Chlamydia trachomatis Serovar L2 Can Utilize Exogenous Lipoic Acid through the Action of the Lipoic Acid Ligase LplA1[▽]

Aishwarya V. Ramaswamy and Anthony T. Maurelli*

Department of Microbiology and Immunology, F. Edward Hébert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799

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Lipoic acid is an essential protein bound cofactor that is vital for the functioning of several important enzymes involved in central metabolism. Genomes of all sequenced chlamydiae show the presence of two genes encoding lipoic acid ligases and one gene encoding a lipoyl synthase. However, the roles of these proteins in lipoic acid utilization or biosynthesis have not yet been characterized. The two distinct lipoic acid ligases in *Chlamydia trachomatis* serovar L2, LplA1_{Ct} and LplA2_{Ct} (encoded by the open reading frames *ctl0537* and *ctl0761*) display moderate identity with *Escherichia coli* LplA (30 and 27%, respectively) but possess amino acid sequence motifs that are well conserved among all lipoyl protein ligases. The putative lipoic acid synthase LipA_{Ct}, encoded by *ctl0815*, is ca. 43% identical to the *E. coli* LipA homolog. We demonstrate here the presence of lipoylated proteins in *C. trachomatis* serovar L2 and show that the lipoic acid ligase LplA1_{Ct} is capable of utilizing exogenous lipoic acid for the lipoylation. Therefore, host-derived lipoic acid may be important for intracellular growth and development. Based on genetic complementation in a surrogate host, our study also suggests that the *C. trachomatis* serovar L2 LipA homolog may not be functional *in vivo*.

Members of the family *Chlamydiaceae* are Gram-negative obligate intracellular pathogens that cause significant ocular, respiratory, and sexually transmitted disease (STD) in humans and animals. In humans, chlamydiae cause a wide range of diseases, including psittacosis, pneumonia, trachoma, and STDs. *Chlamydia trachomatis*-mediated genital infections are among the most prevalent of all STDs and result in a wide range of sequelae, including pelvic inflammatory disease, infertility, and ectopic pregnancy, thus posing an enormous public health problem throughout the world (4). *C. trachomatis* also causes trachoma, one of the leading causes of preventable blindness in the developing world (9).

Chlamydiae exhibit a biphasic developmental cycle that involves two morphologically distinct forms (1). The elementary body (EB) is the metabolically inactive form that initiates infection by attaching to and invading host epithelial cells. After invasion, the EBs differentiate into the metabolically active but noninfectious reticulate body (RB) within a vacuole known as the inclusion. After multiple rounds of binary fission ranging from 24 to 72 h, the RBs redifferentiate back into EBs. Eventually, cell lysis results in the release of EBs that can infect surrounding cells or be transmitted to a new host.

As obligate intracellular bacteria, chlamydiae depend on the host for much of their nutrient requirements and in general have undergone significant genome reduction (29). Consistent with this lifestyle, bioinformatic analyses of chlamydiae genomes show that while they possess pathways for the biosynthesis of some nutrients, other pathways are incomplete or entirely absent. Chlamydiae are known to be dependent on the

host for a variety of metabolites, including purines, pyrimidines, cofactors, and amino acids (27) and have evolved several mechanisms, such as the use of specialized transporters, trafficking of host organelles, and modulation of signaling pathways to successfully tap into the nutrient-rich host environment (36). Characterizing the metabolic capabilities of intracellular pathogens is important because it enhances our understanding of the pathogen and its interactions with the host and enables the design of better drugs and vaccines.

Lipoic acid is a sulfur-containing cofactor that is universally required for aerobic metabolism (39). It is required for the activity of enzymes involved in oxidative and single carbon metabolism such as pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (2-OGDH), and branched-chain ketoacid dehydrogenase (BCKDH). These enzyme complexes typically consist of three subunits (E1, E2, and E3), and lipoic acid is attached to a conserved lysine residue in the E2 subunit via an amide bond (34). Another protein that undergoes lipoylation is the GcsH protein of the glycine cleavage system (GCS), which converts glycine to carbon dioxide, ammonia, and 5,10-methylenetetrahydrofolate (11). Protein-bound lipoic acid then serves as a carrier of reaction intermediates between the different subunits of the multienzyme complexes.

Details regarding the biosynthesis of lipoic acid and its utilization were largely obtained from studies conducted on *Escherichia coli* (11). Posttranslation modification of all apo-enzymes can occur by two distinct pathways in bacteria and plants. In the first, exogenous or salvaged lipoic acid is attached to unlipoylated E2 subunits by the action of a lipoic acid ligase (LplA) in an ATP-dependent manner (30) (Fig. 1). The pathway involving *de novo* biosynthesis of the cofactor is primarily mediated through the action of two enzymes, LipA and LipB. LipB is an octanoyl-ACP transferase that transfers an octanoyl group from acyl carrier protein (ACP) to the lipoyl-accepting domain of the apoenzyme, which subsequently becomes the

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799. Phone: (301) 295-3415. Fax: (301) 295-1545. E-mail: amaurelli@usuhs.mil.

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FIG. 1. Lipoylation pathways demonstrated in *E. coli*. Lipoic acid ligase (LplA) activates exogenous lipoic acid in an ATP-dependent manner and catalyzes its transfer to the apo E2-subunit of enzyme complexes that require lipoic acid to form the holo enzyme. *De novo* biosynthesis of lipoic acid involves the action of two enzymes, LipB and LipA. LipB is an octanoyl-ACP (acyl carrier protein) transferase that transfers the octanoyl moiety from octanoyl-ACP to the apo-E2 subunit. LipA uses the octanoylated-E2 domain as the substrate for the synthesis of lipoylated E2. Another pathway for the biosynthesis of lipoic acid involves the transfer of free octanoic acid by LplA to the apo-E2 domain, which then serves as a substrate for LipA to form lipoylated-E2. Enzymes predicted by genome annotation that are present (enclosed in solid line box) or absent (enclosed in dashed line box) in *C. trachomatis* are also indicated.

substrate for sulfur insertion by LipA to form lipoylated holoenzyme (28, 45) (Fig. 1). Lipoic acid synthesis can also occur through the action of LplA which can transfer octanoic acid in an ATP-dependent manner to the apo E2 domain, which then becomes the substrate for the action of LipA (21) (Fig. 1).

Although lipoic acid is a vital cofactor, details regarding its utilization by intracellular pathogens have started to emerge only recently (2, 10, 14, 24, 41). The obligate intracellular bacterium, *C. trachomatis* serovar L2, encodes for four proteins that are predicted to undergo lipoylation. Its genome also encodes for two putative lipoic acid ligases designated LplA1_{Ct} and LplA2_{Ct} and a LipA_{Ct} homolog but no putative LipB homolog, suggesting that it possesses an incomplete pathway for *de novo* lipoic acid biosynthesis. Transcriptome studies of *C. trachomatis* serovar D have shown that both putative ligases genes and the lipoic acid synthase genes are expressed during growth and development (5, 6).

In this study we show the presence of lipoylated proteins in *C. trachomatis* serovar L2 and characterize the activity of the chlamydial lipoic acid ligase, LplA1_{Ct}. Based on the present data, we propose that chlamydiae are capable of utilizing exogenous lipoic acid. However, LipA_{Ct} was found to be non-functional *in vivo* in a heterologous system and did not allow us to determine its role in *de novo* biosynthesis of lipoic acid in the bacterium.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in the present study are listed in Table 1. Strains were grown in Luria-Bertani (LB) medium or M9 minimal salts supplemented with 0.5% mannitol at 37°C. M9 minimal medium was supplemented with 0.5% mannitol as the carbon source, because gene expression under the *ara* promoter in pBAD18 used as the vector for complementation assays is repressed in the presence of glucose. Growth medium was supplemented with antibiotics for selection at the following concentrations: ampicillin (100 µg/ml), kanamycin (50 µg/ml), and chloramphenicol (25 µg/ml). When required, media were also supplemented with arabinose (0.2%), IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM), sodium acetate (10 mM), sodium succinate (10 mM), or lipoic acid (10 µM). Lipoic acid was obtained from Sigma. The lipoylated tripeptide DK_LA was purchased from AnaSpec.

Quantitative determination of plating efficiency of the ligases was done as

follows. Strains from exponential-phase cultures were washed three times in 1× phosphate-buffered saline (PBS; 1.05 mM KH₂PO₄, 154 mM NaCl, 5.6 mM Na₂HPO₄), serially diluted, and plated on M9 mannitol medium without exogenous lipoic acid or M9 mannitol with arabinose and lipoic acid (10 µM). Efficiency of plating was calculated as the percentage ratio between the number of colonies obtained on plates without lipoic acid and those growing on plates with lipoic acid.

For growth curves, single colonies from appropriate LB plates were inoculated into M9 mannitol minimal with 0.2% arabinose, 10 mM sodium acetate, and 10 mM sodium succinate and then cultured overnight at 37°C. About 1 ml of the overnight culture was then washed three times in PBS and inoculated into fresh M9 mannitol medium containing 0.2% arabinose with or without 10 µM lipoic acid. Growth curves were performed by using a Bioscreen growth curve analyzer. The standard deviation represents the growth measurements of five replicates.

Strain and plasmid construction. ATM967 (*E. coli* Δ*lplA* Δ*lipB*::Kan^r) and ATM1102 (*E. coli* Δ*lplA* Δ*lipA*::Kan^r) were constructed by allelic exchange as described previously (12). Briefly, the kanamycin resistance cassette region (*kan*) from pKD4 (Table 1) with 5' and 3' overhangs identical to the 5' and 3' regions of *E. coli* *lplA*, *lipA*, and *lipB* (Table 1) was amplified by PCR. All primers are listed in Table 2. *E. coli* BW25113 containing pKD46 was used as the recipient for linear transformation for mutant construction. Kanamycin-resistant (Kan^r) recombinants were purified and screened via PCR to ensure proper insertion of the cassette. P1L4 lysates grown on the Δ*lplA*::*kan* strain and Δ*lipB*::*kan* strain were used to transduce the standard prototroph, MG1655 with selection for Kan^r. To construct the double mutant, the Δ*lplA*::*kan* allele was transduced into MG1655 and the transductant was transformed with pCP20 which expresses the Fip recombinase and mediates recombination between FRT sequences to excise the *kan* cassette. The Kan^r strain thus obtained was transduced with the Δ*lipB*::*kan* allele and selected on M9 minimal medium supplemented with mannitol, sodium acetate, sodium succinate, and kanamycin. The Δ*lplA* Δ*lipB*::*kan* double mutant was then stocked as ATM967. A P1L4 lysate grown on the BW25113 Δ*lipA* strain was used to transduce the MG1655 Kan^r Δ*lplA* strain to generate the Δ*lplA* Δ*lipA*::*kan* double mutant, which was stocked as ATM1102 (Δ*lplA* Δ*lipA*::*kan*).

All plasmids used in the present study are described in Table 1. Plasmids pLplA_{EC}, pLipA_{EC}, and pLipB_{EC} contain an optimized *E. coli* ribosome binding site (RBS) and complete open reading frames of *E. coli* *lplA*, *lipB*, and *lipA*, respectively, under the control of the *ara* promoter in pBAD18. Similarly, pLplA1_{Ct}, pLplA2_{Ct}, and pLipA_{Ct} contain the same optimized RBS and complete open reading frames of *ctl0537*, *ctl0761*, and *ctl0821*, respectively, from *C. trachomatis* serovar L2 under the control of the *ara* promoter in pBAD18. All constructs were verified by sequencing at the Biomedical Instrumentation Center of the Uniformed Services University.

Protein expression and purification. LplA1_{Ct} and LplA2_{Ct} were amplified by PCR from *C. trachomatis* serovar L2 genomic DNA using Vent (New England Biolabs) and ligated into the XhoI site of the expression vector pGEX-6-P1 (GE

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Source or reference
Strains		
<i>C. trachomatis</i> serovar L2	Biovar lymphogranuloma venereum L2/434/Bu	H. Caldwell
<i>E. coli</i> MG1655	F ⁻ λ^- <i>ilvG rfb50 rph-1</i>	16
BL21(DE3)	F ⁻ <i>ompT hsdSB(r_B⁻ m_B⁻) gal dcm</i> (DE3)	Novagen
DH5 α	<i>fhuA2</i> Δ (<i>argF-lacZ</i>)U169 <i>phoA glnV44</i> ϕ 80 Δ (<i>lacZ</i>)M15 <i>gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	18
BW25113	Δ (<i>araD-araB</i>)567 Δ <i>lacZ</i> 4787(<i>::rrnB-3</i>) λ^- <i>rph-1</i> Δ (<i>rhaD-rhaB</i>)568 <i>hsdR514</i>	12
ATM967	MG1655 Δ <i>lplA</i> Δ <i>lipB::Kan</i> ^r	This study
ATM1098	ATM967 transformed with pLplA _{Ec}	This study
ATM1099	ATM967 transformed with pLipB _{Ec}	This study
ATM1100	ATM967 transformed with pLplA1 _{Ct}	This study
ATM1101	ATM967 transformed with pLplA2 _{Ct}	This study
ATM1102	MG1655 Δ <i>lplA</i> Δ <i>lipA::Kan</i> ^r	This study
ATM1103	ATM1102 transformed with pLipA _{Ec}	This study
ATM1104	ATM1102 transformed with pLipA _{Ct}	This study
ATM974	ATM967 transformed with pGEX-6-P1::LplA1 _{Ct}	This study
ATM975	ATM967 transformed with pGEX-6-P1::LplA2 _{Ct}	This study
ATM1108	ATM967 transformed with pGEX-6-P1::BCKDH-E2	This study
ATM921	DH5 α transformed with <i>E. coli</i> LplA expression construct, pYFJ16 (pQE2::LplA)	22
ATM1112	BL21(DE3) transformed with pYFJ16	This study
Plasmids		
pBAD18	Arabinose-inducible expression vector, pBR322 ori; Amp ^r	17
pKD4	ori R _{6K} <i>bla aphA-3</i>	12
pGE-X-6P1	Protein expression vector, N-terminal GST fusion; Amp ^r	GE Life Sciences
pLplA _{Ec}	pBAD18::lplA, contains coding sequence of <i>E. coli</i> lipoyl transferase under the control of the <i>ara</i> promoter	This study
pLipA _{Ec}	pBAD18::lipA, contains coding sequence of <i>E. coli</i> lipoyl transferase under the control of the <i>ara</i> promoter	This study
pLipB _{Ec}	pBAD18::lipB, contains coding sequence of <i>E. coli</i> lipoyl transferase under the control of the <i>ara</i> promoter	This study
pLipA _{Ct}	pBAD::ctl0821, contains coding sequence of lipoyl transferase from <i>C. trachomatis</i> serovar L2 under the control of the <i>ara</i> promoter	This study
pLplA1 _{Ct}	pBAD18::ctl0537, contains coding sequence of putative lipoyl transferase from <i>C. trachomatis</i> serovar L2 under the control of the <i>ara</i> promoter	This study
pLplA2 _{Ct}	pBAD18::ctl0761, contains coding sequence of putative lipoyl transferase from <i>C. trachomatis</i> serovar L2 under the control of the <i>ara</i> promoter	This study
pGEX-LplA1 _{Ct}	pGEX-6-P1::ctl0537, generates an N-terminal GST-tagged version of CTL0537	This study
pGEX-LplA2 _{Ct}	pGEX-6-P1::ctl0761, generates an N-terminal GST-tagged version of CTL0761	This study
pGEX-BCKDH-E2	pGEX-6-P1::ctl0657, generates an N-terminal GST-tagged version of BCKDH-E2	This study
pYFJ16	<i>E. coli</i> lplA cloned into pQE-2 with an N-terminal His tag for overexpression	22

^a Amp^r, ampicillin resistance; Kan^r, kanamycin resistance.

Life Sciences), verified by sequencing, and transformed into ATM967 (Δ *lplA* Δ *lipB::kan*). ATM967 containing pGEX-6-P1::LplA1_{Ct} or pGEX-6-P1::LplA2_{Ct} was induced with 1 mM IPTG at room temperature overnight. Glutathione S-transferase (GST)-tagged proteins were then purified from bacterial lysates by using GST beads (GE Life Sciences) according to the manufacturer's instructions for batch purification. The N-terminal GST tag was cleaved from the recombinant protein by using PreScission protease (GE Life Sciences) and dialyzed against buffer containing 50 mM sodium phosphate (pH 7.6), 2 mM EDTA, 100 mM NaCl, 10 mM MgCl₂, and 10% glycerol. The BCKDH-E2 subunit encoded by *ctl0657* was amplified from *C. trachomatis* serovar L2 by PCR and also cloned into pGEX-6-P1 by using the XhoI restriction site. Recombinant BCKDH-E2 subunit was purified as described above, and the cleaved protein was dialyzed into 1× PBS plus 10% glycerol. LplA_{Ec} was purified as a His-tagged protein according to a previously described report (22). Briefly, *E. coli* BL21DE3 expressing LplA_{Ec} with an N-terminal His tag was grown at 37°C until reaching an optical density at 600 nm (OD₆₀₀) of ~0.5 and induced with 1 mM IPTG for 3 h at the same temperature. LplA_{Ec} was then purified under native conditions using HisPur cobalt resin (Pierce) according to the manufacturer's recommendations. Purified LplA_{Ec} was then dialyzed into buffer containing 20 mM sodium phosphate (pH 7.6), 2 mM EDTA, and 5% glycerol. All purified enzymes were stored at -80°C.

The purification of apo-PDH from ATM967 (Δ *lplA* Δ *lipB::kan*) was adapted from that of an earlier study (23). Briefly, ATM967 was grown in 100 ml of M9

minimal medium supplemented with mannitol, acetate, and succinate at 37°C. The cells were pelleted and resuspended in 12 ml of 0.1 M potassium phosphate (pH 7), lysed by sonication, and centrifuged for 30 min at 39,000 × g at 4°C. The supernatant was then centrifuged at 236,000 × g in a Beckman 45 Ti rotor for 8 h. The resulting pellet was resuspended in 5 ml of 50 mM potassium phosphate buffer and centrifuged for an additional 8 h at 236,000 × g. The supernatant was discarded, and the pellet was resuspended in 1 ml of potassium phosphate buffer containing 10% glycerol and stored at -80°C. Concentrations of all recombinant proteins were measured by using a BCA protein assay kit (Pierce).

Western blot analysis. For Western blot analyses, samples were resolved by SDS-PAGE and blotted. Lipoylated proteins were detected using α -lipoyl acid antibody (1:10,000; Calbiochem), followed by the addition of donkey anti-rabbit IgG antibody conjugated to horseradish peroxidase (1:1,000; GE Life Sciences). Western blots were developed using the Pico Super Signal system (Pierce) and visualized by using a Fuji Intelligent Dark Box and Image Reader LAS-3000 software. For equal loading of samples, the protein concentration (*A*₂₈₀) was determined by using a NanoDrop ND-1000 spectrophotometer.

Lipoic acid ligase assays. For lipoic acid ligase activity, purified LplA1_{Ct} (1 μ M) or LplA_{Ec} (0.3 μ M) was incubated in 10 mM sodium phosphate (pH 7) containing 5 mM ATP, 0.3 mM dithiothreitol, and 5 mM MgCl₂. *E. coli* apo-PDH/2-OGDH (1 μ g) and chlamydial BCKDH-E2 (2 μ M) were used as substrates in the reaction, and lipoic acid was used at a concentration of 1 mM. The

TABLE 2. Primers used in this study

Primer function	Sequence (5'–3') ^a	
	Forward	Reverse
Inactivation of <i>lplA</i>	CCC ATT ACT ACA AGA AAG GAA ATC GTT ATG TCC ACA TTA CGC CTC TGT GTA GGC TGG AGC TGC TTC	AAA GTT GCC CGC ATG GGC GGG TAA CTA CCT TAC AGC CCC CGC CAT CAT ATG AAT ATC CTC CTT AGT TCC
Inactivation of <i>lipB</i>	CTC ATT CTC CAC GGA GAT ACC GTT TTG TAT CAG GAT AAA ATT CTT TGT GTA GGC TGG AGC TGC TTC	AAA TTG GGC CAT TGA TGT ATG GAA TTA AGC GGT AAT ATA TTC GAA CAT ATG AAT ATC CTC CTT AGT TCC
Inactivation of <i>lipA</i>	ATT CGC AAC TGG AAC ACG CAC GCT ATG AGT AAA CCC ATT GTG ATG TGT GTA GGC TGG AGC TGC TTC	CAG ATG TAA GTA ATT ATT ACA GGA TTA CTT AAC TTC CAT CCC TTT CAT ATG AAT ATC CTC CTT AGT TCC
Clone <i>lplA</i> into pBAD18	<u>AGGAGGAATTCACCATGTCCACATTAC GCCT</u> GCTC	ACGCGTCGACCTACCTTACAGCCCCCG C
Clone <i>lipA</i> into pBAD18	<u>AGGAGGAATTCACCATGAGTAAAC CCATTGTG</u> ATGG AA	ACG CGT CGA CTT ACT TAA CTT CCA TCC CTT TCG C
Clone <i>lipB</i> into pBAD18	<u>AGG AGG AAT TCA CCT TGT ATC AGG ATA</u> AAA TTC TTG TC	ACG CGT CGA CTT AAG CGG TAA TAT ATT CGA AGT C
Clone <i>lplA1_{Ct}</i> into pBAD18	<u>AGG AGG AAT TCA CCA TGA GAA CGC GTG</u> TCA TTG AT	ACG CGT CGA CTT AAA TCC CCT CCT GCA TAA A
Clone <i>lplA2_{Ct}</i> into pBAD18	<u>AGG AGG AAT TCA CCA TGC TCA TTA ATT</u> GCG TTT TTG TT	ACG CGT CGA CTT ATA GGA TTT GTG TAG CTT TTC G
Clone <i>lipA_{Ct}</i> into pBAD18	<u>AGG AGG AAT TCA CCA TGA CCG ATT CAG</u> AAT CTC CTA TT	ACG CGT CGA CTT A AT CTT TAT TCG GAA GTA GCG C
Clone CTL0537 into pGEX6P1	CCGCTCGAGATGAGAACGCGTGTTCATTGATTCT	CCGCTCGAGTTAAATCCCCTCCTGCATA AAGAT
Clone CTL0761 into pGEX6P1	CCGCTCGAGATGCTCATTAAATTGCGTTTTTGTTC	CCGCTCGAGTTATAGGATTGTGTAGCT TTTCGGTG
Clone BCKDH-E2 (SucB2) into pGEX6P1	CCGCTCGAGATGAAAGGGCAAAAGTATTAT	CCGCTCGAGCTAGGGTTCTAAAGA AAAG

^a Restriction sites are indicated in boldface. Optimized RBS sites in each primer are underlined.

reaction was incubated at 37°C for 1 h and analyzed by SDS-PAGE, followed by Western blotting with anti-lipoic acid antibody as described above.

RESULTS

Sequence alignments of LipA_{Ct}, LplA1_{Ct}, and LplA2_{Ct}

Genes encoding for putative enzymes involved in *de novo* lipoic acid biosynthesis (*lipA*) and lipoic acid salvage (*lplA*) were identified by BLASTp using the *E. coli* homologs against the *C. trachomatis* serovar L2 genome (42). The LipA homolog (LipA_{Ct}) shares 43 and 42% identity with lipoyl synthases from *E. coli* and *Bacillus subtilis*, respectively. LipA_{Ct} possesses both sets of conserved cysteine residues involved in coordinating iron-sulfur (Fe-S) clusters, the first of which is common to all radical SAM superfamily enzymes and the second of which is unique to lipoic acid synthases (40) (Fig. 2A).

C. trachomatis serovar L2 also possesses two lipoic acid ligases, LplA1_{Ct} and LplA2_{Ct} (encoded by *ctl0537* and *ctl0761*), that are only 30 and 27% identical to *E. coli* LplA (Fig. 2B) as determined by BLASTp analyses. These enzymes possess the typical BPL_LplA_LipB domain, which is characteristic to this family of enzymes (35). Although LplA1_{Ct} showed homology to ligases from only prokaryotic organisms, LplA2_{Ct} appeared to be more closely related to plant lipoic acid ligases (8). However, the two ligases did not show any similarity to the *E. coli* LipB transferase. Orthologs of both ligases have been identified in all of the sequenced chlamydiae, suggesting that they are a well-conserved family of genes (Table 3).

Evidence of lipoylation in *C. trachomatis* serovar L2 EBs and RBs. Chlamydiae possess at least three proteins that are predicted to undergo posttranslational modification with lipoic

acid, including the E2 subunits of PDH, 2-OGDH, and BCKDH (encoded by *ctl0499*, *ctl0311*, and *ctl0657*). All of the enzyme subunits are predicted to possess a single lipoyl domain (13). In order to examine whether *C. trachomatis* serovar L2 possesses lipoylated proteins, renografin purified RBs and EBs were analyzed with a polyclonal antibody specific for lipoylated proteins (37). Western blot analysis showed that the anti-lipoic acid antibody recognized lipoylated proteins in both RB and in EB fractions (Fig. 3). The sizes of three of these lipoylated proteins are close to the predicted sizes of the chlamydial E2 subunits of PDH (~46 kDa), 2-OGDH (~40 kDa), and BCKDH (~42 kDa). The lipoylated protein (~14 kDa) present only in RBs is consistent with the predicted size of the GcsH subunit encoded by *ctl0534*.

Functionality of *lipA_{Ct}*, *lplA1_{Ct}*, and *lplA2_{Ct}*. Currently, the necessary tools to generate targeted knockout mutants in chlamydiae is lacking; thus, *E. coli* is used as the surrogate host for functional characterization of relevant genes. Toward this end, we first constructed a Δ *lplA* Δ *lipA* mutant of *E. coli* that was deficient in lipoic acid synthesis (Δ *lipA*) and utilization of exogenous lipoic acid (Δ *lplA*) but possesses a functional *lipB*. The resulting strain, ATM1102 (Δ *lplA* Δ *lipA::kan*) has an absolute requirement for acetate and succinate which provide metabolic bypasses to enzymes that require lipoic acid and served as the background for determining the functionality of the chlamydial lipoic acid synthase (*lipA_{Ct}*). Because ATM1102 (Δ *lplA* Δ *lipA::kan*) possess a functional *lipB* gene, complementation with *E. coli* *lipA* (*lipA_{Ec}*) or functional chlamydial lipoyl synthase (*lipA_{Ct}*) should restore *de novo* lipoic acid biosynthesis, thereby allowing bacteria to grow on M9 mannitol

E.c --MSKPIVMERGKVYKRADAKMALIPVKNVAITEREALLRKPEWMIKIKLPAIDSTRIQGIKAAMRKNGLHSVCEEASCPNLAECFNHG-----TATFMILGAI 96
B.s -----MAK-----KDEHLRKPEWLKIKLNTNEN-YTGLKKLMRENNLHTVCEEAKCPNIHECWAVRR-----TATFMILGSV 97
H.s -STWDEYKGN-----LRQKG-----ERLRLLPPLWKTEIPLMGKN-YNKLNLTLRLNLHNTVCEEARCPNIGECWGGEYATATATIMLMGDT 66
S.c -KASKMILDP-----LEKARQNT--EEAKKLPRWLKVPIPKGTN-YHKLGKDVKELGLSTVCEEARCPNIGECWGGKDKSKATATIMLLGDI 83
A.t -KKSEYPYGG-----MPKMGPFFGRDPPNVKPKAWLRQKAPQGER-FQEVKESLSRLNLNTVCEEACPNIGECWNGGGDG VATATIMVLGDI 85
LipAct MTDSESPTPK-----KSIPARFPKWLRQLKLPLGRV-FAQTNDTIKNKGLPTVCEEASCPNRTHCWSRH----TATYLALGDA 72
Clustal Consensus || : * *: * :***** ** *: **: : *

E.c CTRRCPPFCDVAHGR-FVAPADANEFVKLAQTLADMALRYVVITSVDRRDLRDGGAQHFDACITAIRESKPQIKIETILVPDFRGMRDRALDIITATPPDVFN 192
B.s CTRACRFCAVKKTGL-PTELDLQEPERVADSV ALMNLKHAVITAVARDQKDDGGAGIAETVRAIRKSPFTTIEVLPSDMGGN DY-NLKTLM DTRPDILN 16
H.s CTRGCRFCSVKLTARNPPPIDASEPYNTAKATAEWGLDYVVLTVSDRRDMDPGGAEEHIAKIVSYLKERNPKILVECLTPDFRGDLK-AIEKVASGLDVYA 179
S.c CTRGCRFCSVKTNRTPSKDPDEM PENTAEAKRWGLGYVVLTTVDRRDLVDGGANHAEITVRKIQKAPTNLVETLSGDFRGDLK-MVDIMAQCGLDVYA 182
A.t CTRGCRFCVAKTSRNPPPPDPMEPENTAKAIASWGVDYIVITSVDRRDIPDGGSGHFAQTVKAKRHKPDIMECLTSDFRGDLE-AVDITVHSGLDVFA 184
LipAct CTRRCGFCDIDFTRNPLPDPDEEGAKIAESA KALGLKHIVITMVSRDDLBDGGASALVHIETLHTELETATIEVLASDFEGNIA-ALHHLLDAHAIYN 171
Clustal Consensus *** ** * : * * * . *: : :!* * ** * **: .. : :: * : * * * * : . : : :

E.c HNLENVPRIYQVR-PGADYNWSLKLLERFKEAHF EIPTKSGLMVGLGETNEEII EVMRDLRRHGVMTMLTGQYLQPSRHHLPVQRYVSPDEFDEMKAEE 291
B.s HNLIETVRRILTFRVR-ARATYDRSLFLIRRAEMQDPIPTKSSIMIGLTEKEBIEVMDDLANNVDIMAICQYLYPTKKHLKVQKYHPDEF AELKEIA 263
H.s HNVETVPELQSKVRDP RVNFQSLRV LKHAKKVQFDVTSKTSIMLGLGENDEQVYATMKALREADVDCLTLTGQQYMPTRRHLKVVEEYITPEKPKYWEKVG 279
S.c HNLIETVESLTPHYVDRRATYRQSLSVLERA KATVPSLITKTSIMLGLGETDEQITQTLKDLN IQCDVVTFGQYMRPTKRHMKVVEYV KPEKFDYWKERA 282
A.t HNVETVKRLQRLVRDPKATYREQSMSVLKHA KISKPMITKTSIMLGLGETDEBLKFA MADLRADVIDLTITGQYLQPTPLHLTKVKEYITPEKFDYWKYSV 284
LipAct HNVETVERLTPFVR-HKATYRSLSMMLLENA AKYLPNLMTKSGIMVGLGEQESVVKQITLEDLDHGVKILVTIQYLRPSRRHIPVKSVSPETFDYRYSVG 270
Clustal Consensus **:.* : ** : : * :.. * : :*:.:***** :.. : : * :::*****: : * * * * * . . 108

E.c LAMGFTHAACGPFVRRSSYHA-DLQA KGMVK----- 321
B.s MQKGFSHC EAGPLVRSSYHA-DEQVNEASKK RQAQ----- 298
H.s NELGFHYTASGPLVRSSYKAGEFFLKNLVAKRKTKDI----- 316
S.c LIMGFAYCASGPLVRSSYKAGEAFFL ENVLKKNMK----- 317
A.t ESIGFRYVASGPLVRSSYRAGELFVKTMVKESYSKSL----- 322
LipAct ESLG-LFIYAGCFVRRSSFNADSVTEAMQRKETSTSSL PNKD 311
Clustal Consensus * .**.****:* . : :

B

E.c -MSTLRLLISDSYDPWFNLAVEECIFRQPMPATQR-VLF LWRNA--DTVVIGRAQNPWKECNTRRM EEDNVRLARRSSGGGAVFHDLGNTCTTFMAGKPEY 96
T.a. -MEGRLLLELTPGNT RMSLAYDEAIYRSFYQGDKPI LRIFYRHD--RSVILGYFQVAEEVVDLDYMKNGIMLARRYTGGGAVYHDLGDLNFSVVRSSDDM 97
LplA2Ct MLINCVFHVCEGLPIFKOIQLEEALLR--TSSQNFLVNTHLP--EAVVLGISRKPERDLHVEHLKEDGPIPIRRYSGGGTVFLDADSLMVSWIINSFTP 96
LplA1Ct ---MTRVRIDSIRGTPKDLMQDRLLDLSLKGGEVILHLYEWEGIPPLYTCGCIKPERPLKAN-WESLGVSAASRP TGGGVTFHNS-DYAFSLLVSS--- 92
Clustal Consensus : . * . : : . * : : : : : * :***. : . : : ..

E.c DKT----ISTSI VLNALNALGVSAEASGRNDLVKT-----VEGRDVRSGSAYRET KD RGPHHGILL LNADLSRLANYLNPDKKKLAAKGITSVRSRV I 186
T.a. DIGSMFRTMNEAVVNSLRILGLDAREGELNDVSI PVNKKT DIMAGEKKIMGAAGAMRKGA KLWHAAMLVHTDLDMLS AVLKV PDEKFRDKIAKSTRERVA 197
LplA2Ct SPS-----SKDLLQWTQDIYAPIPTGFKITENDY-----TPLDKKIGGNAQYIQKYRWVHHTTFLWNMNP KK LARYLPTPEIQPSYRQNRSHDBFLT 184
LplA1Ct -----EHPLYQDSL ANIYHTVNRFLKT-----~INKFLGLEGLSLPIEVSTDRAESSNFCAKTSKYDVDLIGNRKVGGAAGR-SVKQGF 170
Clustal Consensus : : : : : * : :

E.c NLTELLPGITHEQVCEAITAEFFAHYGERVEAEIISP N-KTPDLPNFAETFAQRSSW EWNFQ QAPAFSHLLDERFTWGGVELHFDVKEGHITRAQVF TDS 285
T.a. NVTD FVDV-VSIDEVRNALRGFSETLHLDREDTITEK-EESLARELFD--KKYSTBEWNMG-----LLRKEVW----- 262
LplA2Ct TIYELFD-----SREDFLSQLKQSAASKMWVEQGSQTITLTPMLS LPHRKATQIL----- 233
LplA1Ct LHQGSIFLSGNSLDFYRNILLPDLVDKIGPEVEKSAFFPLGMEASSTVLKEVRKEVKDSL MRIFMQEGI----- 239
Clustal Consensus :. : :

E.c LNPAPLEALAGRLQGCLYRADMLQQECEALLVDFPEQEKELREL SAWMAGAVR 338
T.a. ----- 262
LplA2Ct ----- 233
LplA1Ct ----- 239
Clustal Consensus -----

FIG. 2. CLUSTAL W sequence alignments of chlamydial proteins predicted to be involved in lipoate utilization and synthesis with representative proteins. (A) The putative chlamydial LipA (LipA_{CL}) was aligned with lipoic acid synthases from *Escherichia coli* (E.c.), *Bacillus subtilis* (B.s.), *Saccharomyces cerevisiae*, (S.c), plant *Arabidopsis thaliana* (A.t.), and *Homo sapiens* (H.s.). Residues common to SAM radical super family of enzymes (I) and residues specific for lipoic acid synthases (II) are highlighted in gray. (B) Alignment of the putative lipoic acid ligases from *C. trachomatis* serovar L2 (LplA1_{CL} and LplA2_{CL}) with ligases from *E. coli* (E.c.) and *Thermoplasma acidophilum* (T.a.). Conserved regions are highlighted in gray, and residues involved in lipoyl-AMP binding are indicated by an inverted triangle (▼).

minimal medium in the presence of arabinose. ATM1102 was transformed with pLipA_{Ec} or pLipA_{Ct} expressing *lipA_{Ec}* and *lipA_{Ct}*, respectively, under the control of the arabinose-inducible promoter in pBAD18. Transformants were selected on M9 mannitol medium supplemented with acetate, succinate, and ampicillin. Select transformants were subsequently examined for growth in the presence of arabinose but in the absence of exogenous lipoic acid. Our complementation studies showed that upon induction with arabinose, *lipA_{Ec}* was able to com-

plement the mutant strain (100% by efficiency of plating), whereas no complementation was observed with *lipA_{Cr}* ($<10^{-4}\%$ by efficiency of plating) under similar induction conditions. Similar results were obtained when complementation analysis was repeated with *lipA_{Cr}* cloned into a high-copy vector (data not shown).

Next, we sought to assess the functionality of the two chlamydial ligase genes, *lplA1_{Cr}* and *lplA2_{Cr}*. For this, a second double mutant of *E. coli* ($\Delta lplA \Delta lipB$) was generated, and it

TABLE 3. Amino acid sequence identities of chlamydial lipoi-
c acid ligases and lipoi- acid synthase with the respective
E. coli homologs

Species	% Sequence identity ^a to:		
	LplA _{Ec}		LipA _{Ec}
	LplA1	LplA2	
<i>Chlamydia trachomatis</i> L2	27	30	43
<i>Chlamydia muridarum</i>	25	27	44
<i>Chlamydia pneumoniae</i>	25	26	44
<i>Chlamydia caviae</i>	22	25	41
<i>Chlamydia felis</i>	23	23	41
<i>Protochlamydia amoebophila</i>	25	26	44

^a Sequence identities were determined by using BLASTp.

was designated ATM967. This strain lacks the ability to synthesize lipoi- acid ($\Delta lipB$), as well as utilize exogenous lipoi- acid ($\Delta lplA$), and provides a better background for assessing ligase activity. To assay for putative ligase activity, ATM967 ($\Delta lplA \Delta lipB::kan$) was transformed with pLplA_{Ec}, pLplA1_{Ct}, and pLplA2_{Ct} expressing *lplA_{Ec}*, *lplA1_{Ct}*, and *lplA2_{Ct}*, respectively, under the control of arabinose-inducible promoter in pBAD. Transformants were screened for their ability to grow on M9 minimal medium in the presence of mannitol, 10 μ g of lipoi- acid/ml, and 0.2% arabinose. LplA_{Ec} was able to restore the ability to utilize exogenous lipoi- acid (100% by efficiency of plating), while LplA1_{Ct} complemented at an efficiency of 94% determined by the same method. However, no complementation was observed with LplA2_{Ct} (<10⁻⁵%).

In order to further examine the functionality of the ligases and to see whether there was a delayed growth of the LplA2_{Ct} transformed strain, the growth rates of ATM967 ($\Delta lplA \Delta lipB::kan$) expressing LplA_{Ec}, LplA1_{Ct}, and LplA2_{Ct} were measured in minimal medium with or without lipoi- acid. As seen in Fig. 4A, in the absence of lipoi- acid, the pLplA1_{Ct} and the pLplA_{Ec} transformed strains showed growth kinetics that

FIG. 3. Detection of lipoylated proteins in *C. trachomatis* serovar L2 RBs and EBs. Equal concentrations of Renografin-purified EBs and RBs were resolved by SDS-PAGE blotted and analyzed by Western blotting with anti-lipoi- acid antibody. The sizes of E2 subunits of pyruvate dehydrogenase (PDH-E2), 2-oxoglutarate dehydrogenase (2-OGDH-E2), branched-chain keto-acid dehydrogenase (BCKDH-E2) and that of the glycine cleavage system protein H (GcsH) are indicated on the blot.

FIG. 4. Growth of lipoi- acid ligase mutant and transformed strains in the presence or absence of lipoi- acid. Strains (see inset) were grown in M9 mannitol medium containing 0.2% arabinose at 37°C in the absence (A) or presence of 10 μ M lipoi- acid (B). The absorbance (OD₆₀₀) was measured in a Bioscreen growth curve analyzer and plotted against time (hours). Error bars represent the standard deviation from five replicates.

were identical to each other and grew faster than MG1655 transformed with pBAD18, presumably due to overexpression of the ligases. The MG1655 transformed with pBAD18 exhibited a longer lag most likely due to the presence of only a single copy of the chromosomal ligase. However, the pLplA_{Ec}- and pLplA1_{Ct}-expressing ATM967 and the MG1655 control strains all had the same slope in exponential phase. A similar trend was seen for these strains even in the presence of lipoi- acid (Fig. 4B), with growth kinetics of the LplA_{Ec}- and LplA1_{Ct}-expressing strains being slightly faster than that of the MG1655 control. The growth rate of the pLplA2_{Ct} transformed strain was similar in the presence or absence of lipoi- acid, reaching an OD₆₀₀ of only ~0.3 even after 46 h of growth. The double mutant, ATM967, transformed with pBAD18 showed no growth throughout the experiment with or without lipoi- acid.

In order to confirm protein lipoylation *in vivo*, lysates of ATM967 ($\Delta lplA \Delta lipB::kan$) expressing *lplA_{Ec}*, *lplA1_{Ct}*, and *lplA2_{Ct}* were blotted with the anti-lipoi- acid antibody. Wild-type *E. coli* is known to possess three lipoylated proteins: PDH-E2 (65 kDa), 2-OGDH-E2 (45 kDa), and GcsH protein (~15 kDa). As shown in Fig. 5, two lipoylated proteins corresponding to PDH-E2 and 2-OGDH-E2 were detected in the *E. coli* MG1655 lysates and in strains expressing *lplA_{Ec}* and *lplA1_{Ct}*. The glycine cleavage protein GcsH was not observed in these lysates because the cultures were not induced by glycine. Interestingly, in lysates expressing *lplA_{Ec}* and *lplA1_{Ct}*, lipoylation was also seen in the absence of exogenous lipoi- acid, suggesting that the LplA1_{Ct} may also be capable of utilizing endogenous octanoic acid as a substrate. The octanoylated

FIG. 5. Detection of lipoylated proteins in lipoic acid ligase mutant and transformed strains. Lysates of transformed strains (LplA_{EC}, LplA1_{Ct}, and LplA2_{Ct}), MG1655 (positive control), and ATM967 (negative control) grown in minimal medium with or without lipoic acid were analyzed for the presence of lipoylated proteins. Equal concentrations of the lysates were separated by SDS-PAGE, blotted, and probed with anti-lipoic acid antibody. Lipoylated proteins corresponding to PDH-E2 and 2-OGDH-E2 of *E. coli* were observed in the strains expressing LplA_{EC} and LplA1_{Ct} in the presence or absence of lipoic acid but not in the LplA2_{Ct}-expressing strain.

apo-domain may then be modified into lipoylated holo-domain by the functional LipA in ATM967 ($\Delta lplA \Delta lipB::kan$) (Fig. 1). This observation is consistent with our growth curve studies and previous reports that have shown that LplA_{EC} can also utilize free octanoic acid as a substrate (21, 28, 30). Therefore, LplA1_{Ct} appears to function in a manner similar to that of LplA_{EC}.

We also examined the ability of LplA2_{Ct} to utilize exogenous octanoic acid. ATM967 ($\Delta lplA \Delta lipB::kan$) was transformed with pLipB_{EC}, pLplA_{EC}, or pLplA2_{Ct} and examined for its ability to grow on M9 mannitol medium supplemented with 50 μ M octanoic acid. Although pLipB_{EC} (98% efficiency of plating) and pLplA_{EC} (80% efficiency of plating) were able to complement the double mutant upon induction with arabinose, no complementation was observed with pLplA2_{Ct} ($<10^{-5}$ % efficiency of plating). *In vivo* lipoylation was not seen in cells expressing LplA2_{Ct} in the presence or absence of lipoic acid (Fig. 4) and confirms the negative complementation data.

In vitro lipoylation activity of LplA1_{Ct}. For *in vitro* assays, we used lysates of ATM967 ($\Delta lplA \Delta lipB::kan$) enriched for apo-

PDH and 2-OGDH and recombinant chlamydial BCKDH-E2 as lipoate acceptors. The latter was overexpressed in ATM967 as a GST-tagged protein so that it could purified in the apo-form. Consistent with our *in vivo* complementation data, recombinant LplA1_{Ct} was able to lipoylate apo-PDH and 2-OGDH-E2 subunits purified from *E. coli* ATM967 (Fig. 6A, left) as detected by Western blotting with anti-lipoic acid antibody. LplA1_{Ct} was also able to lipoylate the recombinant chlamydial BCKDH-E2 subunit (Fig. 6B, left). Purified LplA_{EC} served as a positive control for both reactions (Fig. 6A and B, right). Recombinant LplA2_{Ct} was unable to lipoylate either substrate, suggesting that this enzyme was inactive under the conditions in which it was tested (data not shown).

Previous reports have described the ability of lipoic acid ligases from *Listeria monocytogenes* to enable the utilization of lipoylated tripeptides as a source of lipoic acid (24). In order to determine whether the chlamydial ligases could function in a similar manner, we used a lipoylated tripeptide consisting of aspartate-lysine [lipoic acid]-alanine (DK_{Lip}A) as a source for lipoic acid in the *in vitro* assay. However, LplA1_{Ct} and LplA2_{Ct}

FIG. 6. *In vitro* lipoic acid ligase activity. Purified LplA_{EC} (positive control) and LplA1_{Ct} were assayed for activity in the presence or absence of various components as indicated. Purified LplA1_{Ct} was assayed for activity using a mixture of *E. coli* apo-PDH/2-OGDH (panel A, left) and recombinant chlamydial BCKDH-E2 (panel B, left) as substrates at 37°C for 1 h. Reactions were analyzed by SDS-PAGE and blotted, and lipoylated proteins were detected using anti-lipoic acid antibody. Purified LplA_{EC} served as a positive control and was also tested with apo-PDH/2-OGDH (panel A, right) and chlamydial BCKDH-E2 (panel B, right).

were not able to utilize DK_{LipA} to lipoylate the apo-enzyme substrates (data not shown).

DISCUSSION

Lipoic acid is an essential cofactor that is required for the posttranslational modification of several enzyme complexes involved in central metabolic processes. Organisms can obtain lipoic acid either by *de novo* biosynthesis or by salvage of lipoic acid from the environment. For example, *E. coli*, which has served as the model for unraveling the various steps involved in lipoic acid biosynthesis, is capable of synthesizing lipoic acid and utilizing exogenously available lipoic acid. Organisms such as *Rickettsia* spp. do not possess an LplA homolog but are predicted to synthesize lipoic acid through the action of LipA/B homologs (3). Others, such as *Listeria* spp., are lipoic acid auxotrophs (24). Genes involved in the synthesis or utilization of salvaged lipoic acid appear to play an important role in the growth and replication of some intracellular bacterial pathogens such as *Burkholderia pseudomallei* (33) and *Listeria monocytogenes* (32). Parasites such as *Toxoplasma* and *Plasmodium* have an absolute requirement for lipoic acid from host cells even though they possess a functional pathway for the *de novo* biosynthesis of the cofactor, which is produced and sequestered in the apicoplast (10, 14, 15). In *Mycobacterium tuberculosis*, the *lipB* gene is considerably upregulated in multiple drug-resistant strains, and attempts to construct a *lipB* mutant proved unsuccessful, suggesting that *lipB* is indispensable for growth (25). These examples underscore the importance of lipoic acid in the growth and replication of bacterial pathogens.

C. trachomatis serovar L2 possesses a putative lipoic acid synthase and two predicted lipoic acid ligase homologs, and this raised some intriguing questions about the biosynthesis and utilization of lipoic acid. Bacteria typically possess between three and four lipoylated proteins. Western blot analyses of *C. trachomatis* serovar L2 EB and RB lysates show the presence of lipoylated chlamydia proteins that correspond to predicted sizes of E2 subunits of PDH, 2-OGDH, and BCKDH. However, we were unable to confirm their identities due to the lack of specific antibodies. RB lysates showed the presence of an additional small lipoylated protein (<15 kDa) that may correspond to the GcsH subunit (*ctl0534*) of the glycine cleavage system. Interestingly, analysis of genome sequences by BLAST shows that the other subunits that form the glycine cleavage complex are absent in chlamydiae. In *Saccharomyces cerevisiae*, lipoylated GcsH is required for the lipoylation of PDH-E2 and 2-OGDH-E2 (38). Orphan GcsH proteins have also been reported in other systems (41). The presence of a putative lipoylated GcsH subunit in chlamydiae suggests that it may have a different role in metabolism. Alternately, this band may represent a breakdown product of one of the larger lipoylated subunits.

The presence of two lipoic acid ligases is not uncommon in bacteria and has been previously reported in lipoic acid auxotrophs such as *L. monocytogenes* wherein the ligases are paralogous, and each ligase is proposed to utilize different sources of lipoic acid (24). In organisms such as *Toxoplasma* and *Plasmodium*, the two ligases appear to be quite divergent at the sequence level and exhibit specificity for PDH-E2 or

2-OGDH-E2 subunits, respectively (2). Of the two putative ligases in *C. trachomatis* serovar L2, LplA1_{Ct} was found to be functional *in vitro* and *in vivo*, whereas LplA2_{Ct} appeared to be inactive in both the surrogate *E. coli* host and in *in vitro* lipoylation assays. The detection of lipoylated proteins in the LplA1_{Ct}-expressing strain even in the absence of octanoic acid suggests that it can utilize free octanoic acid-like LplA_{Ec} (21).

Transcriptome studies of *C. trachomatis* serovar D, which has a similar intracellular progression as *C. trachomatis* L2, have shown that *lplA1_{Ct}* gene expression is detected about 8 h postinfection, coincident with the differentiation of EBs into metabolically active RBs. However, the expression of *lplA2_{Ct}* is detected only 16 h postinfection (6). Overall, relative expression of *lplA1_{Ct}* is slightly higher than that of *lplA2_{Ct}* throughout the developmental cycle (6), suggesting that it may play a more active role. As described earlier, LplA1_{Ct} is more similar to other bacterial ligases, whereas LplA2_{Ct} is more related to plant lipoic acid ligases and could be regarded as a remnant of this ancestral relationship between chlamydiae and cyanobacterial endosymbionts of chloroplasts (8). Inactivity of LplA2_{Ct} in our system could be the result of incorrect assay conditions or the lack of a lipoic acid donor or substrate specific to LplA2_{Ct}. Inherent difficulties associated with genetic manipulation of chlamydiae precluded gene inactivation experiments that could have elucidated the function of LplA2_{Ct}.

The *de novo* pathway leading to the formation of lipoic acid involves the action of two enzymes, LipA and LipB. LipA belongs to the radical SAM family of enzymes that also includes enzymes such as biotin synthase (31). LipA binds to iron-sulfur clusters and mediates reductive cleavage of *S*-adenosylmethionine to generate 5'dA[•] radical, which ultimately results in the insertion of two sulfur atoms (presumably from the iron-sulfur cluster) into the octanoylated apodomain to form lipoic acid (11). Although LipA_{Ct} shows a fairly high degree of homology to the *E. coli* LipA and has all of the canonical residues that are typical for this enzyme family, it failed to restore growth of an *E. coli* mutant lacking *lipA*. Homologs of LipA from other organisms have been shown to complement a similar *E. coli* *lipA* mutant, suggesting that heterologous enzymes are capable of functioning *in vivo* and complementing an *E. coli* mutant (26, 44). *Bacillus subtilis* possesses two ligases and a lipoic acid synthase but no putative *lipB*, an arrangement similar to that encountered in chlamydiae (26). The *B. subtilis* *lipA* is essential for growth of the bacteria in minimal medium without exogenous lipoic acid, suggesting that it is capable of *de novo* lipoic acid biosynthesis, even in the absence of a putative LipB transferase (26). The existence of a similar pathway in chlamydiae cannot be ruled out since we have shown that LplA1_{Ct} expressed in ATM967 ($\Delta lplA \Delta lipB::kan$) can lipoylate target proteins even in the absence of exogenous lipoic acid. Due to the limitations imposed by the lack of genetic tools in chlamydiae, we used a lipoic acid synthase-deficient *E. coli* mutant as a surrogate host to examine the functionality of LipA_{Ct}. Our complementation assay in this system suggests that LipA_{Ct} is not functional *in vivo*. However, we cannot rule out the possibility that LipA_{Ct} may not be functionally similar to *E. coli* LipA or that expression in the surrogate host could have resulted in an unstable protein or incomplete assembly of iron-sulfur clusters required

for activity. In fact, several attempts to overexpress and purify LipA_{Ct} in the soluble form proved unsuccessful and did not allow us to conclusively demonstrate the presence of the *de novo* pathway that perhaps utilizes LipA_{Ct} and LplA1_{Ct}.

Taken together, our data suggest that chlamydiae are capable of utilizing exogenous lipoleic acid during growth and differentiation. Given the relatively low concentration of free lipoleic acid in the intracellular environment (39), questions still remain as to the exact mechanism by which chlamydiae are able to gain access to and sequester the cofactor. Chlamydiae are known to alter mitochondrial function (19) and interact with the host mitochondria through transient or indirect interactions (36) and may therefore have access to the limited concentrations of free lipoleic acid. Lipoleic acid is a fairly small molecule (206 Da) and could thus freely diffuse into the inclusions (20). Another source for lipoleic acid, as hypothesized for *Toxoplasma* (10), could be lipoylated peptides from breakdown of host enzymes, which could be taken up by the bacterium through an oligopeptide transporter and subsequently cleaved by nonspecific amidases to release free lipoleic acid for further utilization.

Although antibiotic therapies are currently available for the treatment of ocular and genital tract infections caused by chlamydiae, reports of treatment failure and demonstration of *in vitro* resistance to antibiotics suggest that development of resistance to commonly used antibiotics against chlamydiae is possible (7, 43). Given the importance of lipoleic acid and its role in the growth and virulence of other intracellular pathogens, genes involved in lipoleic acid biosynthesis and utilization make attractive candidates for the development of novel therapeutics (25). In the present study, we have demonstrated the activity of the chlamydial lipoleic acid ligase, LplA1_{Ct}, which relies on exogenous lipoleic acid obtained from the host. Therefore, LplA1_{Ct} presents a potential target that can be exploited for the development of novel anti-infectives against chlamydiae.

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