Received: 28 June 2021 | Revised: 25 April 2022 | Accepted: 25 April 2022

DOI: 10.1111/mmi.14913

RESEARCH ARTICLE

WILEY

Functional characterization of the first lipoyl-relay pathway from a parasitic protozoan

Albertina Scattolini[1,2] | Antonela Lavatelli[1,3] | Paola Vacchina[1] | Daniel A. Lambruschi[1] | María C. Mansilla[1,2] | Antonio D. Uttaro[1,2]

[1]Instituto de Biología Molecular y Celular de Rosario (IBR), Consejo Nacional de Investigaciones Científicas y Técnicas, Rosario, Argentina

[2]Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

[3]Centre for Research in Agricultural Genomics (CRAG), Consejo Superior de Investigaciones Científicas, Barcelona, Spain

Correspondence
María C. Mansilla and Antonio D. Uttaro, Instituto de Biología Molecular y Celular de Rosario (IBR), Consejo Nacional de Investigaciones Científicas y Técnicas, Rosario, Santa Fe 2000, Argentina.
Email: mansilla@ibr-conicet.gov.ar and uttaro@ibr-conicet.gov.ar

Funding information
Consejo Nacional de Investigaciones Científicas y Técnicas, Grant/Award Number: P-UE-00392016; Fondo para la Investigación Científica y Tecnológica, Grant/Award Number: PICT 2016-1853; Ministerio de Ciencia, Tecnología e Innovación Productiva, Grant/Award Number: EULACH 16/T02-0161

Abstract

Lipoic acid (LA) is a sulfur-containing cofactor covalently attached to key enzymes of central metabolism in prokaryotes and eukaryotes. LA can be acquired by scavenging, mediated by a lipoate ligase, or de novo synthesized by a pathway requiring an octanoyltransferase and a lipoate synthase. A more complex pathway, referred to as "lipoyl-relay", requires two additional proteins, GcvH, the glycine cleavage system H subunit, and an amidotransferase. This route was described so far in *Bacillus subtilis* and related Gram-positive bacteria, *Saccharomyces cerevisiae*, *Homo sapiens*, and *Caenorhabditis elegans*. Using collections of *S. cerevisiae* and *B. subtilis* mutants, defective in LA metabolism, we gathered evidence that allows us to propose for the first time that lipoyl-relay pathways are also present in parasitic protozoa. By a reverse genetic approach, we assigned octanoyltransferase and amidotransferase activity to the products of Tb927.11.9390 (*TblipT*) and Tb927.8.630 (*TblipL*) genes of *Trypanosoma brucei*, respectively. The *B. subtilis* model allowed us to identify the parasite amidotransferase as the target of lipoate analogs like 8-bromo-octanoic acid, explaining the complete loss of protein lipoylation and growth impairment caused by this compound in *T. cruzi*. This model could be instrumental for the screening of selective and more efficient chemotherapies against trypanosomiases.

KEYWORDS
amidotransferase, chemotherapy, lipoic acid, trypanosomatids

1 | INTRODUCTION

Lipoic acid (6,8-dithiooctanoic acid; LA) is the cofactor of several enzyme complexes, which are involved in key metabolic pathways in prokaryotic and eukaryotic cells, like: pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (ODH), branched-chain 2-oxoacid dehydrogenase (BCDH), the glycine cleavage system (GCS), acetoin dehydrogenase and 2-oxoadipate dehydrogenase (Danhauser et al., 2012; Douce et al., 2001; Perham, 2000; Spalding & Prigge, 2010). LA is covalently attached to the N^6 amino group of a lysyl residue in the lipoyl domains located in the E2 subunits of dehydrogenases and H protein of GCS (GcvH).

LA is synthesized using octanoic acid as a precursor. *Escherichia coli* presents the simplest biosynthetic pathway, in which GcvH or E2 subunits are acylated by an octanoyltransferase (OT, LipB), using octanoyl-ACP as substrate (Figure 1). Subsequently, the lipoate synthase LipA introduces two sulfur atoms at carbons 6 and 8 of the resulting octanoamide moiety (Cronan, 2016). A more complex pathway was found in *Bacillus subtilis*. In this Gram-positive bacterium, the OT LipM octanoylates only GcvH, then LipA inserts the sulfur atoms, and an additional enzyme with amidotransferase activity, LipL, transfers lipoyl moieties from GcvH to E2 subunits

Albertina Scattolini and Antonela Lavatelli considered joint first author.

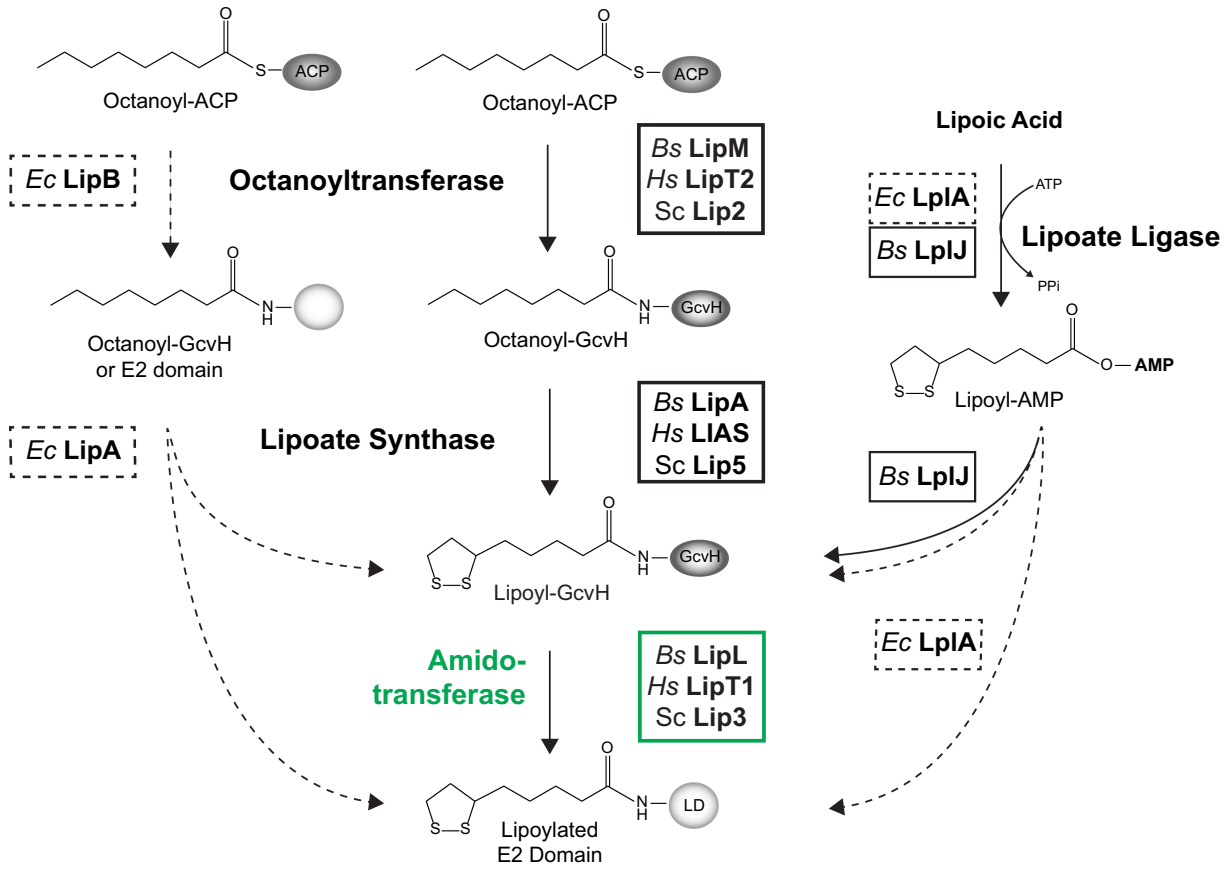

FIGURE 1 Known enzymes involved in protein lipoylation. *Escherichia coli* octanoyltransferase LipB and lipoate ligase, LplA, can use GcvH and all the E2 lipoyl domains as substrates (classic pathway, striped rectangles). In contrast, organisms that possess the lipoyl-relay pathway require the activity of an amidotransferase to modify E2 subunits. *Bacillus subtilis* lipoate ligase can transfer the activated lipoyl moiety also to E2o. *Bs*, *B. subtilis*; *Hs*, *H. sapiens*; *Sc*, *S. cerevisiae*; *Ec*, *E. coli*

(Christensen, Martin, et al., 2011; Martin et al., 2011) (Figure 1). This biosynthetic route involving an amidotransferase was called the lipoyl-relay pathway (Cao, Hong, et al., 2018) and was also identified in important Gram-positive pathogens, like *Staphylococcus aureus* (Zorzoli et al., 2016).

Some organisms acquire LA by a salvage pathway, requiring the action of lipoate-protein ligases, like LplA in *E. coli* or LplJ in *B. subtilis*. These ligases activate and transfer lipoyl moieties to GcvH and E2 subunits (Cronan, 2016). However, the *B. subtilis* ligase is able to transfer the lipoyl moiety only to GcvH and E2o (the lipoylable subunit of ODH). Modification with exogenous LA of E2p and E2b, the corresponding subunits of PDH and BCDH, requires a lipoyl-relay involving the amidotransferase activity of LipL (Rasetto et al., 2019) (Figure 1). A similar pathway of LA utilization was described in *S. aureus* and *Listeria monocytogenes* (Christensen, Hagar, et al., 2011; Laczkovich et al., 2018).

Saccharomyces cerevisiae Lip2, Lip3, and Lip5 are functional orthologues of LipM, LipL, and LipA, respectively. Mutations in each of these genes render yeasts unable to grow on non-fermentable carbon sources such as glycerol (Schonauer et al., 2009). This growth defect cannot be bypassed by supplementing the media with LA, indicating that *S. cerevisiae* lacks the salvage pathway. It was previously proposed that Lip2 is an OT specific for GcvH, using octanoyl-ACP as substrate, whereas Lip3 acylates E2 subunits but using octanoyl-CoA (Hermes & Cronan, 2013). The evidence for such conclusions came partially from in vitro experiments where the LA synthesis was reconstituted by purified enzymes and putative substrates. New experiments, where yeast mutants deficient in LA metabolism were complemented with *Homo sapiens* genes, indicate that Lip3 has amidotransferase activity, indicating that *S. cerevisiae* has also a lipoyl-relay pathway (Pietikäinen et al., 2021). The reinterpretation of old experimental results and the physiology of individuals undergoing metabolic diseases associated with defects in enzymes of LA metabolism indicate that humans lack LA salvage but have amidotransferase activity. Biochemical assays directly demonstrate that human LIPT1 functions as an amidotransferase, instead of lipoate ligase as proposed earlier, and LIPT2 has OT activity only toward GcvH (Cao, Zhu, et al., 2018). A similar LA biosynthetic route was found in the nematode *Caenorhabditis elegans* (Lavatelli et al., 2020).

Trypanosomatids like *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania* spp. are flagellated protists responsible for several neglected diseases, with elevated morbidity and mortality if not treated. The repertoire of available treatments is limited and most of the drugs used are toxic and, in some cases,

ineffective, rendering urgent development of new chemotherapies (Bhattacharya et al., 2020). These parasites have digenetic life cycles, that alternate between a mammalian host and an insect vector where they encounter notably diverse nutrients, so they need to adapt their metabolic network accordingly (Michels et al., 2021). Due to the involvement of LA in the relevant metabolic pathways described above, disruption of LA biosynthesis could represent a novel strategy in order to develop new chemotherapies against trypanosomiases. We have recently shown that *T. cruzi* was susceptible to treatment with LA analogs like 8-bromo-octanoic acid (BrO), and particularly its methyl ester derivative (mBrO), validating the pathway as a good target for intervention. These analogs interfered with the lipoylation of trypanosome E2 subunits, inhibiting PDH, BCDH, and ODH activities and cell growth. The addition of LA did not bypass such inhibition. *T. cruzi* was shown to incorporate very inefficiently small monocarboxylic acids like LA, BrO, or octanoic acid (Vacchina et al., 2018), suggesting the lack of the salvage pathway. Indirect experimental evidence indicated the absence of salvage also in *T. brucei* (Stephens et al., 2007), ruling out the existence of LA ligases in trypanosomatids.

The trypanosomatid genomes show the presence of genes encoding all subunits of PDH (E1p, E3Bp, E2p), ODH (E1o, E2o), BCDH (E1b, E2b), and GCS (P, H, and T proteins), and a sole dihydrolipoamide dehydrogenase (DHLDH), involved in re-oxidation of dihydrolipoamide to lipoamide. DHLDH is probably shared by the four complexes (Spalding & Prigge, 2010). Interference of DHLDH expression had a strong proliferation defect in *T. brucei* followed by rapid cell death (Roldán et al., 2011). However, no data about the effect of inhibition on LA synthesis is available. Although LA analogs seem to interfere with protein lipoylation (Vacchina et al., 2018) we cannot anticipate which step is inhibited. A survey of trypanosomatid genomes also revealed the presence of enzymes putatively involved in LA synthesis and utilization (Spalding & Prigge, 2010), including orthologues of lipoate synthases, OT, amidotransferases and/or lipoate ligases. In order to shed light on LA metabolism in trypanosomatids and gain insight for future development of chemotherapeutic strategies, here we show a series of experiments that support the presence of a lipoyl-relay pathway in *T. brucei*. This was achieved in vivo, by complementing mutants in key LA metabolic enzymes of both *S. cerevisiae* and *B. subtilis* as model organisms. The bacterial model allowed us to identify the amidotransferase as the target of LA analogs, which could be instrumental in the screening of drugs against these parasites.

2 | RESULTS

2.1 | *T. brucei* encodes putative enzymes involved in lipoate synthesis and utilization

T. brucei has a sole gene encoding a putative OT (locus Tb927.11.9390, protein accession number EAN79891) (Spalding & Prigge, 2010); we named it TbLipT. The protein shares 68 and 56% identity with *T. cruzi* and *Leishmania major* orthologues, respectively. TbLipT also shares a significant identity with functionally characterized OT from *E. coli* (LipB, 27.6%), *Mycobacterium tuberculosis* (LipB, 30%), and *S. cerevisiae* (Lip2, 23%) (Figure S1). Phylogenetic analysis revealed that the trypanosomatid enzymes are related to OT from plants, like *Arabidopsis thaliana* LIP2, but are more distant from *B. subtilis* LipM (less than 13% identity), which was used as an outgroup root in our phylogenetic tree (Figure S2).

The gene product of Tb927.8.630 (AAZ12834) encodes a protein of 512 amino acid residues. Structural modeling suggests that its N-terminal domain (residues 1–290) belongs to the cofactor transferase family, Pfam03099, composed of lipoate and biotinyl ligases, octanoyl, and amidotransferases. It was annotated as a putative lipoate ligase (TbLipL) since the encoded protein has 29.5% and 23.6% identity to *E. coli* and *B. subtilis* ligases, respectively, although there is no significant similarity between the C-terminal domain of TbLipL and the lipoate-activating domains of lipoate ligases (underlined in Figure S3). Experimental evidence suggests that trypanosomatids are unable to utilize LA (Stephens et al., 2007; Vacchina et al., 2018). In addition, TbLipL also shows 19.5% and 22.6% identity to amidotransferases of *B. subtilis* and *S. cerevisiae*, respectively (Figure S4), so the true activity of this protein should be confirmed empirically.

The polypeptide encoded by Tb927.10.15010 (EAN78865) was annotated as a putative lipoate synthase (TbLipA). Lipoate synthases are highly conserved proteins belonging to the "radical SAM enzymes" superfamily. They have two [4Fe-4S] clusters and the characteristic CX_3CX_2C motif (Cronan, 2016). TbLipA shares 78% identity (88% similarity) with trypanosomatid orthologues and 54%, 50%, and 48% identity with synthases from eukaryotic organisms as diverse as mammals, yeasts, and plants, respectively. Due to such considerable conservation, we assumed it to be the *T. brucei* lipoate synthase and then focused our attention on TbLipT and TbLipL for further characterization, as this information would allow us to decipher the pathway present in trypanosomatids.

2.2 | *T. brucei* LipT rescued the octanoyltransfer activity of a yeast *lip2*-null mutant

We tested if TbLipT could functionally replace the enzymes involved in octanoyl or lipoyl transfer in yeasts. *TblipT* was expressed in *lip2*- and *lip3*-null mutants of *S. cerevisiae*, which lack, respectively, octanoyl-ACP:GcvH octanoyltransferase and amidotransferase. These mutant cells are unable to grow on non-fermentable carbon sources, due to the essentiality of functional PDH and ODH for respiratory growth, but can grow using a fermentable carbon source, such as glucose, bypassing the lack of lipoylated proteins (Hermes & Cronan, 2013). Besides, Δ*lip2* yeasts are unable to use glycine as a nitrogen source, since their GCS cannot produce the cleavage of this amino acid to release NH_3, due to the lack of GcvH lipoylation (Nagarajan & Storms, 1997). We found that *TblipT* was able to rescue the growth of Δ*lip2* yeasts in media containing

different non-fermentable carbon sources like glycerol and succinate (Figure 2a). This means that the complemented yeast strains had active PDH and ODH, rendering a functional TCA cycle. The ability of Δ*lip2/lipT* cells to grow on glycine as a nitrogen source (Figure 2b) was evidence of an active GCS, indicating lipoylation of GcvH. These results indicate that TbLipT is most likely an OT that requires octanoyl-ACP and GcvH as substrates, although we cannot rule out that the E2 subunits could also be acylated by this protein, as occurs with the OT of the classical pathways of protein lipoylation (i.e., *E. coli* LipB). If this is the case, expression of TbLipT would also restore the growth of Δ*lip3* cells on non-fermentable carbon sources. As shown in Figure 2a, the expression of *T. brucei* LipT did not rescue the growth of the Δ*lip3* cells on glycerol, which requires the activity of both ODH and PDH for energy production. The growth of these cells was impaired also in media supplemented with succinate that would bypass the need for functional ODH (Figure 2a). Similar results were obtained when ethanol was added to the growth medium, a compound that would be metabolized to acetate, bypassing PDH activity (Figure S5). Consequently, we can conclude that TbLipT is not transferring the octanoyl moiety to any of the E2 subunits of *S. cerevisiae*.

A Western blot analysis of Δ*lip2* mutants complemented with *TblipT*, using an anti-lipoate antibody, detected both lipoyl-E2 subunits in agreement with the presence of functional PDH and ODH (note that *S. cerevisiae* lacks BCDH) (Figures 2c and S6). Restoration of protein lipoylation can be explained by the combined action of TbLipT, which octanoylates GcvH; ScLip5, which introduces the sulfur atoms producing lipoyl-GcvH; and ScLip3 which transfers the lipoyl moiety to E2p and E2o.

2.3 | TbLipT functionally complements a *B. subtilis* mutant deficient in OT activity

We wondered if TbLipT could replace the activity of the phylogenetically distant OT LipM since the *B. subtilis* model would be a very useful tool for our research. We have a series of characterized single and double mutants in *B. subtilis* genes involved in lipoate metabolism that would help to gain deep insight into TbLipT activity.

B. subtilis strains deficient in either the OT (Δ*lipM*) or amidotransferase (Δ*lipL*) are unable to grow in a minimal medium unless they are provided with acetate and branched-chain fatty acid precursors (BCFAP) that allow them to bypass the lack of PDH and BCDH activity, respectively. The addition of succinate, to circumvent the lack of ODH activity, is not required to complement the growth of lipoate auxotrophs in a minimal medium (Martin et al., 2009) since this bacterium is able to produce enough succinate via the reductive tricarboxylic acid cycle when provided with a good carbon source. When TbLipT expression was induced it functionally complemented a Δ*lipM* strain, enabling its growth in a minimal medium (Figure 3a), indicating that both E2p and E2b are being lipoylated. Modification of these apoproteins was indeed confirmed by a Western blot assay (Figures 3b and S7). Besides, expression of the parasite LipT allowed Δ*lipM* cells to use glycine as a nitrogen source, indicating that the GCS was active, meaning that GcvH is also a good substrate for TbLipT (Figure 3a). Growth with glutamate as the sole nitrogen source is GCS-independent and, therefore, it was used as a positive growth control.

To assess if TbLipT was modifying GcvH and then the bacterial amidotransferase was in charge of transferring the lipoyl moiety to

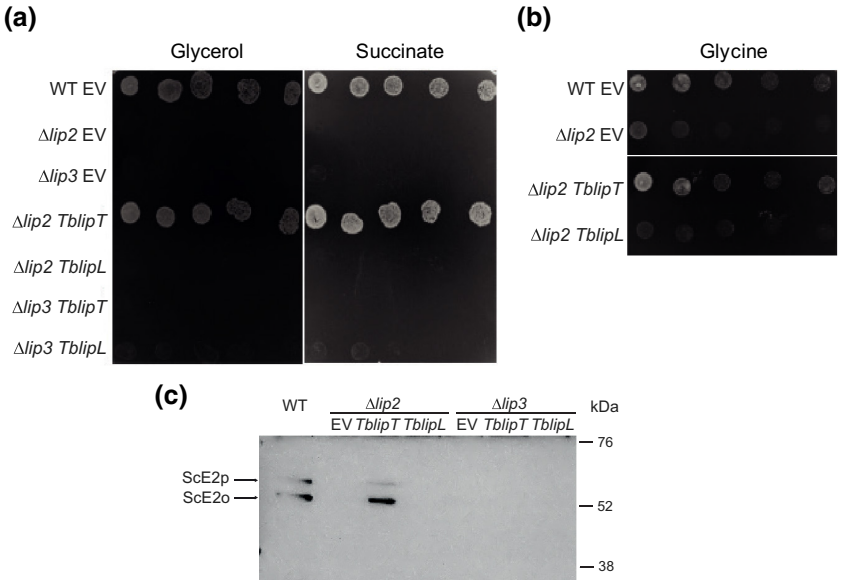

FIGURE 2 Expression of TbLipT and TbLipL in *Saccharomyces cerevisiae*. (a) Growth of yeast wild type strain (BY4741, WT), Δ*lip2* and Δ*lip3* null mutants transformed with plasmids p426GPD (EV), pTbLipT, or pTbLipL in complex medium supplemented with different carbon sources. Cells were grown overnight, and upon standardization by OD_{600}, 1:2 dilutions were spotted in YP agar plates supplemented with 3% glycerol or 3% succinate, as indicated. (b) Growth of yeast WT or Δ*lip2* mutant transformed with plasmids p426GPD (EV), pTbLipT, or pTbLipL in minimal medium supplemented with glycine as nitrogen source. (c) Western blot analysis of protein extracts from yeast cells grown in YP medium supplemented with glucose, using antibodies against LA

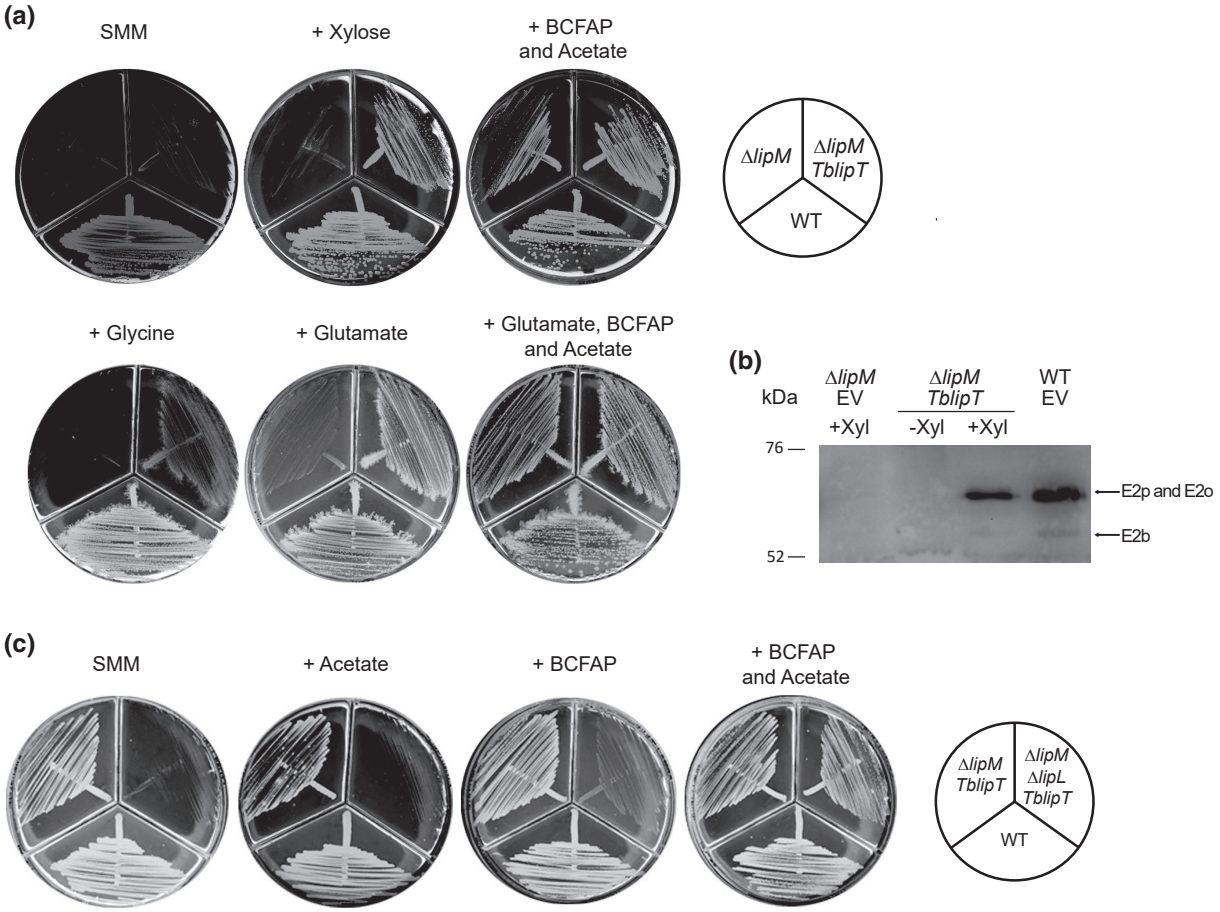

FIGURE 3 Expression of TbLipT functionally complements a *Bacillus subtilis* Δ*lipM* strain. (a) Growth of *B. subtilis* WT (JH642) or Δ*lipM* (NM57) strains transformed with either the empty vector (EV) or pAL31, a plasmid with the coding sequence for TbLipT under the control of a xylose-inducible promoter. Strains were streaked in Spizizen minimal medium (SMM) (upper panel) or modified minimal medium without ammonium source and 0.8% xylose (lower panel) containing the supplements indicated above and incubated for 48 h at 37°C. BCFAP: branched-chain fatty acid precursors. (b) Protein extracts from WT or Δ*lipM* strains transformed with either the EV or pAL31, grown in SMM supplemented with acetate and BCFAP, with or without xylose, were analyzed by Western blot with antibodies against LA. (c) Growth of *B. subtilis* WT, Δ*lipM*, or Δ*lipM* Δ*lipL* (CM28) strains transformed with either the EV or pAL31. Strains were streaked in SMM containing 0.8% xylose and the supplements indicated above. Plates were incubated for 48 h at 37°C

E2p and E2b, or if TbLipT was able to recognize all *B. subtilis* E2s as substrates, as occurs with *E. coli* LipB (Martin et al., 2011), the parasite protein was expressed in a Δ*lipM* Δ*lipL* strain. In this case, TbLipT did not restore the growth of bacterial cells in a minimal medium, even when provided with acetate or BCFAP separately (Figure 3c). This result indicates that neither E2p nor E2b are TbLipT substrates, as we have observed with *S. cerevisiae* E2s.

2.4 | Cysteine 195 and lysine 161 are critical residues for TbLipT catalysis

OT-mediated reactions proceed through an acyl-enzyme intermediate in which the octanoyl moiety forms a thioester bond with the thiol of a conserved cysteine residue (Zhao et al., 2005). It has been reported that in *E. coli* and *M. tuberculosis* such a conserved residue (C169 and C176, respectively) was essential for function in vivo (Ma et al., 2006; Zhao et al., 2005). Sequence alignments suggest that the corresponding cysteine residue of the parasite protein is C195 (Figures 4a and S1). To assess its role during catalysis, we constructed a mutant version of the parasite OT in which C195 was replaced with a serine residue (TbLipT-C195S). We could effectively demonstrate its essentiality, as the mutated version of the enzyme was unable to restore the growth of a *B. subtilis* Δ*lipM* strain in the minimal medium, even after 96 h of cultivation (Figure 4b). This behavior is coincident with the absence of protein lipoylation in cell extracts of this mutant expressing the TbLipT-C195S allele (Figures 4c and S8).

Members of the cofactor transferase family (Pfam03099) have a strictly conserved lysine. Its essentiality during *M. tuberculosis* OT catalysis has been shown, since the K142S mutant protein was inactive, both in vivo and in vitro (Ma et al., 2006). The change of the corresponding residue to serine in *T. brucei* OT only weakened its catalytic activity, since after prolonged incubation the K161S mutant protein allowed the growth of *B. subtilis* Δ*lipM* cells in a minimal medium (Figure 4b). However, although E2 lipoylation was restored, the observed bands were faint (Figure 4c), in concordance with the

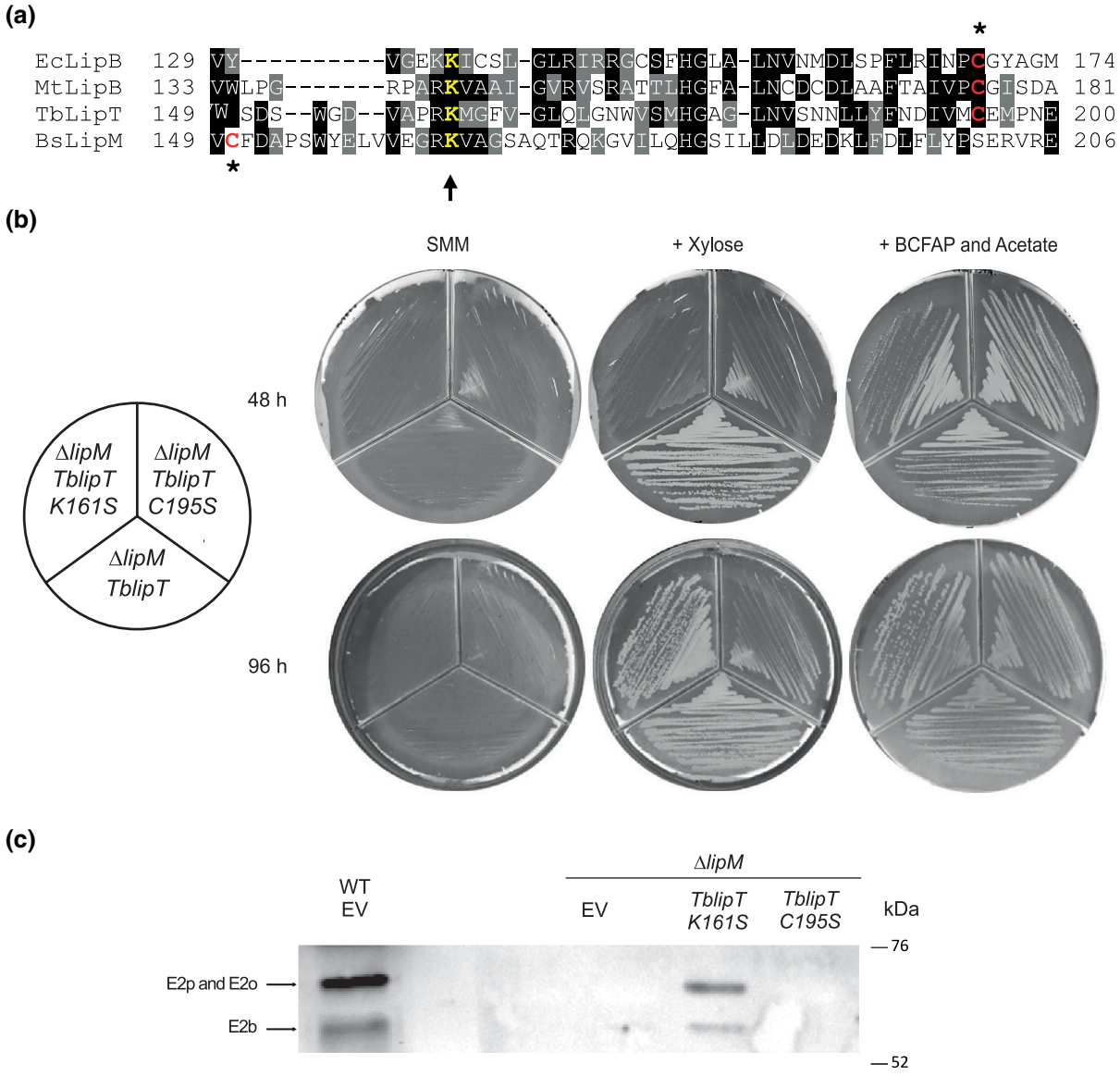

FIGURE 4 C195 and K161 are involved in TbLipT activity. (a) Sequence alignment of TbLipT and bacterial octanoyltransferases. Identical residues are shown highlighted in black and similar residues are highlighted in gray. The conserved lysine residues are indicated by an arrow. (*) Cysteine residues involved in catalytic activity. Ec: *E. coli*; Bs: *B. subtilis*; Mt: *M. tuberculosis*; Tb: *T. brucei*. (b) Growth of *B. subtilis* Δ*lipM* (NM57) transformed with plasmids that allow expression of either wild type TbLipT (pAL31) or mutated versions of this protein under the control of a xylose-inducible promoter. Strains were streaked in Spizizen minimal medium (SMM) and the supplements indicated above. Plates were incubated for 48 or 96 h at 37°C. BCFAP: branched-chain fatty acid precursors. (c) Protein extracts from NM57 cells transformed with either the EV or plasmid expressing mutant alleles of TbLipT, grown in SMM supplemented with acetate, BCFAP and xylose, were analyzed by Western blot with antibodies against LA

observed delayed growth of the complemented strain, highlighting the involvement of this residue in the biological activity of TbLipT.

2.5 | TbLipL is the *T. brucei* amidotransferase

TbLipL has been annotated as a lipoate ligase. However, there is compelling evidence that, instead, it might function as an amidotransferase. First, the apparent inability of trypanosomatids to utilize exogenously provided LA dismisses its ligase activity. Second, as TbLipT only transfers octanoate to GcvHs, both in yeast cells and *B. subtilis*, it implies that another enzyme should be necessary to modify the parasite E2s. To test this hypothesis, we expressed TbLipL in *S. cerevisiae* mutants unable to synthesize LA. The parasite protein was unable to rescue the growth of Δ*lip2* cells in media containing different non-fermentable carbon sources, indicating that it is unable to transfer octanoate to the yeast E2s (Figures 2a and S5). Besides, it was also unable to transfer octanoate to GcvH, since its expression could not restore the growth of Δ*lip2* cells in media containing glycine as the sole nitrogen source (Figure 2b).

Expression of TbLipL in a Δ*lip3* mutant did not allow growth of the cells in media supplemented with glycerol or succinate (Figure 2a). An examination of the lipoylation pattern of Δ*lip3 TblipL* cells by Western blot did not show modified proteins (Figure 2c), indicating inefficient E2 lipoylation. This could be explained by an ineffective targeting to the yeast mitochondria of the heterologous trypanosome protein and/or a poor recognition of yeast E2s by this amidotransferase. In any case, it indicates that the yeast model is not useful for the characterization of this enzyme.

Then, we performed functional complementation experiments on lipoylation-deficient *B. subtilis* cells unable to both synthesize and ligate LA (Δ*lipM* Δ*lplJ*). The expression of TbLipL in this strain did not restore its growth in SMM supplemented with LA, which argues against its ability to ligate exogenous LA to apoproteins (Figure 5a). In contrast, the growth of the *B. subtilis* Δ*lipL* mutant was restored by the expression of TbLipL (Figure 5b). Western blot analysis of extracts from complemented cells grown in the presence of the inducer xylose, using anti-LA antibodies, showed the characteristic lipoylation pattern of E2s, in contrast to the absence of lipoylated proteins in the Δ*lipL* strain (Figures 5c and S9). Together, these results strongly suggest that *T. brucei* LipL functions as an amidotransferase.

The amidotransferase of *B. subtilis*, LipL, can transfer the lipoyl moiety from lipoyl-GcvH to E2s but is also able to use lipoyl-E2o as the cofactor donor, which makes LA utilization GcvH-independent if E2o is present (Rasetto et al., 2019). To test if the parasite protein shares this property, TbLipL was expressed in a *B. subtilis* Δ*lipL* Δ*gcvH* strain (NM27). Growth of this double mutant is strictly dependent on supplementation with acetate and BCFAP since it cannot synthesize LA due to the absence of GcvH, or ligate exogenous lipoate due to the absence of LipL. Expression of TbLipL did not restore the growth of the Δ*lipL* Δ*gcvH* strain in SMM supplemented with lipoate (Figure 6a). This observation correlates with the detection of only one of the two lipoylated bands in the immunoblots, corresponding to E2o (Figures 6b and S10). These results indicate that *B. subtilis* LplJ can ligate exogenous lipoate to E2o but then TbLipL cannot transfer the lipoyl residue to E2p and E2b, showing that the parasite's amidotransferase only uses lipoyl-GcvH as the donor for the amidotransferase reaction. Furthermore, as *B. subtilis* lipoate synthase can

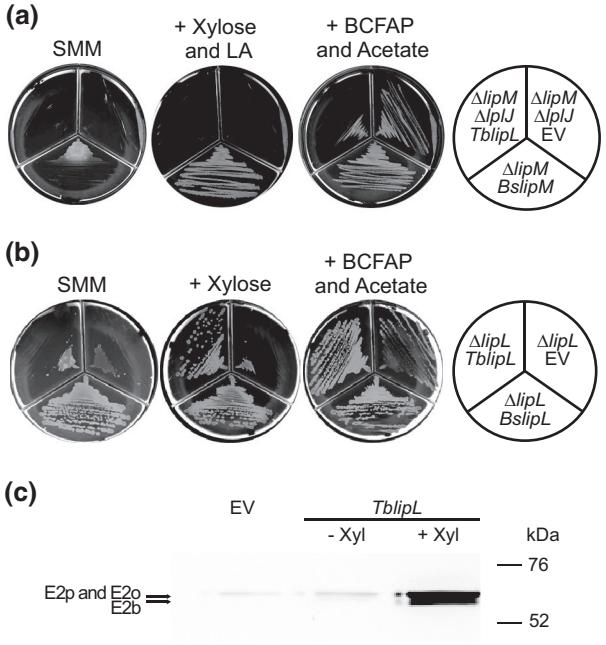

FIGURE 5 TbLipL functions as an amidotransferase. (a) Growth of *Bacillus subtilis* Δ*lipM* Δ*lplJ* (NM65) or Δ*lipM* (NM57) strains transformed with either the empty vector (EV), or a plasmid containing the coding sequence of TbLipL under the control of the xylose-inducible promoter P*xylA* (pAS4). Strain NM08 (Δ*lipM amyE*::*lipM*) was used as positive growth control. Cells were streaked in Spizizen minimal medium (SMM) containing the supplements indicated above. BCFAP: branched-chain fatty acid precursors. (b) Growth of *B. subtilis* Δ*lipL* strain (NM51) transformed with either the empty vector (EV) or plasmid pAS4. Strain NM13 (Δ*lipL amyE*::*lipL*) was used as positive growth control. Cells were streaked in SMM containing the supplements indicated above. Plates were incubated for 48 h at 37°C. (c) Protein extracts from NM51 cells transformed with either the EV or a plasmid containing TbLipL under P*xylA*, grown in SMM supplemented with BCFAP and acetate, with or without the inducerr xylose, were analyzed by Western blot with antibodies against LA

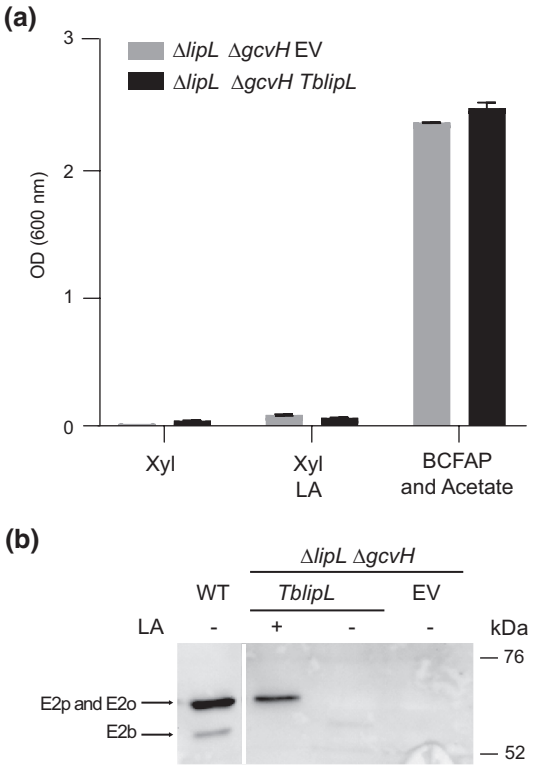

FIGURE 6 TbLipL only uses lipoyl-GcvH as the donor for the amidotransferase reaction. (a) Growth of *Bacillus subtilis* Δ*lipL* Δ*gcvH* (NM27) cells transformed with either the empty vector (EV) or pAS4, a plasmid with the coding sequence for TbLipL under the control of the xylose-inducible promoter P*xylA*. Strains were inoculated in Spizizen minimal medium (SMM) containing the indicated supplements and incubated for 15 h at 37°C. Each bar is the mean ± *SD* from three independent experiments. BCFAP: branched-chain fatty acid precursors. (b) Protein lipoylation pattern of *B. subtilis* strains grown in SMM supplemented with BCFAP and acetate, in the presence of xylose, with or without LA. Relevant lanes are shown. The uncropped blot is shown in Figure S10

insert sulfur atoms into octanoyl moieties attached to E2o (Rasetto et al., 2019), the absence of a band corresponding to lipoyl-E2o in Δ*lipL* Δ*gcvH* protein extracts of cells grown in the absence of LA addition (Figure 6b) leads us to conclude that TbLipL cannot transfer octanoyl moieties from ACP to this apoprotein.

It is remarkable that TbLipL lacks a cysteine residue equivalent to C150 of *B. subtilis*, identified as essential for the amidotransferase reaction (Christensen, Martin, et al., 2011), and conserved in bacterial proteins (Figure 7a; see also Figure S4). This seems to be a common characteristic of eukaryotic amidotransferases, which only share the conserved lysine present in the biotin/lipoyl protein ligase family (Figure 7a; see also Figures S3 and S4). The involvement of this conserved residue of TbLipL in the amidotransferase reaction was demonstrated using a K169S mutant. This protein was unable to restore the growth in the minimal medium of a *B. subtilis* Δ*lipL* strain, underscoring its participation in the catalytic activity of TbLipL (Figure 7b).

2.6 | A lipoic acid analog inhibits amidotransferase activity

It was previously shown that LA analogs, such as BrO and mBrO inhibited the growth of cultured *T. cruzi* epimastigotes, the replicative form of the parasite present in the vector's midgut (Vacchina et al., 2018). Although it was demonstrated that treatment with these compounds completely inhibited E2 lipoylation, and consequently the activity of the three dehydrogenase complexes, the mechanism of this inhibition was not elucidated. When we tested the effect of BrO on *B. subtilis* we observed that it did not affect the growth of wild type cells, and in fact it promoted the growth of Δ*lipM* mutants in a minimal medium (Figure 8a), suggesting that they were using this compound as a source of octanoate via the scavenging pathway. Taking advantage of the finding that BrO had no inhibitory effects on *B. subtilis*, we tested the influence of the LA analog in a Δ*lipL* mutant expressing the enzyme of *T. brucei* (Figure 8b). We observed that the growth of cells expressing the parasite's amidotransferase was strongly affected in the presence of 10 mM BrO, while this effect was not observed when these cells expressed the *B. subtilis* amidotransferase. This deleterious effect was not observed when *B. subtilis* Δ*lipM* cells expressing the OT of the parasite grew in the presence of BrO. Conversely, the growth of these cells was enhanced upon BrO supplementation (Figure 8b).

The growth deficiency of Δ*lipL* cells expressing TbLipL in the presence of 10 mM BrO correlates with the absence of lipoylation of E2s, as observed in Western blot analysis of protein extracts using anti-LA antibodies (Figures 8c and S11). In contrast, the presence of 10 mM BrO in the media did not affect the characteristic lipoylation

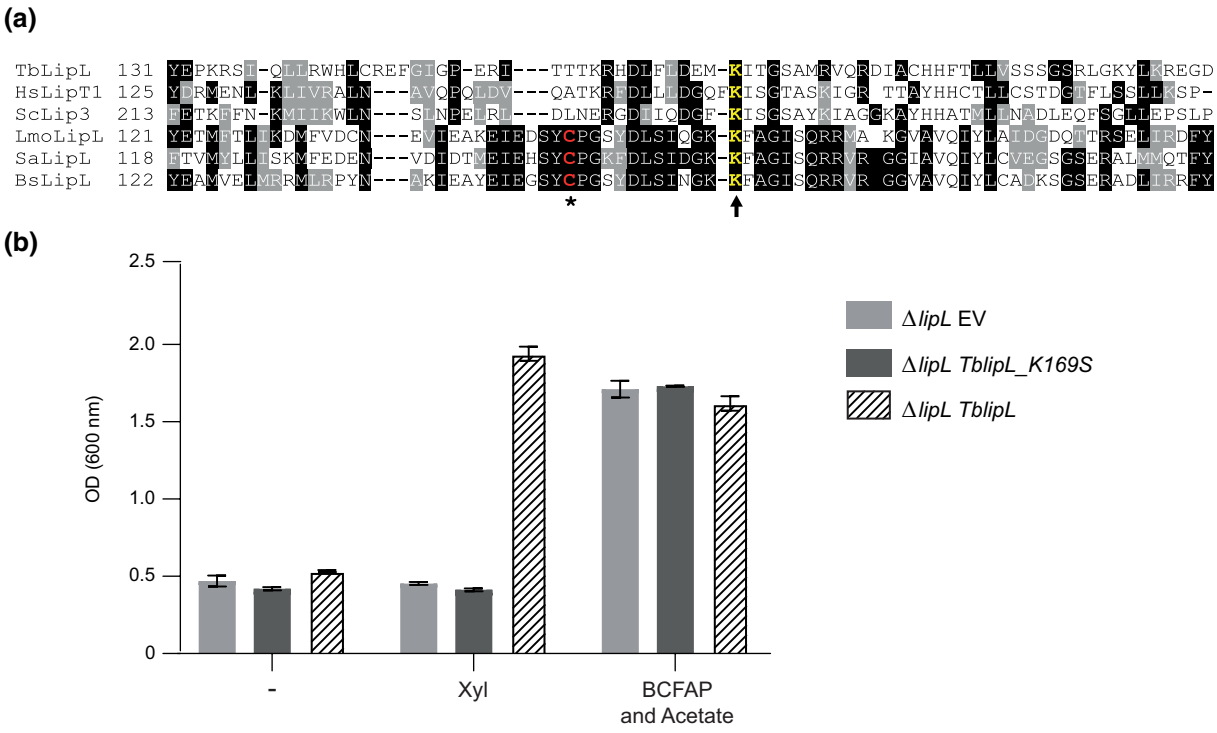

FIGURE 7 A lysine residue present in the active site of TblipL is essential for catalysis. (a) Sequence alignment of the region containing essential catalytic residues of bacterial and eukaryotic amidotransferases. Identical residues are shown highlighted in black and similar residues are highlighted in gray. The strictly conserved Pfam03099 lysine residues are indicated by an arrow. The bacterial cysteine residues involved in catalysis are marked with an asterisk. Bs: *B. subtilis*; Hs: *H. sapiens*; Lmo: *L. monocytogenes*; Sa: *S. aureus*; Sc: *S. cerevisiae*; Tb: *T. brucei*. (b) Growth of *B. subtilis* Δ*lipL* strain (NM51) transformed with a plasmid containing *TblipL_K169S* under P*xylA* (pAS9), in Spizizen minimal medium (SMM) containing the indicated supplements. Strains transformed with either a plasmid containing the coding sequence of TbLipL under P*xylA* (pAS4) or the empty vector (EV) were used as controls. Strains were incubated for 15 h at 37°C. Each bar is the mean ± *SD* from three independent experiments

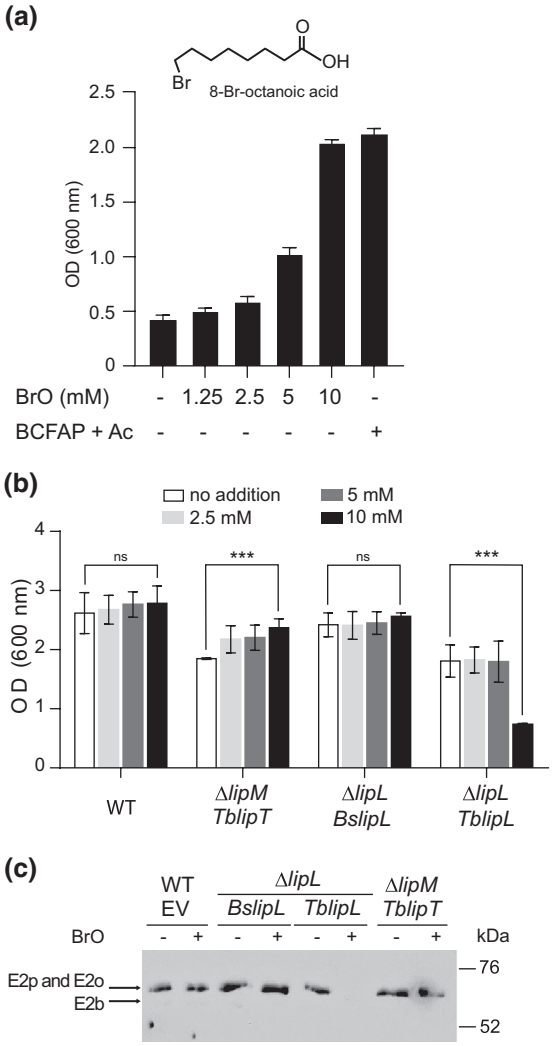

FIGURE 8 *Trypanosoma brucei* amidotransferase is the target of a lipoic acid analog. (a) *Bacillus subtilis* uses BrO as a source of octanoate. *B. subtilis* Δ*lipM* (NM57) cells were inoculated in Spizizen minimal medium (SMM), either with different concentrations of BrO or BCFAP and acetate. (b) BrO affects TbLipL activity. Growth of *B. subtilis* Δ*lipL* strains expressing TbLipL (NM51/pAS4) or BsLipL (NM13) under the control of the xylose-inducible promoter P*xylA*, and Δ*lipM* expressing TbLipT under P*xylA* (pAL31). The wild-type strain JH642 was included as growth control. Strains were inoculated in SMM containing 0.8% xylose and different concentrations of BrO, as indicated. Cultures were incubated for 15 h at 37°C. Each bar is the mean ± *SD* from at least three independent experiments. NS, no significant difference between growth at 0 and 10 mM BrO; ***$p < .001$ (Student's two tail *t* test). (c) LA-containing proteins of *B. subtilis* strains grown in SMM supplemented with BCFAP and acetate, treated or not with BrO were analyzed by Western blot with antibodies against LA

pattern of E2s when this mutant strain expressed BsLipL. In agreement with their growth behavior, protein lipoylation in Δ*lipM* cells expressing the OT of the parasite was also not affected by the presence of the LA analog (Figure 8c).

In a Δ*lipL* mutant background, lipoyl-GcvH is still produced by the sequential action of the OT and lipoate synthase, but since amidotransferase activity is lacking, E2s cannot be modified. The absence of growth and lipoylated E2s upon the addition of 10 mM BrO to Δ*lipL* cells that express the amidotransferase of the parasite, but not when the parasite OT is expressed in Δ*lipM* cells, indicates that this compound only affects TbLipL activity. Our results suggest that this enzyme would be the target of the reported inhibition by BrO in epimastigotes (Vacchina et al., 2018), positioning the trypanosomatid amidotransferase as a valid target for drug intervention.

3 | DISCUSSION

The cofactor transferase family Pfam03099 is composed of lipoate and biotinyl ligases and octanoyl and amidotransferases. All the members of this family have a strictly conserved lysine residue. Regarding octanoyltransferases, they are divided into two groups: the ones similar to the *E. coli* LipB and the ones known as "short lipoate ligases", like *B. subtilis* LipM (Christensen & Cronan, 2010). Loss of the charge of the conserved lysine residue in members of the first group of OTs inactivated the enzyme (Ma et al., 2006). In contrast, the replacement of this lysine residue with alanine in *B. subtilis* LipM partially reduced the OT activity (Christensen & Cronan, 2010). Although TbLipT may be grouped with the "LipB type" OT (Figures S1 and S2), replacement of the conserved lysine residue with serine (TbLipT-K161S) showed a behavior similar to the *B. subtilis* OT mutants (Figure 4). Probably other positively charged lysine or arginine residues, located in the surroundings of the conserved lysine in both mutant proteins, would stabilize the substrate oxyanion, enabling partial activity retention. In addition, TbLipT, as all the characterized OTs, contains a Cys residue in the active site, which is essential for catalysis (Figure 4).

The model organism *B. subtilis* allowed us to identify the amidotransferase TbLipL as the target of BrO in *T. brucei*, and most probably in other trypanosomatids, explaining the growth reduction seen for *T. cruzi*. In this parasite, LA analogs, like BrO and mBrO, completely blocked protein lipoylation and the activity of the lipoate-dependent enzymes. It was expected to produce a pleiotropic deleterious effect in the cell, affecting several pathways at a time. It was evidenced by a severe impairment in the growth of the parasite epimastigote stage, not rescued by LA supplementation (Vacchina et al., 2018). BrO inhibited the growth of *B. subtilis* Δ*lipL* mutants expressing the parasite amidotransferase but did not have an effect when the bacterial enzyme was expressed. The high concentration of BrO (10 mM) required for a significant deleterious effect (Figure 8b) is probably due to the fact that *B. subtilis* is able to metabolize BrO. So, its effective concentration on the parasite target is likely to be significantly lower. We have previously shown that *B. subtilis* can use lipoic acid as a sulfur source (Mansilla & de Mendoza, 1997), indicating this organism should be able to release the sulfur atoms and be left with the octanoate. A similar situation would occur with the bromine atoms from BrO. Remarkably, our results indicate that BrO can act as a precursor for octanoic acid production in these bacteria (Figure 8a).

TABLE 1 Strains and plasmids used in this study

Bacterial strains		
Saccharomyces cerevisiae		
BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	EUROSCARF
lip2	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lip2::kanMX4*	Schonauer et al. (2009)
lip3	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lip3::kanMX4*	Schonauer et al. (2009)
Bacillus subtilis		
JH642	*trpC2 pheA1*	Laboratory stock
NM51	JH642 *lipL::Sp*^{r}	Martin et al. (2011)
NM13	JH642 *lipL::Sp*^{r} *amyE*::P*xylA-lipL*	Martin et al. (2011)
NM27	JH642 *gcvH:: Km*^{r} *lipL:Sp* ^{r}	N. Martin (unpublished)
NM57	JH642 *lipM::Km*^{r}	Martin et al. (2011)
CM28	JH642 *lipL::Sp*^{r} *lipM::Km*^{r}	Martin et al. (2011)
NM08	Δ*lipM amyE*::P*xylA-lipM*	Martin et al. (2011)
NM65	JH642 *lplJ::Sp*^{r} *lipM::Km*^{r}	Martin et al. (2011)
Plasmids		
p426GPD	pRS424 carrying the *URA3* marker gene, the 2-μ *ori*, and the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter	Mumberg et al. (1995)
pTbLipT	p426GPD containing *TblipT* gene	This study
pTbLipL	p426GPD containing *TblipL* gene	This study
pLarC1	pHPKS containing P*xylA* and *xylR*, MCL^r	Trajtenberg et al. (2014)
pAL31	*TblipT* cloned in pLarC1	This study
pAS2	*TblipT_C195* cloned in pLarC1	This study
pAS3	*TblipT_K161S* cloned in pLarC1	This study
pAS4	*TblipL* cloned in pLarC1	This study
pAS9	*TblipL_K169S* cloned in pLarC1	This study

Characterization of TbLipL as an amidotransferase ruled out the presence of a lipoyl ligase, which confirms the absence of LA salvage in trypanosomatids (Stephens et al., 2007; Vacchina et al., 2018). This result has implications for the design of effective chemotherapy against these parasites since human serum contains a significant amount, 33–145 ng/ml, of LA bound to albumin (Packer et al., 1995; Teichert & Preiss, 1992). Clearly, the inhibitory effect of drugs on enzymes from the lipoyl-relay should not be bypassed by acquired LA.

Until very recently it was believed that mammals were able to ligate exogenous LA to their apoproteins through a lipoyltransferase, a "half ligase" that required lipoyl-adenylate as substrate (Fujiwara et al., 1994). This activity resulted in an evolutionary remnant of amidotransferases, as experimentally demonstrated for LIPT1, the human amidotransferase (Cao, Zhu, et al., 2018). By measuring lipoyltransferase activity in vitro it was postulated that *Plasmodium falciparum* LipL2 (PfLipL2) lipoylates E2b and E2o, using dihydrolipoyl-AMP (Afanador et al., 2017). This activated form of LA would be generated by PfLipL1, the plasmodia lipoate ligase. This ligase has narrow substrate specificity, similar to the characterized ligases of Gram-positive bacteria since it only transfers LA to GcvH. The homology model of PfLipL2 based on bLT structure (PDB:2E5A, Fujiwara et al., 2007), the *Bos taurus* amidotransferase formerly considered a lipoyltransferase, allowed the identification of a lysine residue (K219), equivalent to the K135 of the mammalian enzyme, essential for the lipoylation reaction (Afanador et al., 2017). We obtained a similar structural homology model for TbLipL using *B. taurus* amidotransferase (Figure S12), and through point mutagenesis, we determined the essentiality of K169 of the parasite enzyme, located in a position equivalent to K219 in PfLipL2. Taking into account the moonlighting lipoyl-adenylate transferase activity of mammalian amidotransferases and the structural similarity of PfLipL2 with them, it would be instructive to investigate the possibility of amidotransferase activity in vivo. If this was the case, lipoyl-relay pathways would be more common than previously expected.

Post-translational modification of trypanosomal proteins with LA is required for normal growth and viability (Roldán et al., 2011; Vacchina et al., 2018). The absence of the cofactor bound to apoproteins affects different processes depending on the stage of the parasite. Energy and carbon metabolisms are TCA cycle-dependent in all *T. cruzi* stages, particularly for the bloodstream trypomastigotes and the replicative intracellular amastigotes in the mammal host, which preferentially rely on amino acid catabolism. *Leishmania* spp. promastigotes and amastigotes and the insect procyclic stage of *T. brucei* have also a very active TCA cycle (Michels et al., 2021).

T. brucei bloodstream forms, the causative stage of the African trypanosomiasis, have very low mitochondrial activity; the cells rely on glycolysis for energy production. The three dehydrogenases are expressed but downregulated, in comparison to the procyclic form (Michels et al., 2021; Roldán et al., 2011). Interestingly, interference of DHLDH expression produced a strong proliferation defect and rapid cell death of bloodstream cells grown in conventional media. It was attributed to the lack of functional GCS, which is involved in thymidine synthesis (Roldán et al., 2011). Indeed, the effect of the interference was bypassed by supplementation with a relatively high concentration of thymidine. So, these results indicate that GCS and lipoylated-GcvH (but not dehydrogenases or their E2 subunits lipoylation) is needed for normal growth in conventional media for this parasite stage. Moreover, *T. brucei* bloodstream forms in which both DHLDH alleles were knocked out were unable to infect mice, even considering that mammalian sera contain 0.1–1 μM thymidine (Clarke et al., 2000). This highlights OT and lipoate synthase as putative targets for African trypanosomiasis, as both enzymes are needed for the activation of the apparently essential GCS. In addition, amidotransferase should be a valid target in *T. cruzi* and *Leishmania* spp., as this enzyme is required for the activation of essential dehydrogenases in the mammalian stages of these parasites.

Our results support the presence of a lipoyl-relay pathway, for the first time detected in a parasitic protozoan, expanding to Excavata the range of organisms having this kind of metabolism. At the moment, the existence of a lipoyl-relay has been confirmed in some Gram-positive bacteria, *S. cerevisiae*, *H. sapiens*, and *C. elegans*, although it is suspected to be present in other organisms. Our work also shows the importance of using multiple and well-known models in order to characterize the genes involved in *T. brucei* LA metabolism. This strategy could be useful in revising previous results which suggested the absence of a lipoyl-relay in other species.

4 | MATERIALS AND METHODS

4.1 | Yeasts and bacteria strains and growth conditions

S. cerevisiae strains (see Table 1) were grown at 30°C in YP media (1% (w/v) yeast extract, 2% (w/v) peptone) supplemented with different carbon sources: 2% (w/v) glucose (YPD); 3% (w/v) glycerol (YPG), 3% (w/v) succinate (YPS), or 3% (w/v) ethanol (YPE). Yeast cells were grown also in 0.67% (w/v) yeast nitrogen base without amino acids or ammonium sulfate (YNB, Sigma), 2% (w/v) glucose, 0.5% (w/v) ammonium sulfate, and leucine, isoleucine, methionine, valine, tryptophan, adenine, lysine, and histidine (all at 20 mg/L). Ammonium sulfate was replaced by glycine (1.5% w/v, SDGly) for the assay of GCS functionality (Schonauer et al., 2009).

B. subtilis strains (Table 1) were routinely grown in Lysogeny Broth (LB) (Sambrook et al., 1989) at 37°C. Spizizen salts (Spizizen, 1958), supplemented with 0.5% glycerol, 0.005% Casaamino acid vitamin free (CAA-VF), and 0.01% each tryptophan and phenylalanine, were used as the minimal medium (SMM) for *B. subtilis*. Different supplements, including 25 nM DL-α-LA, 0.5 mM octanoic acid, 5 mM sodium acetate, and 0.1 mM each branched-chain fatty acid precursors (BCFAP: isobutyric acid, isovaleric acid, and 2-methylbutiric acid) were added as needed. In experiments involving gene expression under the control of the xylose inducible promoter P*xylA*, 0.8% xylose was added to the media. Antibiotics were added to the media at the following concentrations: chloramphenicol, 5 $\mu g ml^{-1}$; kanamycin, 25 $\mu g ml^{-1}$; spectinomycin, 50 $\mu g ml^{-1}$; erythromycin 0.5 $\mu g ml^{-1}$, and lincomycin 12.5 $\mu g ml^{-1}$. BrO (Sigma) was dissolved in ethanol and added to the cultures in the concentrations indicated in each experiment. To assay the growth of *B. subtilis* cultures in liquid media, strains were incubated overnight on SMM containing 0.05% CAA-VF, supplemented with acetate and BCFAP. Cells were centrifuged and washed with SMM and used to inoculate fresh media at an OD_{600} of 0.1–0.15. Cell growth was measured using a Bioscreen C with 200 μl per well with continuous and medium shaking.

4.2 | Genetic and molecular biology techniques

T. brucei DNA from the Lister 427 cell line 90–13 was a gift of Dr. Paul A. M. Michels. It was used for PCR amplification and cloning of the parasite LipT and LipL full-length genes, including their N-terminal mitochondrial targeting signals. PCRs were carried out using Q5 high fidelity DNA polymerase with oligonucleotides TblipT-Fw-BamHI and TblipT-Rv-EcoRI or TblipL-Fw-BamHI and TblipL-Rv-XhoI (Table S1). Cloned sequences were ligated into the *BamH*I/*Eco*RI or *BamH*I/*Xho*I sites of p426GPD, the 2-μ yeast expression vector containing a glyceraldehyde-3-phosphate dehydrogenase promoter (Mumberg et al., 1995) rendering plasmids pTbLipT and pTbLipL (Table 1). This vector has a selectable marker gene, which confers uracil prototrophy in the host. The resulting plasmid constructs and the vector alone were introduced by electroporation into the *S. cerevisiae* mutant strains (Table 1). Transformed yeasts were selected on minimal agar plates lacking uracil (Ausubel & Frederick, 1991).

For expression of mature TbLipT in *B. subtilis*, a DNA fragment was amplified using oligonucleotides mTblipT-Fw-Sma, which includes a bacterial ribosome binding site, and TblipT-Rv-Xba. The resulting 700 bp fragment was inserted into *Sma*I and *Xba*I sites of vector pLarC1, which allows expression of cloned genes under a xylose-inducible promoter (Trajtenberg et al., 2014), rendering plasmid pAL31. In order to obtain a mutant copy of the OT gene, in which Cys195 is replaced by a Ser residue, oligonucleotides mTblipT-Fw-Sma and TBLipT-Dw-C195S were used to amplify the 5´fragment of *lipT* gene while oligonucleotides TBLipT-Fw-C195S and TblipT-Rv-Xba, to amplify the 3´end. Both fragments were used as a template for an overlap extension PCR in which after 10 cycles of extension, oligonucleotides mTblipT-Fw-Sma and TblipT-Rv-Xba were added. The product obtained, *lipTC195S*, was inserted into *Sma*I and *Xba*I sites of vector pLarC1, resulting in plasmid pAS2. Using the same strategy, a mutant copy of the OT gene in which Lys161 is replaced by Ser was obtained. In this case oligonucleotides mTblipT-Fw-Sma

and TbLipT-Dw-K161S were used to amplify the 5´fragment of *lipT* gene, and oligonucleotides TbLipT-Fw-K161S and TblipT-Rv-Xba, to amplify the 3´end. The resulting plasmid, pAS3, contained *lipTK161S* inserted into *Sma*I and *Xba*I sites of vector pLarC1. To express TbLipL in *B. subtilis*, a 1570 bp fragment was amplified using oligonucleotides TblipL-Fw-Sma, which includes a bacterial ribosome binding site, and TblipL-Rv-Xba. This fragment was inserted into *Sma*I and *Xba*I sites of vector pLarC1, rendering plasmid pAS4. A *lipL-K169S* mutant was created by site-directed mutagenesis, using oligonucleotides TbLipL-Fw-Sma and TbLipL-Dw-K169S to amplify the 5′ fragment of *lipL* and TbLipL-Fw-K169S and TblipL-Rv-Xba to amplify the 3′ end. After 10 cycles of overlap extension PCR using the resulting fragments as templates, TbLipL-Fw-Sma, and TblipL-Rv-Xba were added to amplify the 1570 bp fragment corresponding to full-length *lipLK169S*. This fragment was inserted into *Sma*I and *Xba*I sites of vector pLarC1, resulting in plasmid pAS9.

Transformation of *B. subtilis* was accomplished by the method of Dubnau and Davidoff-Abelson (Dubnau & Davidoff-Abelson, 1971). Plasmids were extracted using the Promega Wizard® Plus SV Minipreps-DNA Purification System. Restriction enzymes and ligases were from Promega and New England Biolabs. DNA sequencing was performed by the DNA Sequencing Facility of the University of Maine. Plasmids used in this work are listed in Table 1.

4.3 | Protein extracts and Western blots

For the preparation of crude cell lysates, yeasts were grown in the corresponding medium, and proteins were extracted after cell lysis. Briefly, after harvesting, cells were washed once by centrifugation with ice-cold phosphate saline buffer (PBS) and lysed in buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris–HCl, pH 8) in the presence of a cocktail of protease inhibitors (Complete Mini tablets, Roche Applied Science).

B. subtilis strains were grown overnight in SMM supplemented with acetate and BCFAP at 37°C. Cells were resuspended in fresh media of the same composition, with or without the inducer xylose, and cultured at 37°C. A 1 ml aliquot of each culture was harvested after 48 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 Tris–HCl pH 8.0, 1 mM phenylmethylsulfonyl fluoride, PMSF) 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.

Protein concentration was determined using BSA as standard. Samples were run in 12% SDS-PAGE. Coomassie staining was performed to evaluate protein patterns and equivalent loading of samples using Coomassie Brilliant blue R 250 stain reagent (Sigma-Aldrich). Amersham LMW Calibration kit (GE Healthcare) or Amersham ECL Rainbow Marker (Cytiva) were used as molecular weight markers. Proteins were transferred to a nitrocellulose membrane and lipoylated E2 subunits were detected using rabbit anti-lipoate antibody (Calbiochem) 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).

4.4 | Sequence analyses

Sequences were retrieved from the databases of the trypanosomatid genome projects and analyzed using tools available online (http://www.genedb.org and http://www.ncbi.nlm.nih.gov/BLAST). Protein sequences were aligned using CLUSTALW (Thompson et al., 1994). Positions with gaps were removed. Phylogenetic analyses were carried out using the program MEGA7 (Kumar et al., 2016). Maximum likelihood, neighbor-joining, and minimum evolution methods were used with 10,000 bootstrap samplings, arriving at similar results. Protein alignments were also generated with T-Coffe (Notredame et al., 2000) and formatted with the 3.21 BoxShade program.

ACKNOWLEDGMENTS

We wish to thank Dr. Paul A.M. Michels for kindly providing trypanosome chromosomal DNA and Dra. Natalia Martin for construction of strain NM27. M.C.M and A.D.U. are members of the Carrera del Investigador Científico del Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. A.S. is a doctoral fellow from CONICET, A.L and D.A.L were doctoral fellows and P.V. was a career member from the same institution. This work was supported by Fondo para la Investigación Científica y Tecnológica (Grant PICT 2016-1853); Ministerio de Ciencia Tecnología e Innovación Productiva (Grant EULACH 16/T02-0161); and Consejo Nacional de Investigaciones Científicas y Técnicas (Grant P-UE-0039 2016).

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

María C. Mansilla and Antonio D. Uttaro designed and supervised; Albertina Scattolini, Antonela Lavatelli, Paola Vacchina and Daniel A. Lambruschi carried out the experiments; María C. Mansilla and Antonio D. Uttaro wrote the manuscript. Albertina Scattolini, Antonela Lavatelli, María C. Mansilla and Antonio D. Uttaro edited the manuscript. All authors have read and approved the final version.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Albertina Scattolini https://orcid.org/0000-0001-7421-2039
Antonela Lavatelli https://orcid.org/0000-0001-7164-213X
Paola Vacchina https://orcid.org/0000-0001-5866-3657
Daniel A. Lambruschi https://orcid.org/0000-0001-5787-5711
María C. Mansilla https://orcid.org/0000-0002-1444-8661

REFERENCES

Afanador, G.A., Guerra, A.J., Swift, R.P., Rodriguez, R.E., Bartee, D., Matthews, K.A. et al. (2017) A novel lipoate attachment enzyme is shared by *Plasmodium* and *Chlamydia* species. *Molecular Microbiology*, *106*(3), 439–451.

Ausubel, F.M. & Frederick, M. (1991) *Current protocols in molecular biology*. New York: Wiley.

Bhattacharya, A., Corbeil, A., do Monte-Neto, R.L. & Fernandez-Prada, C. (2020) Of drugs and trypanosomatids: new tools and knowledge to reduce bottlenecks in drug discovery. *Genes (Basel)*, *11*(7), 722.

Cao, X., Hong, Y., Zhu, L., Hu, Y. & Cronan, J.E. (2018) Development and retention of a primordial moonlighting pathway of protein modification in the absence of selection presents a puzzle. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(4), 647–655.

Cao, X., Zhu, L., Song, X., Hu, Z. & Cronan, J.E. (2018) Protein moonlighting elucidates the essential human pathway catalyzing lipoic acid assembly on its cognate enzymes. *Proceedings of the National Academy of Sciences of the United States of America*, *155*(30), 7063–7072.

Christensen, Q.H. & Cronan, J.E. (2010) Lipoic acid synthesis: a new family of octanoyltransferases generally annotated as lipoate protein ligases. *Biochemistry*, *49*(46), 10024–10036.

Christensen, Q.H., Hagar, J.A., O'Riordan, M.X. & Cronan, J.E. (2011) A complex lipoate utilization pathway in *Listeria monocytogenes*. *Journal of Biological Chemistry*, *286*(36), 31447–31456.

Christensen, Q.H., Martin, N., Mansilla, M.C., de Mendoza, D. & Cronan, J.E. (2011) A novel amidotransferase required for lipoic acid cofactor assembly in *Bacillus subtilis*. *Molecular Microbiology*, *80*(2), 350–363.

Clarke, S.J., Farrugia, D.C., Aherne, G.W., Pritchard, D.M., Benstead, J. & Jackman, A.L. (2000) Balb/c mice as a preclinical model for raltitrexed-induced gastrointestinal toxicity. *Clinical Cancer Research*, *6*, 285–296.

Cronan, J.E. (2016) Assembly of lipoic acid on its cognate enzymes: an extraordinary and essential biosynthetic pathway. *Microbiology and Molecular Biology Review*, *80*(2), 429–450.

Danhauser, K., Sauer, S.W., Haack, T.B., Wieland, T., Staufner, C., Graf, E. et al. (2012) DHTKD1 mutations cause 2-aminoadipic and 2-oxoadipic aciduria. *American Journal of Human Genetics*, *91*(6), 1082–1087.

Douce, R., Bourguignon, J., Neuburger, M. & Rébeillé, F. (2001) The glycine decarboxylase system: a fascinating complex. *Trends in Plant Science*, *6*(4), 167–176.

Dubnau, D. & Davidoff-Abelson, R. (1971) Fate of transforming DNA following uptake by competent *Bacillus subtilis*. I. Formation and properties of the donor-recipient complex. *Journal of Molecular Biology*, *56*(2), 209–221.

Fujiwara, K., Hosaka, H., Matsuda, M., Okamura-Ikeda, K., Motokawa, Y., Suzuki, M. et al. (2007) Crystal structure of bovine lipoyltransferase in complex with lipoyl-AMP. *Journal of Molecular Biology*, *371*, 222–234.

Fujiwara, K., Okamura-Ikeda, K. & Motokawa, Y. (1994) Purification and characterization of lipoyl-AMP:N epsilon-lysine lipoyltransferase from bovine liver mitochondria. *Journal of Biological Chemistry*, *269*(24), 16605–16609.

Hermes, F.A. & Cronan, J.E. (2013) The role of the *Saccharomyces cerevisiae* lipoate protein ligase homologue, Lip3, in lipoic acid synthesis. *Yeast*, *30*(10), 415–427.

Kumar, S., Stecher, G. & Tamura, K. (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874.

Laczkovich, I., Teoh, W.P., Flury, S., Grayczyk, J.P., Zorzoli, A. & Alonzo, F., III. (2018) Increased flexibility in the use of exogenous lipoic acid by *Staphylococcus aureus*. *Molecular Microbiology*, *109*(2), 150–168.

Lavatelli, A., de Mendoza, D. & Mansilla, M.C. (2020) Defining *Caenorhabditis elegans* as a model system to investigate lipoic acid metabolism. *Journal of Biological Chemistry*, *295*(44), 14973–14986.

Ma, Q., Zhao, X., Nasser Eddine, A., Geerlof, A., Li, X., Cronan, J.E. et al. (2006) The *Mycobacterium tuberculosis* LipB enzyme functions as a cysteine/lysine dyad acyltransferase. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(23), 8662–8667.

Mansilla, M.C. & de Mendoza, D. (1997) L-cysteine biosynthesis in *Bacillus subtilis*: identification, sequencing and functional characterization of the gene coding for phospho-adenylylsulfate sulfotransferase. *Journal of Bacteriology*, *179*(3), 976–981.

Martin, N., Christensen, Q.H., Mansilla, M.C., Cronan, J.E. & de Mendoza, D. (2011) A novel two-gene requirement for the octanoyltransfer reaction of *Bacillus subtilis* lipoic acid biosynthesis. *Molecular Microbiology*, *80*(2), 335–349.

Martin, N., Lombardía, E., Altabe, S.G., de Mendoza, D. & Mansilla, M.C. (2009) A *lipA* (*yutB*) mutant, encoding lipoic acid synthase, provides insight into the interplay between branched-chain and unsaturated fatty acid biosynthesis in *Bacillus subtilis*. *Journal of Bacteriology*, *191*(24), 7447–7455.

Michels, P.A.M., Villafraz, O., Pineda, E., Alencar, M.B., Cáceres, A.J., Silber, A.M. et al. (2021) Carbohydrate metabolism in trypanosomatids: new insights revealing novel complexity, diversity and species-unique features. *Experimental Parasitology*, *224*, 108102.

Mumberg, D., Muller, R. & Funk, M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene*, *156*, 119–122.

Nagarajan, L. & Storms, R.K. (1997) Molecular characterization of GCV3, the *Saccharomyces cerevisiae* gene coding for the glycine cleavage system hydrogen carrier protein. *Journal of Biological Chemistry*, *272*(7), 4444–4450.

Notredame, C., Higgins, D.G. & Heringa, J. (2000) T-coffee: a novel method for fast and accurate multiple sequence alignment. *Journal of Molecular Biology*, *302*, 205–217.

Packer, L., Witt, E.H. & Tritschler, H.J. (1995) Alpha-lipoic acid as a biological antioxidant. *Free Radical Biology and Medicine*, *19*, 227–250.

Perham, R.N. (2000) Swinging arms and swinging domains in multifunctional enzymes: catalytic machines for multistep reactions. *Annual Review in Biochemistry*, *69*, 961–1004.

Pietikäinen, L.P., Rahman, M.T., Hiltunen, J.K., Dieckmann, C.L. & Kastaniotis, A.J. (2021) Genetic dissection of the mitochondrial lipoylation pathway in yeast. *BMC Biology*, *19*(1), 14.

Rasetto, N.B., Lavatelli, A., Martin, N. & Mansilla, M.C. (2019) Unravelling the lipoyl-relay of exogenous lipoate utilization in *Bacillus subtilis*. *Molecular Microbiology*, *112*(1), 302–316.

Roldán, A., Comini, M.A., Crispo, M. & Krauth-Siegel, R.L. (2011) Lipoamide dehydrogenase is essential for both bloodstream and procyclic *Trypanosoma brucei*. *Molecular Microbiology*, *81*(3), 623–639.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, 2nd edition, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schonauer, M.S., Kastaniotis, A.J., Kursu, V.A., Hiltunen, J.K. & Dieckmann, C.L. (2009) Lipoic acid synthesis and attachment in yeast mitochondria. *Journal of Biological Chemistry*, *284*(35), 23234–23242.

Spalding, M.D. & Prigge, S.T. (2010) Lipoic acid metabolism in microbial pathogens. *Microbiology and Molecular Biology Review*, *74*(2), 200–228.

Spizizen, J. (1958) Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proceedings of the National*

Academy of Sciences of the United States of America, *44*(10), 1072–1078.

Stephens, J.L., Lee, S.H., Paul, K.S. & Englund, P.T. (2007) Mitochondrial fatty acid synthesis in *Trypanosoma brucei*. *Journal of Biological Chemistry*, *282*(7), 4427–4436.

Teichert, J. & Preiss, R. (1992) HPLC-methods for determination of lipoic acid and its reduced form in human plasma. *International Journal of Clinical Pharmacology, Therapy, and Toxicology*, *30*, 511–512.

Thompson, J.D., Higgins, D.G. & Gibbson, T.J. (1994) ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research*, *22*, 4673–4680.

Trajtenberg, F., Albanesi, D., Ruétalo, N., Botti, H., Mechaly, A.E., Nieves, M. et al. (2014) Allosteric activation of bacterial response regulators: the role of the cognate histidine kinase beyond phosphorylation. *mBio*, *5*, e02105.

Vacchina, P., Lambruschi, D.A. & Uttaro, A.D. (2018) Lipoic acid metabolism in *Trypanosoma cruzi* as putative target for chemotherapy. *Experimental Parasitology*, *186*, 17–23.

Zhao, X., Miller, J.R. & Cronan, J.E. (2005) The reaction of LipB, the octanoyl-[acyl carrier protein]:protein N-octanoyltransferase of lipoic acid synthesis, proceeds through an acyl-enzyme intermediate. *Biochemistry*, *44*(50), 16737–16746.

Zorzoli, A., Grayczyk, J.P. & Alonzo, F.I.I.I. (2016) *Staphylococcus aureus* tissue infection during sepsis is supported by differential use of bacterial or host-derived lipoic acid. *PLoS Pathogens*, *12*(10), e1005933.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Scattolini, A., Lavatelli, A., Vacchina, P., Lambruschi, D. A., Mansilla, M. C. & Uttaro, A. D. (2022). Functional characterization of the first lipoyl-relay pathway from a parasitic protozoan. *Molecular Microbiology*, *117*, 1352–1365. https://doi.org/10.1111/mmi.14913