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LplA1-dependent utilization of host lipoyl peptides enables *Listeria* cytosolic growth and virulence

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Summary

The bacterial pathogen *Listeria monocytogenes* replicates within the cytosol of mammalian cells. Mechanisms by which the bacterium exploits the host cytosolic environment for essential nutrients are poorly defined. *L. monocytogenes* is a lipoate auxotroph and must scavenge this critical cofactor, using lipoate ligases to facilitate attachment of the lipoyl moiety to metabolic enzyme complexes. Although the *L. monocytogenes* genome encodes two putative lipoate ligases, LplA1 and LplA2, intracellular replication and virulence require only LplA1. Here we show that LplA1 enables utilization of host-derived lipoyl peptides by *L. monocytogenes*. LplA1 is dispensable for growth in the presence of free lipoate, but necessary for growth on low concentrations of mammalian lipoyl peptides. Furthermore, we demonstrate that the intracellular growth defect of the Δ*lplA1* mutant is rescued by addition of exogenous lipoic acid to host cells, suggesting that *L. monocytogenes* dependence on LplA1 is dictated by limiting concentrations of available host lipoyl substrates. Thus, the ability of *L. monocytogenes* and other intracellular pathogens to efficiently use host lipoyl peptides as a source of lipoate may be a requisite adaptation for life within the mammalian cell.

Introduction

Nutrient scavenging is critical for replication and persistence of intracellular bacterial pathogens. The facultative Gram-positive bacterium *Listeria monocytogenes* establishes a replicative niche within the host cytosol, where it is protected from humoral immunity. Cytosolic growth of *L. monocytogenes* is rapid, approximating the doubling time of the bacterium in rich broth culture (Marquis *et al*., 1993). Upon entry into the host cell, *L. monocytogenes* escapes from the phagosome by secreting a cholesterol-dependent cytolysin, Listeriolysin O (LLO) (Portnoy *et al*., 2002). LLO-deficient bacteria remain within the vacuole, do not proliferate and are rapidly cleared by the host immune response (Bouwer *et al*., 1992). Thus, *L. monocytogenes* must access the cytosol in order to replicate within an infected host. Although the host cytosol contains many nutrients that support its own growth, non-adapted bacterial species like *Bacillus subtilis* or *Yersinia enterocolitica* placed in the cytosolic environment replicate poorly (Portnoy *et al*., 1992; Goetz *et al*., 2001). These observations suggest that *L. monocytogenes* has adaptations for survival and nutrient acquisition that facilitate rapid cytosolic growth; however, relatively little is known about how *L. monocytogenes* exploits the biochemical environment of the host cell.

The requirements for *in vitro* replication of *L. monocytogenes* are well defined. For optimal growth in defined medium, the bacterium requires nine essential amino acids, vitamins (biotin, lipoic acid, riboflavin and thiamine), as well as a carbon source such as glucose (Phan-Thanh and Gormon, 1997; Tsai and Hodgson, 2003). The mechanisms of nutrient acquisition during *L. monocytogenes* intracellular growth have not been extensively studied. At this time, several processes have been empirically demonstrated to directly promote intracellular replication of *L. monocytogenes* via synthesis or transport of essential nutrients. These include hexose phosphate transport, aromatic amino acid biosynthesis and activation of pyruvate dehydrogenase (PDH) by the lipoate ligase-like protein, LplA1. The hexose phosphate transporter, *hpt*, contributes to optimal intracellular growth by *L. monocytogenes* through uptake of glucose phosphates, which are abundant in the host cytosol (Chico-Calero *et al*., 2002). *Hpt* mutant bacteria are approximately 10-fold less virulent than wild-type bacteria in a mouse model of infection. Genes involved in aromatic amino acid biosynthesis (*aroA*, *aroB* and *aroE*) are also important during intracellular growth; deletion of any one of these genes results in a 10^4-fold decrease in virulence (Stritzker *et al*., 2004). Lastly, the lipoate ligase-like protein, LplA1, is necessary for intracellular, but not

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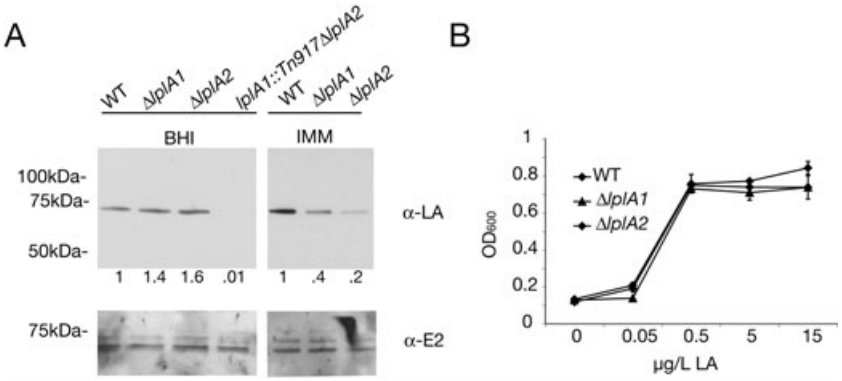

Fig. 1. *L. monocytogenes* has two functional lipoate ligase activities.
A. Equivalent numbers of stationary phase wild type (WT), Δ*lplA1*, Δ*lplA2* and *lplA1::Tn917*Δ*lplA2 L. monocytogenes*, based on OD_{600}, were pelleted and protein harvested using the FastProtein™ Blue Matrix (MP Biomedicals). Bacterial lysates were analysed by SDS-PAGE, followed by immunoblot with an anti-lipoic acid antibody. The band slightly below 75 kDa has previously been identified by Mass-Spectrometry as lipoyl-E2-PDH (O'Riordan *et al*., 2003). Relative intensities of the bands to wild type were calculated using ImageJ. Loading of equivalent bacterial lysates was confirmed by stripping the blot and reprobing with an anti-E2 antibody (Stein and Firshein, 2000). Bacteria were grown in both the rich medium BHI and IMM, a medium that is limiting for lipoate; *lplA1::Tn917*Δ*lplA2* was only studied in BHI as it exhibited impaired growth in IMM.
B. Wild-type *L. monocytogenes* and the Δ*lplA1* and Δ*lplA2* mutant strains were grown in IMM containing different concentrations of lipoic acid. The OD_{600} was measured after bacteria had reached stationary phase (30 h) and plotted against lipoic acid concentration. The OD_{600} was measured in a Bioscreen Growth Curve Analyzer.

extracellular growth (O'Riordan *et al*., 2003). The LD_{50} of LplA1-deficient *L. monocytogenes* is 250-fold greater than the wild-type parental strain in C57BL/6 mice demonstrating the importance of LplA1 for virulence.

The function of lipoate ligases has been extensively characterized in the model organism *Escherichia coli* (Morris *et al*., 1994; 1995; Fujiwara *et al*., 2005). Lipoate, a thiol-containing cofactor, is essential for the oxidative decarboxylation reactions of aerobic metabolism, and also acts as an antioxidant (Jordan and Cronan, 1997; Bast and Haenen, 2003). Lipoate ligases catalyse formation of an amide linkage between lipoate and a conserved lysine residue within target apoenzymes that include components of the PDH, α-ketoglutarate dehydrogenase (KGDH) and glycine H cleavage complexes (Perham, 2000; Zhao *et al*., 2003). *E. coli* can synthesize lipoyl groups *de novo*, but also scavenges extracellular lipoate by a pathway dependent on the lipoate protein ligase LplA (Morris *et al*., 1995). *L. monocytogenes* is a lipoate auxotroph and does not encode the genes necessary for lipoate biosynthesis (Welshimer, 1963; Glaser *et al*., 2001). However, the *L. monocytogenes* genome encodes two proteins, termed LplA1 and LplA2, that share 54% and 49% similarity, respectively, with *E. coli* LplA (Glaser *et al*., 2001). The presence of two LplA-like enzymes in *L. monocytogenes* suggests the possibility that the bacterium uses two distinct external sources of lipoic acid. Deletion of the *lplA1* gene impairs bacterial replication and lipoylation of *L. monocytogenes* PDH within host cells, but not in rich medium (O'Riordan *et al*., 2003). As LplA1 and LplA2 are not redundant during intracellular replication, we hypothesized that LplA1 might be required for utilization of a host-derived form of lipoate. Here we show that LplA1 enables *L. monocytogenes* to use small host-derived lipoyl peptides, revealing an adaptive mechanism to exploit the host cytosol for essential nutrients.

Results

LplA1 and LplA2 both contribute to PDH lipoylation during extracellular growth

Deletion of *lplA1* impairs growth and lipoylation of bacterial protein in the intracellular environment, but not in a rich complex medium, brain–heart infusion (BHI) broth, suggesting that *L. monocytogenes* encodes a second functional lipoate ligase (O'Riordan *et al*., 2003). LplA1 shares significant amino acid identity and similarity with LplA2 (*lmo0764*) and *E. coli* LplA (Fig. S1) (Glaser *et al*., 2001). To determine whether *lplA2* contributes to lipoylation during extracellular growth, we constructed strains containing an in-frame deletion of *lplA2*, or a disruption of both *lplA1* and *lplA2*, and examined modification of bacterial proteins after growth in BHI (Fig. 1A). The Δ*lplA1* and Δ*lplA2* mutant strains exhibited lipoyl modification of a 75 kDa protein, previously identified as the E2 subunit of *L. monocytogenes* PDH (*Lm*PDH), indicating that either enzyme could function during growth in rich medium (O'Riordan *et al*., 2003). In contrast, the double mutant (*lplA1::Tn917*Δ*lplA2*) showed no detectable lipoylation. Both the Δ*lplA1* and Δ*lplA2* mutant strains grew similarly

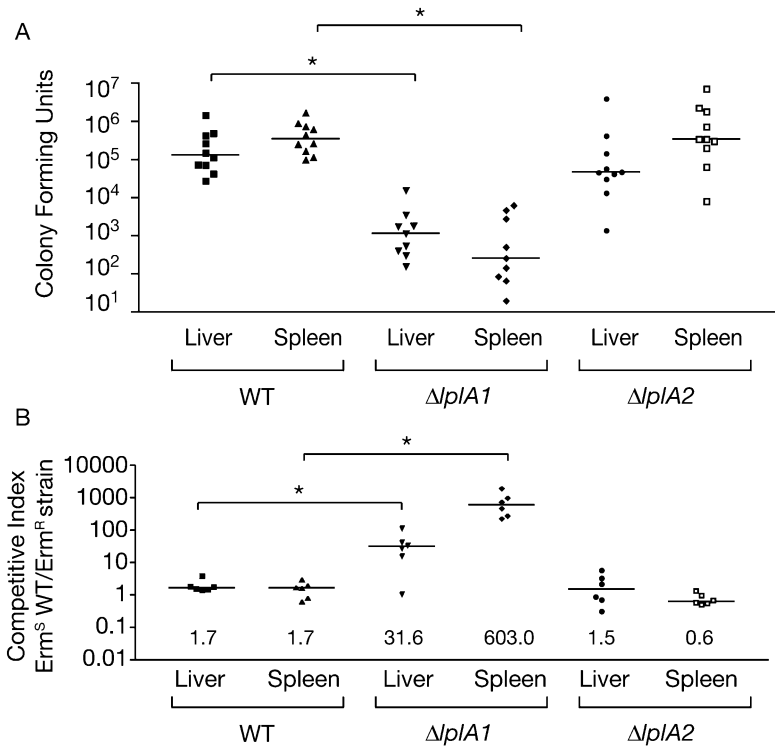

Fig. 2. Bacterial virulence *in vivo* requires LplA1, but not LplA2.
A. 2×10^5 total cfu of exponentially growing cultures of wild-type and mutant *L. monocytogenes* were injected i.p. into ten 5- to 7-week-old male C57BL/6 mice. After 72 h, spleens and livers were harvested, homogenized and plated onto LB.
B. Exponentially growing cultures of wild-type and mutant *L. monocytogenes* were mixed at a 1:10 (WT : Δ*lplA1*) or 1:1 ratio (WT : Δ*lplA2*) and 2×10^5 total cfu injected i.p. into six 5- to 7-week-old male C57BL/6 mice. After 72 h, spleens and livers were harvested, homogenized and plated onto LB with or without 1 μg ml^{-1} erm. The competitive index was calculated by dividing the number of wild-type strain cfu (erm^S) by the number of mutant cfu (erm^R). The horizontal line represents the median value. Statistically significant differences between two groups were determined by the Student's *t*-test at $P < 0.05$, as indicated by the symbol '*'.

to the parental wild-type *L. monocytogenes* strain in BHI, but the *lplA1::Tn917*Δ*lplA2* strain exhibited slower growth (data not shown). Modification of *Lm*PDH was also measured in bacteria grown in improved minimal medium (IMM) containing free lipoic acid (Fig. 1A). *Lm*PDH lipoylation was decreased in the single mutants compared with the wild-type strain. However, the single mutants grew as well as wild-type *L. monocytogenes* in IMM, demonstrating functional redundancy of LplA1 and LplA2 in medium containing free lipoic acid (Fig. 1B). While we could observe limited growth of the double mutant in BHI, this strain did not grow in IMM, thus the *lplA1::Tn917*Δ*lplA2* strain was not used for further analysis in this study (data not shown). These data demonstrate that at least one of these enzymes, LplA1 or LplA2, is necessary for lipoyl modification in *L. monocytogenes*.

LplA1, but not LplA2, contributes to bacterial virulence in a mouse model of infection

As both LplA1 and LplA2 enabled lipoate ligase activity in the extracellular environment, we used an animal model of infection to define their respective contributions to *in vivo* growth and virulence. C57BL/6 mice were infected intraperitoneally (i.p.) with wild-type and mutant *L. monocytogenes*, and colony-forming units (cfu) from spleen and liver were enumerated at 72 h (Fig. 2A). The bacterial burden in mice infected with wild-type or Δ*lplA2* bacteria was several orders of magnitude higher than mice infected with the Δ*lplA1* mutant strain (Fig. 2A). We also assessed the ability of Δ*lplA1* and Δ*lplA2* mutants to compete against wild-type *L. monocytogenes* by performing a competitive index (CI) analysis. Mice were co-infected with wild-type and mutant *L. monocytogenes* and cfu were enumerated at 72 h (Fig. 2B). The CI was calculated by dividing the number of wild-type cfu (antibiotic sensitive) by the mutant cfu (antibiotic resistant). While the CI of the Δ*lplA1* strain was approximately 32 and 603 in liver and spleen respectively, the CI of the Δ*lplA2* strain was close to 1 for both liver and spleen. These data indicate that LplA2 does not contribute to bacterial fitness or virulence *in vivo*. In contrast, successful replication of *L. monocytogenes* in a mouse model of infection required LplA1, even in the presence of LplA2.

LplA1 is required for intracellular growth using host-derived lipoate

We hypothesized that attenuation of virulence and loss of lipoylation in the Δ*lplA1* mutant strain was due to inability of the remaining ligase-like protein, LplA2, to use lipoate from the host cytosol. To ensure that intracellular growth was dependent upon host-derived lipoate, we starved wild-type, Δ*lplA1* and Δ*lplA2* strains in IMM without lipoic acid prior to intracellular infection. *Lm*PDH was extensively lipoylated when the bacteria were grown in the presence of free lipoic acid, but loss of PDH modification was observed in the absence of exogenous lipoate

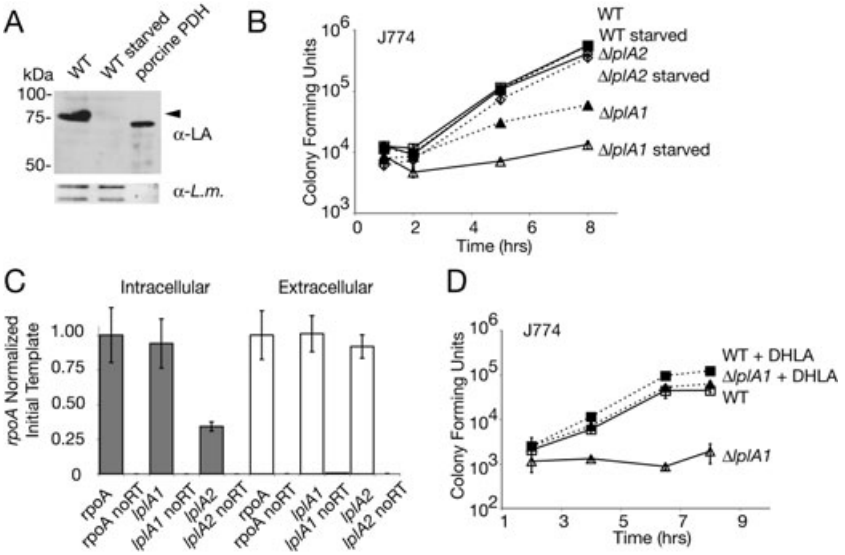

Fig. 3. LplA1, but not LplA2, is essential for intracellular growth.
A. A culture of wild-type *L. monocytogenes* grown in IMM with or without lipoic acid was grown at 37°C to stationary phase, and protein was harvested as in Fig. 1. Bacterial lysates were analysed by immunoblot using an anti-lipoic acid antibody. The 75 kDa band (black arrowhead) corresponds to the *L. monocytogenes* E2-PDH. Porcine PDH was used as a positive control for the anti-lipoic acid antibody. Loading of equivalent bacterial lysates was confirmed by reprobing the blot with polyclonal anti-*Listeria* antibody.
B. Wild-type, Δ*lplA1* and Δ*lplA2* bacterial strains were grown in IMM in the presence (dashed lines) or absence (solid lines) of lipoic acid overnight at 37°C, and used to infect J774 cell cultures. Intracellular growth was quantified by enumerating cfu.
C. Wild-type bacteria were grown in BHI overnight at 37°C and used to infect J774 cells. At 6 h post infection, WT infected J774 cells were lysed and bacteria isolated. Quantitative RT-PCR was performed in triplicate on isolated bacterial RNA to determine template quantities of *rpoA*, *lplA1* and *lplA2*. Template quantities were normalized against *rpoA* levels. To control for genomic DNA contamination, a portion of each RNA sample was removed from the reaction prior addition of reverse transcriptase (labelled 'no RT') and analysed by QRT-PCR.
D. Starved bacterial strains were used to infect J774 cells with (dashed lines) or without (solid lines) 50 μM DHLA, then cells were lysed and cfu were enumerated. For all growth curves, the mean ± SD was calculated for each time point ($n = 3$).

(Fig. 3A). To test the respective contributions of LplA1 and LplA2 towards intracellular growth, bacteria grown with or without lipoic acid were used to infect J774 macrophages. Wild-type and LplA2-deficient *L. monocytogenes* exhibited similar intracellular growth whether or not the bacteria were lipoate starved (Fig. 3B). However, lipoate-starved Δ*lplA1* mutant bacteria no longer replicated within host cells. These results show that LplA2 is dispensable for intracellular growth, but demonstrate an absolute requirement for LplA1 in utilization of host-derived lipoate for proliferation in the macrophage cytosol.

Attenuated intracellular growth by the Δ*lplA1* mutant could be explained by lack of *lplA2* expression or an absence of substrate utilized by LplA2-dependent mechanisms inside the host cell. To examine whether attenuated intracellular growth of the Δ*lplA1* mutant strain was due to lack of intracelluar *lplA2* expression, we measured levels of *lplA1* and *lplA2* transcript by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in wild-type *L. monocytogenes* isolated from J774 macrophages at 6 h post infection. *lplA2* transcripts were present in intracellular bacteria, albeit at lower levels than *lplA1* (Fig. 3C). As there is no detectable free lipoate found in the cytosol, we next investigated if lack of intracellular free lipoate was the limiting factor for growth of the Δ*lplA1* strain in the cytosol (Akiba *et al.*, 1998; Podda *et al.*, 1994). Prior to infection, we incubated J774 macrophages with 50 μM dihydrolipoic acid (DHLA) and assessed the effect of supplementation on intracellular growth (Fig. 3D). DHLA supplementation did not further enhance the intracellular growth of wild-type *L. monocytogenes* but did rescue growth of the Δ*lplA1* mutant. Taken together, these results suggest LplA2 is competent to enable intracellular bacterial growth only if free lipoate is added exogenously. As LplA2 does not normally contribute to intracellular growth, we propose that the requirement for LplA1 during cytosolic replication of *L. monocytogenes* results from the absence of lipoyl substrates used by an LplA2-dependent pathway.

Host-derived lipoyl peptides support LplA1-dependent growth of L. monocytogenes

Lipoyl moieties in mammalian cells are primarily found attached to host proteins rather than as free lipoic acid (Akiba *et al.*, 1998; Perham, 2000). We therefore hypothesized that LplA1, but not LplA2, would permit utilization of lipoyl peptides found in the host cytosol. The predomi-

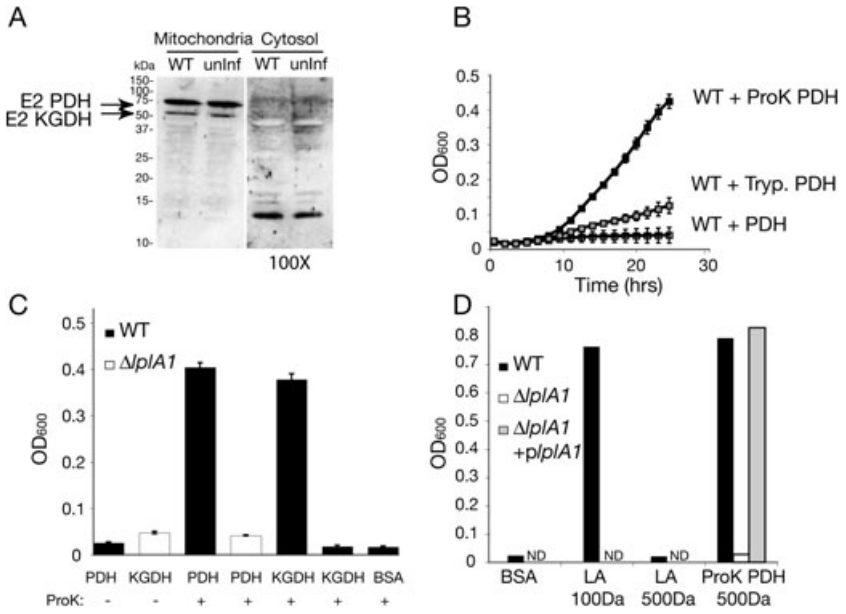

Fig. 4. LplA1 enables utilization of degraded host-derived PDH for bacterial growth.
A. J774 cell lysate was separated into cytosolic and mitochondrial fractions as described in *Experimental procedures.* Mitochondrial fractions and concentrated cytosolic fractions (100×) were analysed by immunoblot using a rabbit polyclonal anti-lipoic acid antibody. The 70 kDa band corresponds to mammalian E2-PDH, while the 55 kDa band corresponds to mammalian E2-KGDH.
B. Wild-type (WT) *L. monocytogenes* was grown in IMM without lipoic acid supplemented with proteinase K (ProK)-digested, trypsin (Tryp)-digested or undigested porcine PDH. Growth was measured by OD_{600} and the mean value ± SD was calculated for each time point ($n = 3$).
C. Wild-type and Δ*lplA1 L. monocytogenes* were grown in IMM containing undigested or ProK-digested porcine PDH or KGDH at 5 mg l^{-1}. After 35 h of growth in the Bioscreen Growth Curve Analyzer, bacterial cultures had reached stationary phase, and the OD_{600} values were plotted for each condition. BSA at 5 mg l^{-1} was also digested with ProK as a negative control. Mean values ± SD were calculated for each time point ($n = 3$).
D. Wild-type, Δ*lplA1* and Δ*lplA1* complemented with a plasmid expressing LplA1 were grown in IMM containing the lipoate sources indicated; ProK-digested BSA at 5 mg l^{-1} was used as a negative control. Free lipoic acid (206 Da) (5 μg l^{-1}) and ProK-digested porcine PDH (5 mg l^{-1}) were dialysed against a 100 Da or a 500 Da MWCO membrane, and the retentate was used to supplement IMM. After 19.5 h of growth in conical tubes, the bacterial cultures had reached stationary phase; OD_{600} values for this time point were plotted for each condition. Growth was not determined (ND) for BSA and LA filtration experiments for Δ*lplA1* and Δ*lplA1* complemented with a plasmid expressing LplA1.

nant lipoyl proteins in J774 macrophages are the E2 subunit of PDH (PDH-E2) and the E2 subunit of α-ketoglutarate dehydrogenase (KGDH-E2), as measured by immunoblot analysis of whole cell lysates using anti-E2/E3 and anti-LA antibodies (data not shown). However, full-length lipoylated PDH-E2 and KGDH-E2 are localized to the mitochondrial matrix and would likely be unavailable to cytosolic bacteria (Margineantu *et al*., 2002). To determine if lipoylated proteins were present in the cytosol of J774 macrophages, cytosolic and mitochondrial fractions were analysed by SDS-PAGE and immunoblot using an anti-lipoic acid antibody (Fig. 4A). We consistently observed a similar profile of low-molecular-weight lipoylated proteins in the cytosol of uninfected or infected J774 cells. The lipoylated proteins in the cytosolic fraction were unlikely to be the result of mitochondrial rupture during preparation as the abundant mitochondrial E2 PDH and KGDH full-length proteins were not observed in the cytosolic fraction, even though it was concentrated 100-fold. These data suggest that *L. monocytogenes* has access to lipoyl groups in the host cytosol in the form of lipoylated polypeptides.

As lipoyl groups in mammalian cells are protein bound, we tested the ability of modified host proteins, such as PDH-E2 and KGDH-E2, to act as a sole source of lipoyl groups for *L. monocytogenes* in defined minimal medium (IMM). After lipoate pre-starvation, lipoylated porcine PDH did not support *L. monocytogenes* growth (Fig. 4B). However, it was possible that *L. monocytogenes* could not transport the large PDH holoenzyme complex. To determine if LplA1 could enable bacterial utilization of smaller lipoylated peptides, we digested porcine PDH with trypsin, a treatment predicted to release a lipoyl peptide of 17 amino acids in length. Trypsin-digested PDH was not able to supplement growth of *L. monocytogenes* (Fig. 4B). Thus, the lipoyl peptides generated by trypsin digestion either were not transported into the bacteria, or were not suitable substrates for LplA1-dependent ligase activity. In contrast, proteinase K (ProK)-digested porcine PDH or KGDH, which would be predicted to contain lipoylated

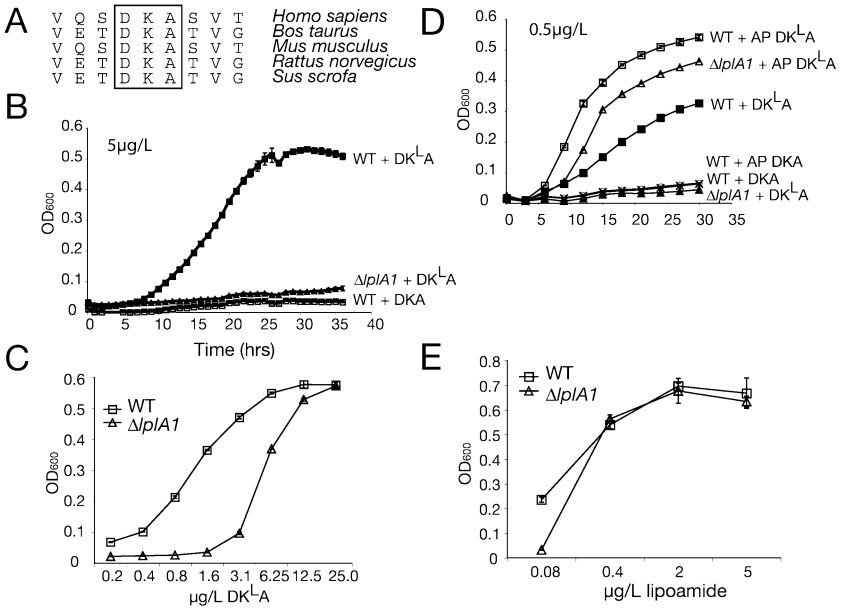

Fig. 5. LplA1 is required for optimal growth on small lipoyl peptides.
A. The amino acid sequences of the dihydrolipoyl transacylase lipoyl domain from *Homo sapiens* (Accession No. AAA64512), dihydrolipoamide S-acetyltransferase lipoyl domain of *Bos taurus* (Accession No. XP_588501), dihydrolipoamide branched chain transacylase E2 lipoyl domain of *Mus musculus* (Accession No. NP_034152), dihydrolipoamide S-acetyltransferase lipoyl domain of *Rattus norvegicus* (Accession No. AAI07441) and the dihydrolipoamide acetyltransferase lipoyl domain of *Sus scrofa* (Accession No. NP_999159) aligned.
B. Wild-type (WT) *L. monocytogenes* and the Δ*lplA1* mutant strain were grown in IMM containing 5 µg l^{-1} $DK^{L}A$, or 5 µg l^{-1} non-lipoylated DKA. The OD_{600} was measured over time in a Bioscreen Growth Curve Analyzer and plotted as a function of time.
C. Wild-type *L. monocytogenes* and the Δ*lplA1* mutant strain were grown as in (B), but in IMM containing different concentrations of $DK^{L}A$. The OD_{600} was measured after bacteria had reached stationary phase (25 h) and plotted against lipoyl peptide concentration.
D. Bacterial growth curves were performed as in (B), but 0.5 µg l^{-1} tripeptide (lipoylated or non-lipoylated) was added with or without prior aminopeptidase M digestion as indicated.
E. Bacterial growth curves were performed as described in (B), but in IMM containing the concentrations of lipoamide indicated. After 30 h of growth, bacteria had reached stationary phase, and OD_{600} values were plotted against lipoamide concentration. The mean value ± SD was calculated for each time point ($n = 3$) in (B–E).

tripeptides (~520 Da), supported growth of wild type but not Δ*lplA1* (Fig. 4B and C). The increase in bacterial growth upon ProK digestion of PDH or KGDH was not due to the presence of contaminating free lipoate in ProK, or an increase in non-lipoylated peptides, as ProK-digested BSA did not support replication.

We next considered the possibility that LplA1-dependent growth using ProK-digested PDH was due to the release of free lipoamide from PDH. To detect free lipoamide that may have been generated by ProK digestion, we dialysed ProK-digested PDH using a membrane with a 500 Da molecular weight cut-off (MWCO), and supplemented minimal medium with the retentate (> 500 Da) as a potential lipoate source. As lipoic acid (206 Da) and lipoamide (205 Da) are both smaller than 500 Da, retentate from a control filtration of free lipoic acid using a membrane with a 500 Da MWCO failed to stimulate bacterial growth. Similarly, filtered ProK-digested PDH-supplemented growth of the wild-type bacteria to the same extent (Fig. 4D). However, the Δ*lplA1* mutant was unable to grow in the presence of the 500 Da retentate, unless complemented by a plasmid containing the *lplA1* gene. As a control for the accuracy of the MWCO of the dialysis membranes, we confirmed that retentate of a free lipoic acid solution after dialysis against a 100 Da MWCO was able to supplement growth of wild-type bacteria. These results demonstrate that LplA1 permits *L. monocytogenes* usage of degraded host lipoyl proteins as a sole source of lipoic acid.

LplA1 is required for utilization of the synthetic lipoyl tripeptide $DK^{L}A$

The predicted lipoyl tripeptide released from porcine PDH and KGDH after ProK digestion shares amino acid identity with human, murine and rat lipoyl domains (Fig. 5A). To establish that LplA1-mediated growth of *L. monocytogenes* was dependent upon lipoyl peptide and not other non-modified peptides, we used synthetic lipoyl DKA ($DK^{L}A$), the smallest lipoyl peptide predicted from ProK digestion of porcine PDH (Fig. 5A, Fig. S2A and B). At 5 µg l^{-1}, $DK^{L}A$ supported growth of wild type but not Δ*lplA1*, demonstrating the specific contribution of LplA1 in utilizing lipoylated peptides for proliferation (Fig. 5B). To

test whether replication required the lipoyl moiety, we also supplemented IMM with non-lipoylated tripeptide (DKA), which did not support *L. monocytogenes* growth in the absence of any lipoyl substrate even at very high concentrations. To determine the optimal concentration of lipoyl peptide for LplA1-mediated multiplication, we performed a dose–response curve by adding $DK^{L}A$ to lipoate-starved bacterial cultures (Fig. 5C). Lower concentrations of lipoyl tripeptide only supported replication of wild-type bacteria, while high concentrations supported growth of both the wild-type and Δ*lplA1* mutant strains. These results imply that LplA2 can also use lipoyl peptides for growth, but only at very high concentrations. Our data suggest that the concentration of available lipoyl peptide in the host cytosol is low, resulting in dependence on LplA1 for intracellular replication of *L. monocytogenes*.

To determine if LplA1 could support growth with smaller lipoyl peptides than lipoylated tripeptides, we digested lipoylated $DK^{L}A$ with the protease aminopeptidase M (Fig. S2C). Unlike the preferential growth of wild-type bacteria mediated by undigested lipoyl $DK^{L}A$, peptide digestion products allowed growth of both the wild-type and Δ*lplA1* strains, suggesting that lipoyl dipeptide or lipoyl lysine does not dictate LplA1-dependent bacterial growth (Fig. 5D). Thus, LplA1 allows bacterial proliferation in low concentrations of lipoyl peptides, and the length of the modified peptide may determine preferential usage by LplA1 over LplA2. We also investigated if the free amide derivation of lipoate, lipoamide, supported LplA1-dependent replication of *L. monocytogenes*. Although there was a slight difference in growth by the Δ*lplA1* mutant strain as compared with wild-type bacteria at suboptimal lipoamide concentrations, both wild type and Δ*lplA1* replicated in lipoamide-containing medium, demonstrating that lipoamide did not define the host-specific requirement for LplA1 (Fig. 5E). Overall, these data indicate that LplA1 facilitates growth of *L. monocytogenes* on lipoyl peptides, the predominant intracellular form of lipoate.

L. monocytogenes *LplA1 exhibits lipoate ligase activity*

Our results suggest a model in which intracellular growth of *L. monocytogenes* using small host-derived lipoyl peptides is dependent upon LplA1. If LplA1 is able to directly use lipoyl substrates, we predicted that the *lplA1* gene would complement an *E. coli* strain deficient in lipoate utilization, TM131, which cannot grow in minimal medium without a functional lipoate ligase (Morris *et al*., 1995). As *E. coli* LplA has been shown biochemically to have lipoate ligase enzymatic activity, this complementation strategy has been used to demonstrate functionality of lipoate ligases from organisms as diverse as the protozoan parasite *Plasmodium falciparum* and *Arabidopsis thaliana* (Wada *et al*., 2001; Allary *et al*., 2007). We grew TM131 in metabolic bypass medium (+succinate +acetate) to allow transformation with an IPTG-inducible plasmid expressing *E. coli lplA*, *L. monocytogenes lplA1* or the vector alone. All transformants grew on bypass medium (Fig. 6A), but only *E. coli lplA* and *L. monocytogenes lplA1* complemented growth of TM131 when acetate and succinate were removed (Fig. 6B and C). However, TM131 expressing *lplA1* grew to a lesser extent, indicating that the *L. monocytogenes* lipoate ligase only partially compensated for the loss of endogenous *E. coli* lipoate ligase activity. We were not able to confirm expression of LplA2 in the TM131 strain transformed with an IPTG-inducible *lplA2* plasmid although the plasmid could direct LplA2 expression in a wild-type *E. coli* strain, thus it is still unknown whether LplA2 can act directly as a functional lipoate ligase (Fig. S3A and data not shown). As expected, expression of both *L. monocytogenes lplA1* and *E. coli lplA* resulted in lipoylation of the E2 subunit of PDH (Fig. S3B). Thus, complementation of the *E. coli* TM131 mutant by *L. monocytogenes lplA1* suggests that LplA1 can act enzymatically as a lipoate ligase.

To explore if there might be a structural basis for the difference in lipoate ligase activity between *L. monocytogenes* LplA1 and LplA2, we modelled the structure of *L. monocytogenes* LplA1 and LplA2 using previously published crystal structures of the *E. coli* and *Streptococcus pneumoniae* LplA proteins (Fujiwara *et al*., 2005). Every residue in the lipoyl AMP-binding pocket predicted to interact with substrate or residues identified as being important for binding of the target apodomain was conserved between the *E. coli* and *L. monocytogenes* enzymes (Fig. S1) (Kim *et al*., 2005). However, the electrostatic surface topology was notably different between the three enzymes, with an overall electrostatic potential for LplA1 of −13, while LplA2 and *E. coli* LplA exhibit electrostatic potentials of −5 and −8 respectively (Fig. 7). Moreover, LplA1 exhibited several regions of clustered negatively charged residues, which might modulate interaction of LplA1 with other proteins or cofactors. These predicted structural differences are consistent with our observations that *L. monocytogenes* LplA1 and LplA2 have overlapping but distinct functions.

Discussion

Intracellular pathogens such as *L. monocytogenes* have evolved mechanisms to take advantage of the biochemical environment of the host cell; the study of these mechanisms can reveal critical parameters of the host–pathogen interaction. The essential nutrient lipoic acid is scarce in the host cell in its free form, but our data demonstrate lipoylated polypeptides are present in low abundance in the cytosol where *L. monocytogenes* replicates. Although

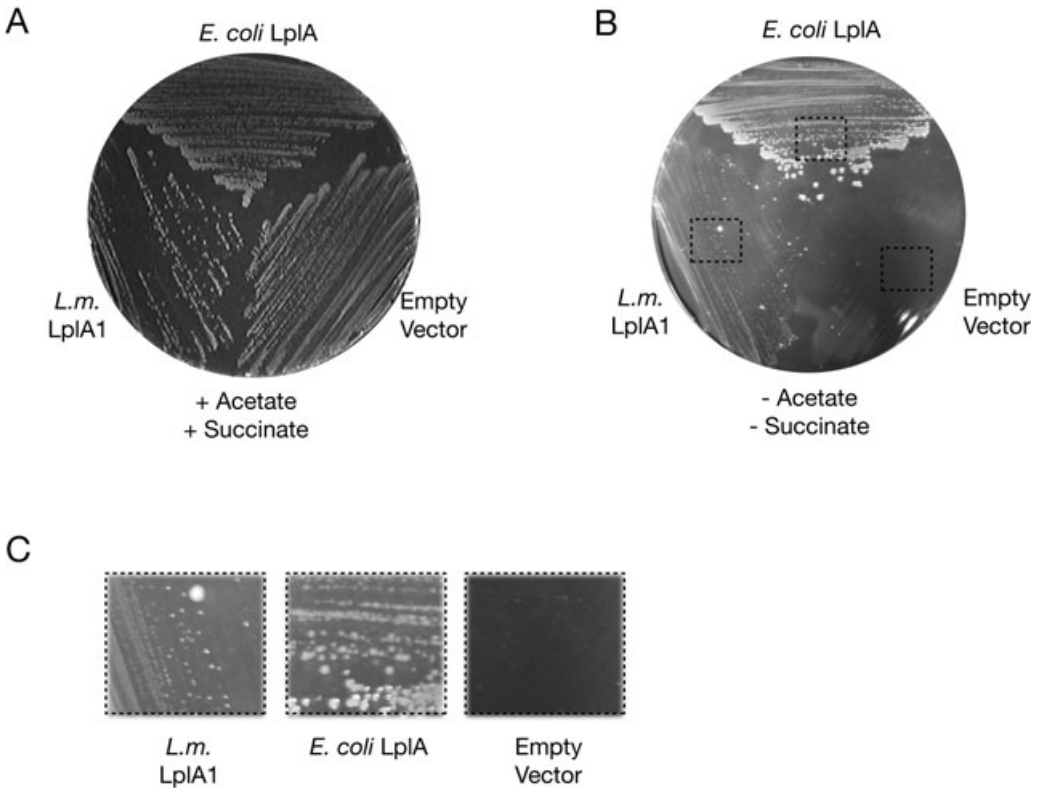

Fig. 6. Complementation of an *E. coli* strain deficient in lipoate utilization by *L. monocytogenes* (*L.m.*) LplA1. *E. coli* TM131 (*lplA*$^{-}$*lipA*$^{-}$) transformed with an empty IPTG-inducible vector, or the same vector expressing *E. coli* LplA or *L. monocytogenes* LplA1. TM131 is deficient in lipoate biosynthesis as well as endogenous LplA; growth requires either exogenous expression of a lipoate ligase, or supplementation with acetate and succinate. Clones expressing either empty vector, *E. coli* LplA or *L. monocytogenes* LplA1 were streaked on M9 minimal medium plates containing IPTG and free lipoic acid. Acetate and succinate were included in (A), but not (B) and (C). The boxes illustrated in (B) are magnified in (C) for viewing of single colonies. Some putative *E. coli* revertants were observed (large white colony observed in the insert of the LplA1-expressing *E. coli* strain).

L. monocytogenes has two lipoate ligases, LplA1 and LplA2, only LplA1 was required for intracellular growth and virulence. LplA2 was sufficient for lipoylation of target proteins and growth in rich broth medium, which contains free lipoate, but was insufficient during intracellular infection by *L. monocytogenes*. We showed that LplA1 was necessary for *L. monocytogenes* growth in low concentrations of lipoyl peptide, yet was dispensable for growth in medium containing free lipoic acid or growth in host cells supplemented with free lipoic acid. These data suggest that optimal replication in the intracellular environment by *L. monocytogenes* requires LplA1-dependent utilization of host-derived lipoyl peptides.

Local concentrations of host lipoyl peptide vary widely depending primarily on the concentration of mitochondria in that tissue (Baker *et al*., 1998). As LplA1 and LplA2 both support growth with high concentrations of lipoyl peptide, tissues that contain high amounts of lipoylated proteins, such as the liver, may be able to support more extensive bacterial growth (Baker *et al*., 1998). The Δ*lplA1* strain was substantially less able to compete with the wild-type strain in spleen when compared with liver; this tissue preference could be due to a higher concentration of lipoyl peptide in the liver supporting more robust replication in the absence of LplA1. However, the spleen also contains more immune effector cells that use oxidative stress as a host defence, which might also contribute to decreased fitness of the Δ*lplA1* mutant. Lipoic acid is a potent antioxidant that may help bacteria survive oxidative stress; by allowing efficient utilization of host lipoyl peptides, LplA1 may promote *L. monocytogenes* survival in the spleen (Bryk *et al*., 2002). Scavenging lipoate is a known growth requirement for several pathogens, such as the auxotrophic bacteria *Enterococcus faecalis* and *L. monocytogenes* (Reed *et al*., 1951). Even pathogenic organisms that synthesize lipoate, such as the protozoan parasites *Toxoplasma gondii* and *Plasmodium falciparum*, still scavenge lipoate from the host environment, as the parasite biosynthetic pathways supply only the apicoplast and not the mitochondrion (Wrenger and Muller, 2004; Crawford *et al*., 2006; Allary *et al*., 2007). Thus, the use of host-derived lipoate may be more widespread among intracellular pathogens than previously appreciated. The ability to utilize nutrients from diverse host pools is a

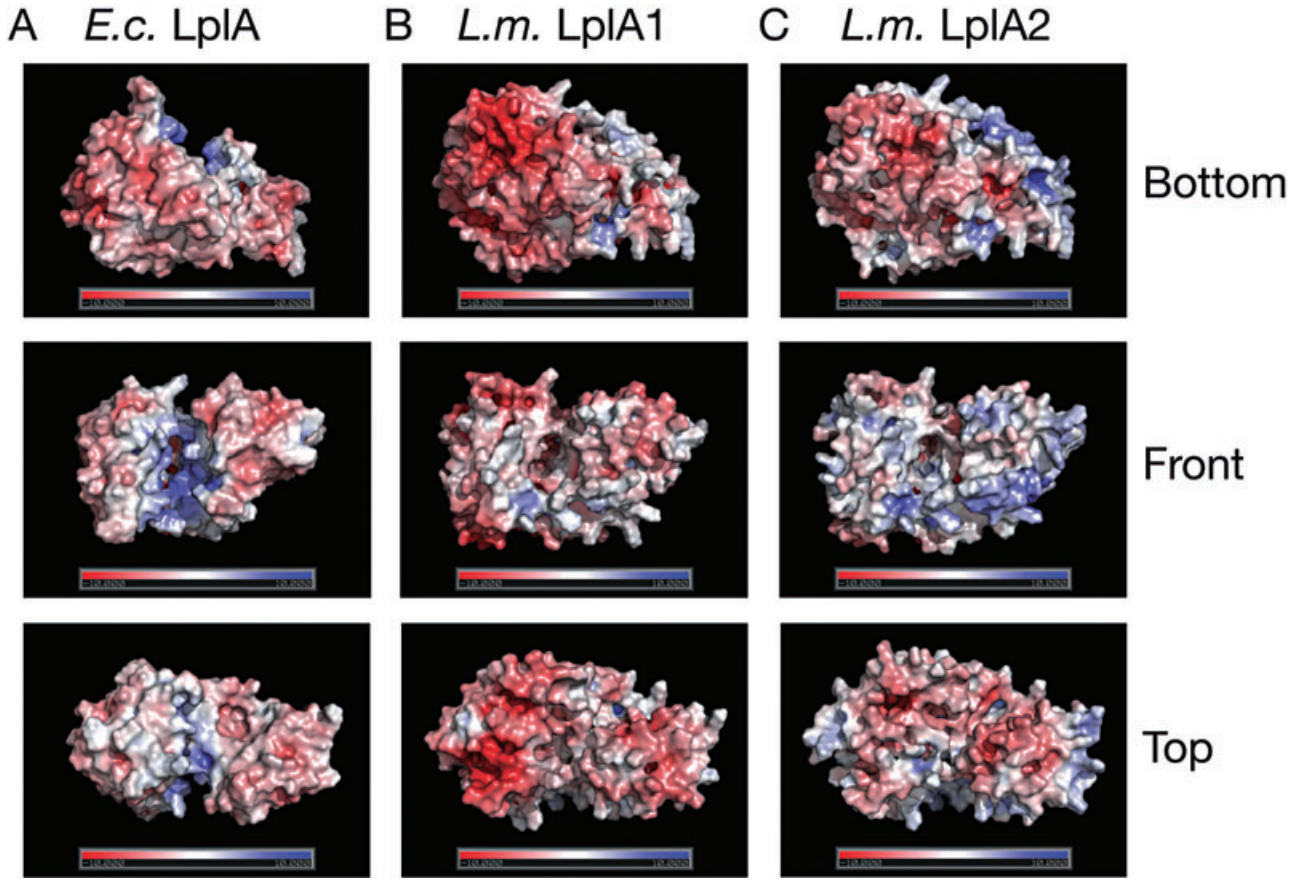

Fig. 7. Structural Modelling of *L. monocytogenes* (*L.m.*) LplA1 and LplA2. Electrostatic surface potentials for the crystallographic structure *E. coli* (*E.c.*) LplA (PDB ID 1X2H) and the modelled structures of LplA1 and LplA2 were calculated using APBS (Baker *et al.*, 2001) and mapped onto their respective solvent accessible surfaces using Pymol (DeLano, 2002). Negative potentials (–10 kT e^{-1}) are shown in red, positive potentials (10 kT e^{-1}) in blue. The views for individual molecules are separated by a 90° rotation about the *x*-axis. The protein structures are shown at the same magnification and orientation for each view.

common theme in pathogenesis, as other essential compounds like iron are scavenged in multiple forms to enable optimal replication (Skaar *et al.*, 2004).

Our structural models of LplA1 and LplA2 suggest that these enzymes share with *E. coli* LplA the basic catalytic mechanism by which free lipoate is activated and transferred to target apoproteins (Morris *et al.*, 1994; 1995). The amino acid residues involved in the binding of the lipoyl group and the target domain appear to be conserved in both *E. coli* LplA and the *L. monocytogenes* lipoate ligases (Fig. S1). However, the enzymatic mechanism by which LplA1 can enable use of low concentrations of lipoyl peptide is still unknown. Utilization of lipoyl peptide by LplA1 would mandate a distinct enzymatic mechanism from ligation of free lipoic acid as synthesis of the activated lipoyl AMP intermediate requires the carboxyl group of lipoate, lacking in lipoyl peptides. The simplest model would predict that, in addition to its LplA-like activity, LplA1 could bind directly to lipoyl peptides and transfer the lipoyl group from the peptide to the target domain, by a mechanism more analogous to LipB-mediated transfer of octanoyl groups from an acyl-carrier protein to the target apoprotein (Zhao *et al.*, 2003; Ma *et al.*, 2006). Although LplA contains no cysteines, which would be required for the lysine/cysteine catalytic dyad characterized in LipB, a cysteine could conceivably be provided by a hybrid interface with an interacting protein (Ma *et al.*, 2006). In this model, LplA1 would bind to lipoyl peptides with higher affinity than LplA2, allowing LplA1 to function efficiently at lower substrate concentrations. As an alternative model, the clustered negatively charged regions on the surface of LplA1 could represent sites of interaction with a critical cofactor. For example, LplA1 may have intrinsic lipoamidase activity or associate with a protein with lipoamidase activity, thus liberating lipoamide from lipoyl peptides and increasing the local concentration of free lipoamide. Such lipoamidase activity would have to be strictly regulated, as it would oppose ligase function (Jiang and Cronan, 2005). Future structure–function studies will elucidate which model more accurately

Table 1. Strains used.

Strains	Genotype/description	Reference
10403S	*L. monocytogenes* serotype 1/2a, parental strain used as wild type	Freitag *et al.* (1993)
MOR125	10403S *lplA1::Tn917ΔlplA2*	This study
MOR129	10403S *ΔlplA2*	This study
DP-L3903	*L. monocytogenes* 10403S::Tn*917*, unknown site of insertion, wild-type phenotype	Auerbuch *et al.* (2001)
DP-L4263	10403S *ΔlplA1*	O'Riordan *et al.* (2003)
MOR142	10403S *ΔlplA1* (DP-L4263) containing the Tn917 insertion transduced from DP-L3903	This study
JK1	*E. coli rpsL*	Morris *et al.* (1995)
TM131	*E. coli rpsL lipA182::*Tn*1000*dKn *lplA148::*Tn*10*dTc	Morris *et al.* (1995)
MOR226	*E. coli rpsL lipA182::*Tn*1000*dKn *lplA148::*Tn*10*dTc + ptac85	This study
MOR229	*E. coli rpsL lipA182::*Tn*1000*dKn *lplA148::*Tn*10*dTc + ptac85-ECLplA	This study
MOR227	*E. coli rpsL lipA182::*Tn*1000*dKn *lplA148::*Tn*10*dTc + ptac85-LMLplA1	This study
MOR228	*E. coli rpsL lipA182::*Tn*1000*dKn *lplA148::*Tn*10*dTc + ptac85-LMLplA2	This study

describes how LplA1 contributes to *L. monocytogenes* pathogenesis.

Experimental procedures

Bacterial culture

Strains used in this study are described in Table 1. For intracellular growth curves, all *L. monocytogenes* strains were grown to mid-log (OD_{600} 0.2–0.6) in IMM at 37°C unless otherwise specified. IMM was prepared as previously described (Phan-Thanh and Gormon, 1997), using the concentrations listed in Table 1 of the referenced paper. Specifically, lipoic acid was used to supplement IMM at 5 μg l^{-1} except when otherwise specified in the figure legends, or substituted where indicated with various concentrations of lipoamide (0–5 μg l^{-1}), $DK^{L}A$ (0–25 μg l^{-1}), digested lipoyl PDH (5 mg l^{-1}) or digested lipoyl KGDH (5 mg l^{-1}). For growth curves in IMM, single colonies from freshly streaked BHI plates were inoculated into IMM without lipoic acid (IMM^{-L}) for 10–14 h at 37°C shaking to OD_{600} 0.2–0.4, back-diluted to an OD_{600} of 0.02 in IMM and cultured at 37°C shaking. Bacterial growth was determined by measuring changes in OD_{600} over time. All IMM growth curves, except the experiment shown in Fig. 4D, were performed in a Bioscreen Growth Curve Analyzer (Growth Curves, USA).

Allelic exchange

A strain containing an in-frame deletion of *lplA2* was generated using homologous recombination. The deletion allele was obtained by amplifying 3′ and 5′ genomic sequences flanking *lplA2* that were then fused by splice overlap extension-PCR (Horton *et al.*, 1990). The 0.8 kb fragments were amplified from the 10403S bacterial genome with Platinum Pfx polymerase (Invitrogen) using primer sequences described in Table S1. The PCR product was subcloned into the allelic exchange vector, pKSV7 (Smith and Youngman, 1992). Allelic exchange was performed as previously described and confirmed by sequencing (O'Riordan *et al.*, 2003). The in-frame deletion resulted in removal of amino acids 30–300 out of 330 amino acids (strain MOR129). The transposon insertion that disrupted *lplA1* in DP-L2214 (O'Riordan *et al.*, 2003) was transduced as previously described (Hodgson, 2000; O'Riordan *et al.*, 2003) into MOR129 to generate a strain deficient in both putative lipoyl ligases (*lplA1Tn::917ΔlplA2*; MOR125). For complementation studies, pAM401 and pAM401*lplA1* were transformed individually into DP-L4263 and 10403S as previously described (O'Riordan *et al.*, 2003).

Mouse infections

Monotypic and CI infections were performed as previously described with the following modifications (Auerbuch *et al.*, 2001). Exponential phase bacterial cultures were diluted in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline, and 2×10^5 bacteria in 200 μl were injected i.p. into 4- to 6-week-old C57BL/6 J mice (Jackson Laboratories, Bar Harbor, ME). At 72 h post infection, the animals were sacrificed, and livers and spleens homogenized in 0.1% NP-40, serially diluted, and plated onto Luria–Bertani (LB) agar. For the CI infections, the homogenization buffer also contained 0.1 μg ml^{-1} erythromycin (erm), and the lysate was plated on LB agar with or without 1 μg ml^{-1} erm. The CI was calculated by dividing the number of wild-type cfu (erm sensitive) by the number of mutant cfu (erm resistant).

Quantitative real-time polymerase chain reaction

After infection of J774 macrophages at a multiplicity of infection (moi) of 1:1 for 6 h, host cells were lysed with 50 mM Tris, pH 8, 150 mM NaCl and 0.05% NP-40 for 10 min on ice. After vortexing and pelleting the host nuclei at 3000 *g* for 30 s, the remaining supernatant was used to isolate bacterial RNA with the Pro-Blue Fast RNA kit (MP Biomedicals) followed by DNase digestion on Qiagen RNeasy minicolumns. cDNA was generated from 2.5 μg of RNA using random primers and M-MLV reverse transcriptase (Invitrogen). Primers for *lplA1*, *lplA2* and *rpoA* (Table S1) were used to amplify cDNA, which was quantified by SYBR Green fluorescence (Stratagene Mx3000p).

Lipoyl substrates

Lipoamide (T5875), PDH (P-7032) and KGDH (K1502) were purchased from Sigma-Aldrich. Prior to using commercial PDH and KGDH, the enzymes were precipitated and washed with Bio-Rad ReadyPrep 2-D Cleanup Kit. Trypsin digestion was performed at 30°C for 4 h in 0.02% Tween-20 and 200 mM ammonium bicarbonate (pH 8.9). The proteins were

digested with a ratio of 1 mg of protein to 8 μg of proteomics grade trypsin in a total volume of 500 μl; the reaction was stopped with 0.0003% trifluoroacetic acid. ProK digestion was performed at a ratio of 5 mg of protein to 20 μg of enzyme (Sigma P-4850) in 20 mM Tris-HCl, 1 mM $CaCl_2$ at pH 7.4 at 25°C for 4 h and heat inactivated for 10 min at 90°C. Enzymatic digestion was confirmed by SDS-PAGE and immunoblotting analysis for lipoylated peptides. For dialysis experiments, ProK-digested samples were loaded in 100 or 500 Da Spectra/Por Micro DispoDialyzer membranes and dialysed against 20 mM Tris-HCl, 1 mM $CaCl_2$ at pH 7.4. Synthetic lipoyl tripeptide $DK^{L}A$ was synthesized by Anaspec using a previously published protocol (Fig. S2A and B) (Konishi *et al*., 1996). Digestion of $DK^{L}A$ with immobilized aminopeptidase M was performed at a ratio of 0.5 U enzyme to 250 μg of peptide for 18 h at 37°C (Pierce; #20238); digestion was confirmed by LC-MS on a nanoAcuity/Qtof premier instrument. The non-digested and digested samples were injected on a C18 column and analysed in positive ion mode (Fig. S2C). Non-lipoylated DKA was synthesized and LC-MS was performed by the Protein Structure Facility of the University of Michigan Medical School.

Subcellular fractionation

Mitochondria were isolated by lysis of 1×10^7 macrophages in 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM PMSF, incubation on ice for 10 min and vortexing for 1 min. After lysis, salts were added to a final concentration of 30 mM HEPES (pH 7.9), 140 mM KCl and 3 mM $MgCl_2$. After removing nuclei by centrifuging at 3000 *g* for 30 s and washing three times, mitochondria were spun at 80 000 *g* for 4 h at 4°C. The remaining supernatant was retained and concentrated in a 3 kDa Ultracel YM-3 Centricon Centrifugal Filter Device (Amicon).

Protein analysis

Bacteria were prepared and analysed by immunoblot with an anti-lipoic acid antibody (Calbiochem) as previously described (O'Riordan *et al*., 2003) except washed exponential phase cultures (OD_{600} 0.5) were used after growth in IMM^{-L} or BHI at 37°C. Protein lysate was prepared with FastProtein Blue Lysing Matrix (MP Biomedicals) by processing in a FastPrep machine for 40 s at a setting of 6.0 in Lysing Matrix B. For cytosolic and mitochondrial peptide analysis by immunoblot, 0.75 M Tris-HCl was used in the resolving gel, running buffer and loading buffer.

Bacterial infections

J774 macrophages were infected as previously described (O'Riordan *et al*., 2003) except washed exponential phase cultures grown at 37°C in IMM^{-L} pre-starved bacteria were used at a moi of 3, resulting in ~10% macrophage infection. In DHLA (Sigma-Aldrich T-8260) supplementation experiments, 50 μM DHLA was added to the J774 macrophage growth medium prior to infection and maintained throughout the infection. Addition of high concentrations of non-reduced lipoic acid resulted in precipitation, as well as non-specific host cell effects. Data points represent the mean with standard deviation.

E. coli *complementation*

TM131 (*rpsL lipA182::*Tn*1000*dKn *lplA148::*Tn*10*dTc) and its parental wild-type strain JK1 (*rpsL*) were transformed with *E. coli lplA*, *L. monocytogenes lplA1* or *lplA2* in the IPTG-inducible ptac85 plasmid and plated onto LB agar containing 0.4% glucose, 5 mM sodium acetate, 5 mM sodium succinate and 100 μg ml^{-1} ampicillin. Primers used to amplify the lipoate ligase genes with Pfx platinum polymerase are listed in Table S1. TM131-transformed strains were also grown in the presence of 50 μg ml^{-1} kanamycin and 125 μg ml^{-1} tetracycline. Transformants were restreaked onto M9 minimal medium plates containing 0.4% glucose, 1 mM IPTG, thiamine (10 μg ml^{-1}), 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and the appropriate antibiotics with or without 5 mM sodium acetate and 5 mM sodium succinate as indicated. Lysates for Western blotting were grown in LB containing 0.4% glucose, 5 mM sodium acetate, 5 mM sodium succinate, 1 mM IPTG and the appropriate antibiotics.

Structural modelling of LplA1 and LplA2

Because of its high homology with LplA1 (65.8%) and LplA2 (66.6%), the structure of LplA from *S. pneumoniae* (PDB ID: 1VQZ; Joint Center for Structural Genomics) was used as the foundation for modelling the LplA1 and LplA2 structures. To model the bound forms of LplA1 and LplA2, the N- and C-terminal domains of 1VQZ were first aligned onto the respective domains of the lipoate-bound form of *E. coli* LplA (PDB ID: 1X2H) using the graphics program O, then the amino acid sequence of 1VQZ was mutated into that of LplA1 (Jones *et al*., 1991; Fujiwara *et al*., 2005). Amino acid insertions and deletions were fit using the lego-loop option in O. The resulting LplA1 model was then placed into a box of waters containing a minimum of two shells of water, minimized and put through simulated annealing using torsion angle dynamics in Crystallography & NMR System (CNS; Brunger *et al*., 1998). The lipoate structure from 1X2H was attached to the NZ atom of a modelled lysine residue using O. CNS parameter and topology files for the lipoate-lysine residue were created via the HIC-Up server (http://xray.bmc.uu.se/cgi-bin/gerard/hicup_server.pl). The tripeptide ($DK^{L}A$) was then created via CNS and modelled into the refined LplA1 model using O. Simulated annealing was then performed on the $DK^{L}A$-bound LplA1 model. To create the LplA2 model, the amino acid sequence of LplA2 was overlaid onto the bound form of LplA1. The resulting $DK^{L}A$-bound LplA2 model was placed into a box of waters and put through simulated annealing.

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