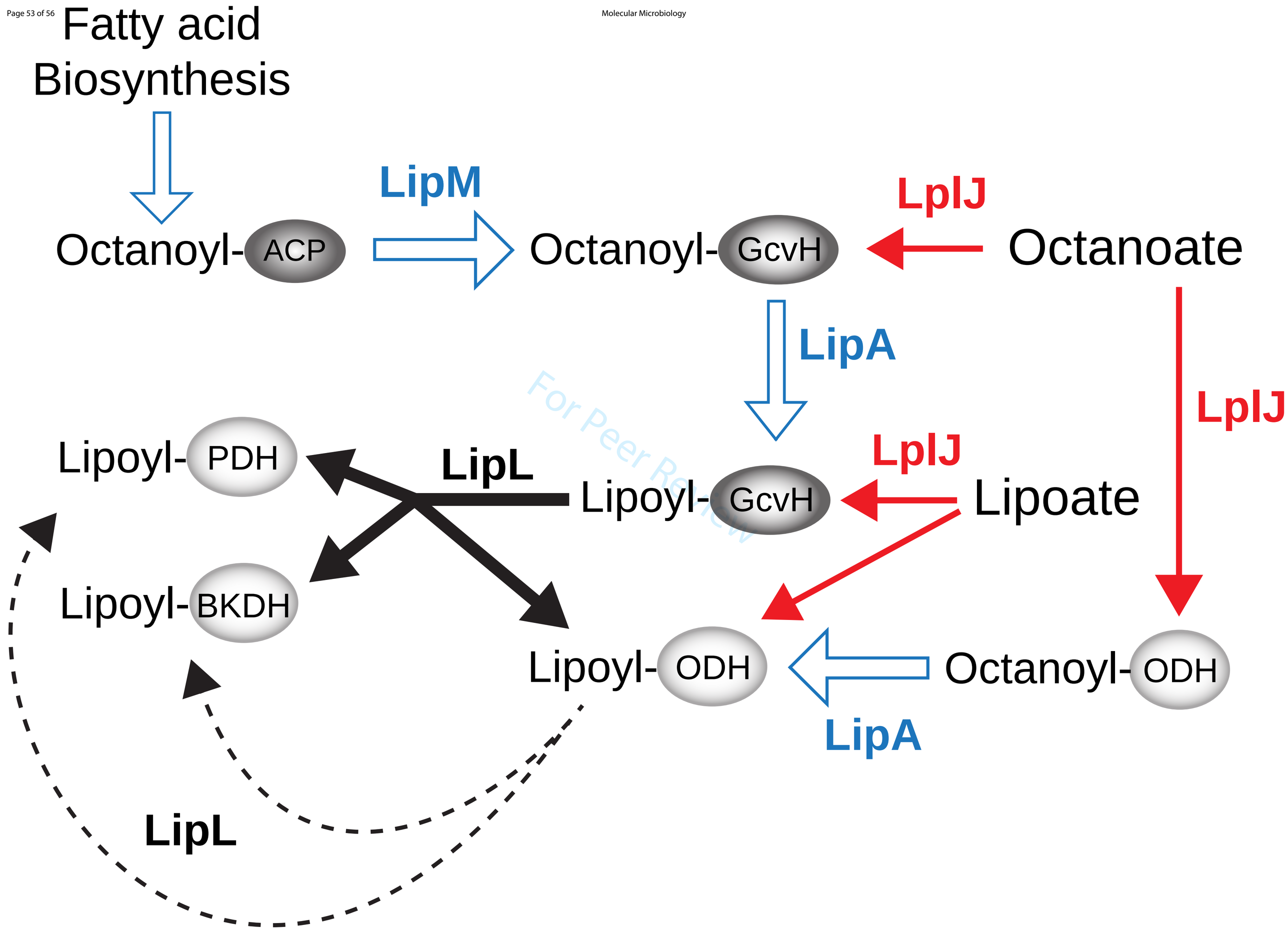
+ LA
+ NaAc
+ BCFAP
ΔgcvH
ΔgcvH
ΔodhB
ΔodhB

B

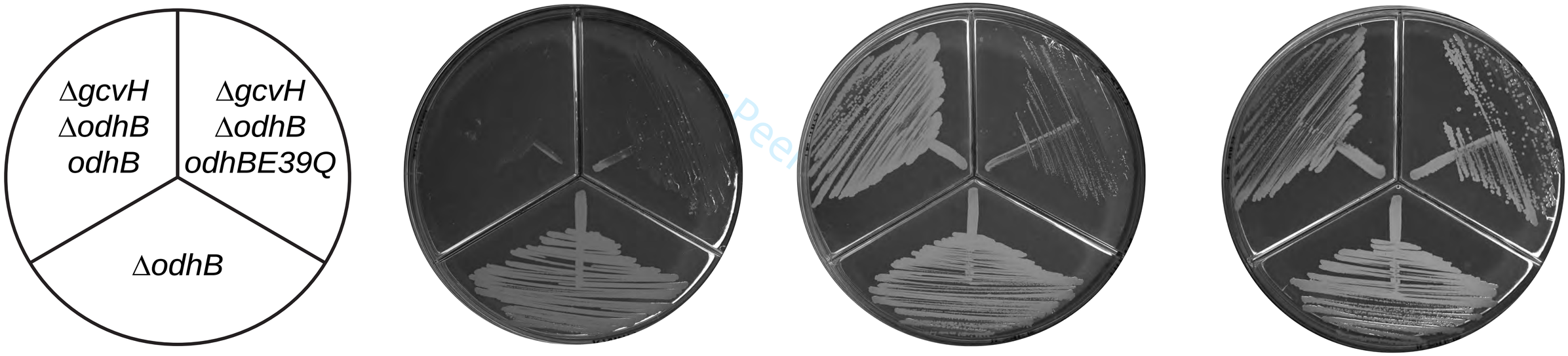

WT
ΔgcvH
ΔgcvH
ΔodhB
ΔodhB
Lipoate
+
-
+
-
+
-
+
kDa
E2p and E2o
E2b
52



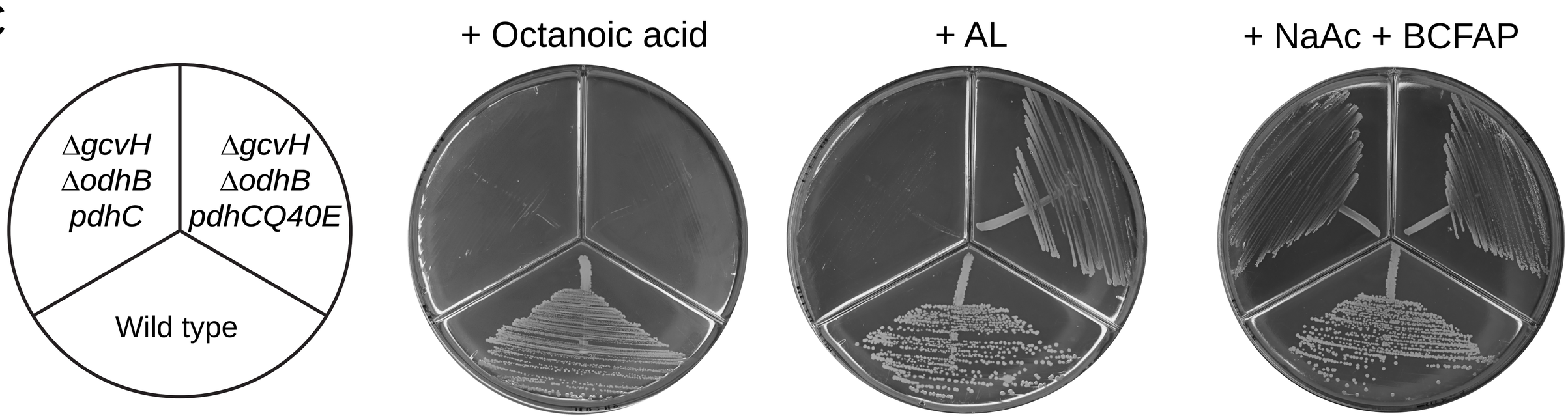
A

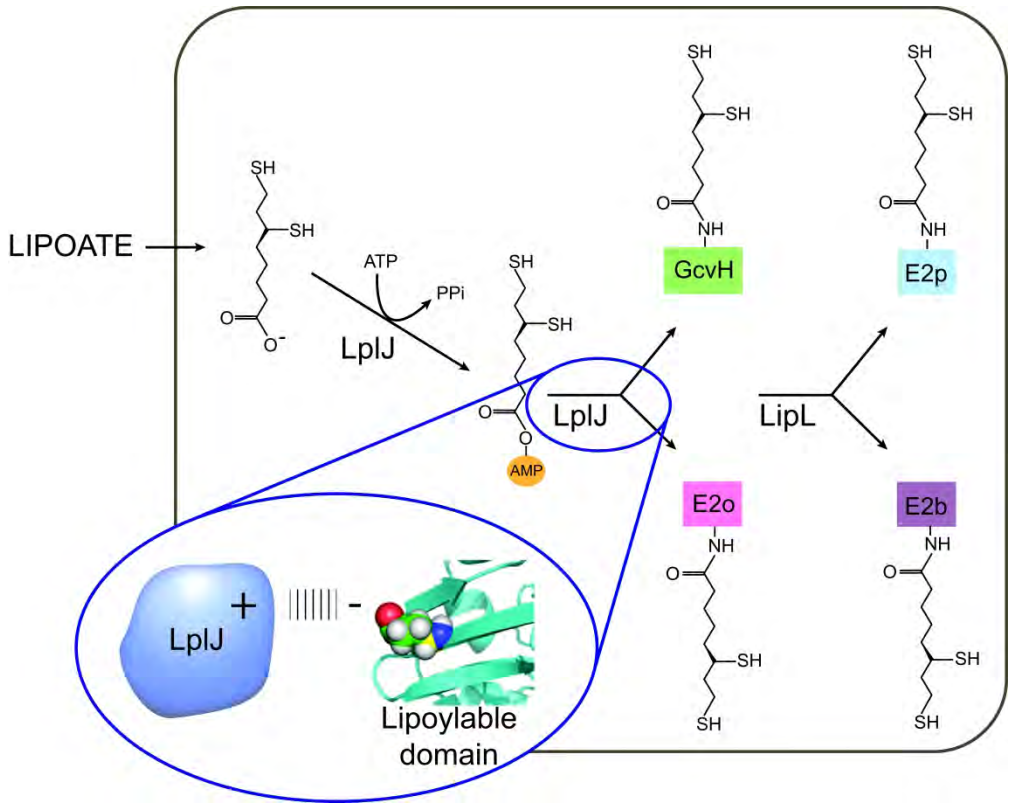
```
BsGcvH     22 KA--RIGITHFAQSELGDIVFVELPEVGAEIKADEPFGSVESVKTVSELYAPINGTVVEV
EcGcvH     23 GTY-TVGITEHAQELLGDMVFVDLPEVGATVSAGDDCAVAESVKAASDIYAPVSGEIVAV
EcODH       1 MSSVDILVPDLPESVADATVATWHKKPGDAVVRDEVLVEIETDKVVLEVPASADGILDAV
EcPDH       1 MAI-EIKVPDIGADEV--EITEILVKVGDKVEAEQSLITVEGDKASMEVPSPQAGIVKEI
BsODH       1 MA--EIKVPELAESISEGTIAQWLKQPGDYVEQGEYLLELETDKVNVELTAEESGVLQEV
BsPDH       1 MAF-EFKLPDIGEGIHEGEIVKWFVKPNDEVDEDDVLAEVQNDKAVVEIPSPVKGKVLEL
BsBKDH      1 MAIEQMTMPQLGESVTEGTISKWLVAPGDKVNKYDPIAEVMTDKVNAEVPSSFTGTITEL
```

B



C





The amidotransferase LipL, previously believed to be involved only in lipoate biosynthesis, plays a crucial role during scavenging of exogenously provided cofactor. Due to the substrate specificity of the ligase LplJ, determined by charge complementarity with the lipoylable subunits, only oxoglutarate dehydrogenase complex E2 and the H protein of the glycine cleavage system are modified. Both lipoylated proteins participate in the lipoyl-relay pathway of lipoate scavenging, that enables modification of the remaining E2 subunits.

582x463mm (600 x 600 DPI)

ABBREVIATED SUMMARY

The amidotransferase LipL, previously believed to be involved only in lipoate biosynthesis, plays a crucial role during scavenging of exogenously provided cofactor. Due to the substrate specificity of the ligase LplJ, determined by charge complementarity with the lipoylable subunits, only oxoglutarate dehydrogenase complex E2 and the H protein of the glycine cleavage system are modified. Both lipoylated proteins participate in the lipoyl-relay pathway of lipoate scavenging that enables modification of the remaining E2 subunits.