The Role of Lipoate Dependent Metabolism During Intracellular Growth and Pathogenesis of *Listeria monocytogenes*

by

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In memory of Allison Lynn Miller

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Abstract

The Gram-positive bacterial pathogen *Listeria monocytogenes* replicates rapidly in the host cytosol, indicating it efficiently utilizes host-derived nutrients. The critical co-factor lipoate is one nutrient this bacterium must scavenge for optimal intracellular growth. Although the *L. monocytogenes* genome encodes two putative lipoate ligases, LplA1 and LplA2, intracellular replication and virulence require only LplA1. The thesis research described here shows that LplA1 enables *L. monocytogenes* to use small host derived lipoyl-peptides, revealing an adaptive mechanism to exploit the host cytosol for essential nutrients. Furthermore, I provide evidence that a single amino acid residue on the surface of LplA1 is required for *L. monocytogenes* growth with lipoyl-peptides, as well as LplA1 mediated intracellular growth and spread. Lipoate dependent metabolism in *L. monocytogenes* controls the amino acid and anteiso-branched chain fatty acid (BCFA) composition of the bacterium, which is critical for intracellular replication. My thesis studies demonstrate that a bacterial enzyme, LplA1, enables usage of host-derived lipoate and plays a key role in the metabolism and pathogenesis of *L. monocytogenes*. The ability to utilize diverse sources of lipoate is likely to be a common theme in pathogenesis, as some parasites, while capable of synthesizing their own lipoate, must also scavenge lipoate from the host

environment. This thesis has illustrated that understanding how microbes specifically alter their metabolism to exploit the host as a replicative environment will lead to a better understanding of disease processes and will reveal targets for development of theraputics.

Chapter 1

ENVIRONMENTAL PREVALENCE OF *Listeria monocytogenes*

The ability of a microbial pathogen to replicate and persist in diverse and varied environments directly impacts the fitness of the organism in both the environment and in its host. The ubiquitous Gram-positive bacterium *Listeria monocytogenes* has been isolated in diverse nutritional environments including soil, water, decaying vegetation, sewage sludge, high salt growth conditions, and both acidic and alkaline conditions. *L. monocytogenes* has also been isolated in birds, fish, shellfish, and a wide variety of mammalian hosts (Gandhi and Chikindas, 2007). The ability of this pathogen to replicate even at refrigeration temperatures makes *L. monocytogenes* contamination of soft cheeses, dairy products, sausages, smoked fish, salads, and other industrially produced ready-to-eat products a major concern in the food industry (Vazquez-Boland *et al.,* 2001). *L. monocytogenes* can survive and even thrive in diverse nutrient conditions, indicating the bacterium may employ multiple mechanisms to adapt its metabolism to exploit different environments. A major aim of this thesis is to characterize how this bacterial pathogen adapts its metabolism to thrive while in one of these growth environments, the mammalian host.

INFECTION, CIRCULATION, AND DISEASE WITHIN THE HUMAN HOST

L. monocytogenes is the etiologic agent of Listeriosis, which can present itself in the elderly, newborn, and immunocompromised human host as meningitis, encephalitis, and septicemia, as well as cause spontaneous miscarriage in pregnant women. While the occurrence of Listeriosis is currently low (3.1 cases/2.5 million), the disease has a mortality rate greater than 20%, far exceeding the mortality rates of other common food-borne pathogens such as *Salmonella enteritidis* (0.38%), *Campylobacter* species (0.02-0.01%) and *Vibrio* species (0.005-0.01%) (Voetsch *et al.,* 2007). Further complicating this illness, the incubation time prior to presenting symptoms in humans is long (20-30 days), making it difficult to identify the pathogen and its source (Riedo *et al.,* 1994).

To cause disease post-ingestion, *L. monocytogenes* must survive the low pH of the stomach, breach the intestinal barrier, and systematically spread within the host through intracellular replication in phagocytic and non-phagocytic cells (Chaturongakul *et al.,* 2008). After crossing the intestinal barrier, *L. monocytogenes* has been isolated replicating in the mesenteric lymph nodes, spleen, and the liver in many different cell types, including macrophages, hepatocytes, epithelial cells, endothelial cells and fibroblasts (Pron *et al.,* 1998; Vazquez-Boland *et al.,* 2001). Infection of the host cell begins by internalization of *L. monocytogenes*, where the bacteria are contained in primary membrane-bound vacuoles that become acidified soon after uptake (Fig. 1.1) (Tilney and Portnoy, 1989). After infection, *L. monocytogenes* mediates its own escape from

the vacuole in a process that is dependent upon secretion of a cholesterol dependent cytolysin, listeriolysin O (LLO). LLO deficient bacteria remain in 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 to replicate within an infected host.

After entry into the macrophage cytosol, the bacterium replicate with a doubling time approximating the doubling time of the bacterium in rich broth medium (Marquis *et al.,* 1993). During cytosolic replication, *L. monocytogenes* expresses and secretes the surface protein ActA, which nucleates polymerization of host actin. Actin polymerization at a bacterial pole enables motility in the cytosolic environment, with subsequent spread after the bacteria push randomly against the cell outer membrane to form long pseudopods that are taken up by adjacent cells. *L. monocytogenes* then escapes from the secondary vacuole directly into the host cytosol, enabling its own spread within the host while minimizing its exposure to extracellular immune effectors such as antibodies and neutrophils (Fig. 1.1)(Tilney and Portnoy, 1989). *L. monocytogenes* is eventually cleared in a non-immunocompromised host by innate and acquired immune mechanisms (Gregory and Liu, 2000; Torres *et al.,* 2005).

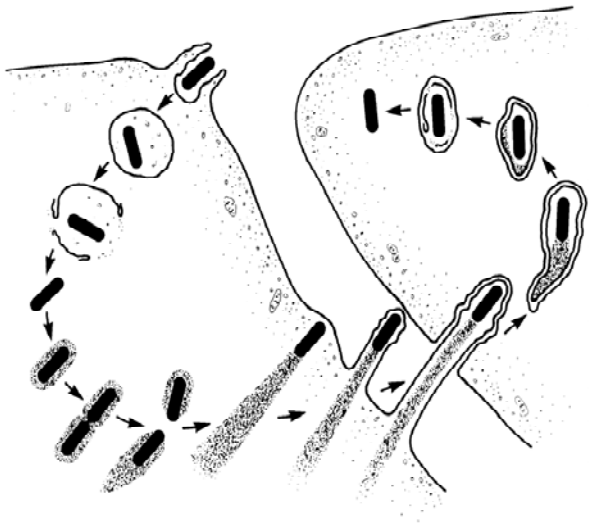


Figure 1.1 Intracellular lifecycle of *Listeria monocytogenes*

From (Tilney and Portnoy, 1989). *L. monocytogenes* is internalized into the host cell and encapsulated by host vacuoles. Once engulfed, the bacteria destroy the vacuole with the aid of the cholesterol dependent cytolysin, Listeriolysin O. Upon escape from the vacuole, *L. monocytogenes* efficiently replicates in the cytosol and secretes ActA, which enables nucleation and polymerization of host actin. After formation of actin tails, the intracellular bacteria are propelled randomly within the cytosolic environment, including against the membrane of its host cell. Finger-like protrusions into neighboring cells facilitate adjacent cell uptake of bacteria, with subsequent escape from the double membrane vacuole.

PRFA-REGULATED VIRULENCE

PrfA, which encodes positive regulatory factor A, is one of several genes that have been implicated in aiding *L. monocytogenes* intracellular growth and spread. PrfA is a transcription factor, which is a protein that binds to specific promoter DNA sequences to control transcription. After *L. monocytogenes* escapes from the host vacuole into the cytosol, PrfA is activated and regulates expression of many virulence factors, including the gene cluster *Listeria* pathogenicity island 1 (LIPI-1). LIPI-1 encodes many of the virulence factors that are critical for pathogenesis, including the *hly* gene encoding LLO, as well as

ActA. Other virulence genes encoded by LIPI-1 include *plcA, plcB,* and *mpl*, which encode two enzymes with phospholipase C (PLC) activity and a metalloprotease, respectively. PrfA can also regulate the expression of two internalins, InlA and InlB, which are required for bacterial internalization into non-phagocytic cells (Lingnau *et al.,* 1995). When point mutants of PrfA that result in constitutive overexpression of PrfA regulated genes were generated, *L. monocytogenes* rapidly utilized glucose-1-phosphate (G-1-P), suggesting that G-1-P utilization was also under control of PrfA (Ripio *et al.,* 1997). It is understood that PrfA is a critical protein encoded by *L. monocytogenes* that contributes significantly to its pathogenesis.

While PrfA regulates the expression of *L. monocytogenes* virulence factors, PrfA expression and activity is regulated by sigma factors and a variety of environmental factors. One of the environmental factors that regulate PrfA is temperature. PrfA dependent transcription of virulence factors was weak at 30°C, but comparatively induced at the higher temperature 37°C. However, even when *L. monocytogenes* was grown at 37°C in the rich broth medium BHI, *prfA* expression was not maximal unless activated charcoal was also added, suggesting there are environmental signals in BHI that need to be removed prior to full expression of this global virulence regulator (Ripio *et al.,* 1996). Carbon availability also regulates PrfA dependent virulence gene expression, as when the bacterium was grown in different carbon sources such as cellobiose, PrfA-dependent transcription of *hly* was reduced, but when grown in glycerol, PrfA

dependent genes were upregulated (Gilbreth *et al.,* 2004; Joseph *et al.,* 2008; Milenbachs *et al.,* 1997). In conclusion, the environmental factors that regulate *L. monocytogenes* virulence are still being characterized. However, a deeper understanding of the nutritional environment that the bacterium encounters while growing in host tissues will provide clues as to how this pathogen regulates its virulence.

ROLE OF THE NUTRIENT ENVIRONMENT ON MICROBIAL PATHOGENESIS

CARBON SOURCE ADAPTATION IS CRITICAL FOR PATHOGENIC SURVIVAL IN DIVERSE ENVIRONMENTS

To fully appreciate the influence upon virulence that the host nutritional environment plays, it is necessary to examine what nutrients pathogens encounter while in their growth compartments. Carbon is a critical nutrient that all living organisms require to support replication. However, carbon can also influence the expression of virulence genes in *L. monocytogenes*. Therefore, pathways for carbon utilization and virulence regulation may be linked. In *V. cholerae,* passage through the intestinal tract induces genes involved in succinate, glycine, and chitin utilization that enhanced or regulated the ability of the pathogen to persist within cholera stool and aquatic environments (Schild *et al.,* 2007). This observation suggests the bacterium could adapt to different carbon environments. Carbon source adaptation also occurs in *Streptococcus pneumoniae,* where two sucrose uptake systems are differentially expressed during lung infection and nasopharynx colonization (Iyer and Camilli, 2007). The

expression of distinct carbon uptake systems in different tissues indicates there may be differences in sucrose containing substrates in both regions. Finally, in *L. monocytogenes*, two glycerol kinases were are important for bacterial replication in the cytosol of one cell type, but not another. Deletion of *glpK-2* and *glpD* impaired impairs *L. monocytogenes* growth in Caco-2 cells but not J774 macrophages, suggesting the glycerol supply may be different in distinct cell types (Joseph *et al.,* 2008). Further studies are needed to determine what adaptive mechanisms *L. monocytogenes* has evolved to utilize the different carbon sources it encounters while replicating in its host.

PATHOGEN COMPARTMENTALIZATION

Within the macrophage, intracellular pathogens can evade microbicidal functions of their host by escaping from the phagosome into the cytosol, stalling phagosome maturation into acidified phagolysosomes, diverting their vacuole from the endocytic pathway, or even replicating within the harsh degradative phagosome. All of these compartments represent unique nutrient environments. For example, in the vacuolar compartment, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) mutants deficient for purine, pyrimidine, aromatic amino acid, and methionine biosynthesis were are avirulent (Fields *et al.,* 1986; Leung and Finlay, 1991). These data suggest the intracellular growth environment for *S.* Typhimurium was limiting for these nutrients. Additionally, during its intracellular lifecycle, *Brucella* sp. replication requireds acquisition of membranes derived from the ER, suggestive that essential nutrients that support

replication were lacking prior to fusion with the ER membrane (Celli, 2006). Finally, after *Coxiella burnetii* was is internalized, the autophagic pathway modulated modulates development of its vacuole (Voth and Heinzen, 2007); . it has been speculated tThis interaction may provide *C. burnetii* with nutrients that are limiting in the vacuole (Voth and Heinzen, 2007). All of these pathogens have likely evolved mechanisms to exploit their distinct environments for nutrients to support replication.

In contrast to many bacterial pathogens that primarily reside in host vacuoles, *L. monocytogenes* can escape into the host cytosol, a nutrient rich environment where it is thought the host cell stores many metabolic intermediates. Because cytosolic pathogens may have better access to host nutrients, initially it was hypothesized that cytosolic pathogens could replicate in this environment only because they have access to the host derived nutrients in the cytosol. However, the bacterium *Bacillus subtilis* grew poorly in this environment when it was engineered to express LLO and enter the cytosol (Goetz *et al.,* 2001; Portnoy *et al.,* 1992). Additionally, when bacteria that normally do not grow in the cytosol, such as *Legionella pneumophila,* uropathogenic *Escherichia coli*, *Yersinia enterocolitica, Listeria innocua,* and *S.* Typhimurium, were directly microinjected into the cytosolic environment of Caco-2 or macrophage cells, they failed to efficiently replicate. In the same study, it was demonstrated bacterial replication could occur when cytosolic pathogens such as *L. monocytogenes*, *Shigella flexneri,* and enteroinvasive *E. coli* were microinjected (Goetz *et al.,* 2001).

Thus, even though cytosolic pathogens such as *L. monocytogenes* appear to have access to relatively more nutrients than vacuolar bound pathogens, cytosolic bacteria have likely evolved specific mechanisms, such as nutrient scavenging as well as innate immune defense mechanisms, to exploit their replicative niche. Understanding what nutrients *L. monocytogenes* requires while growing in this environment will lead to an clearer understanding of how the nutrient environment influences pathogenesis.

NUTRIENT ENVIRONMENT OF *L. monocytogenes*

NUTRIENT REQUIREMENTS OF *L. monocytogenes* *IN VITRO*

The host cytosol contains a complex mixture of nutrients that are often attached to functional host proteins. Therefore, the nutrients the host can potentially offer an intracellular pathogen could be spatially and chemically restricted. As such, it is difficult to know what nutrients are available to *L. monocytogenes* while it grows within the host cytosol. However, much more is known about the requirements for replication of the bacterium when outside the host cytosol in defined minimal medium. *L. monocytogenes* can grow under both aerobic and anaerobic conditions, and requires the vitamins lipoic acid, riboflavin, thiamine and biotin to be provided in its environment for optimal replication (Table 1.1)(Phan-Thanh and Gormon, 1997; Tsai and Hodgson, 2003). The genes that synthesize these four vitamins are missing from the genome of *L. monocytogenes,* and the requirement for these vitamins in defined medium indicates *L. monocytogenes* must scavenge them from its growth environment

(Tsai and Hodgson, 2003). *L. monocytogenes,* as well as many other organisms, then use these essential cofactors to generate their own metabolites to support replication. One of these cofactors, lipoate, is a required component of the E2 subunit of metabolic enzyme complexes such as pyruvate dehydrogenase (PDH) and branched chain α-keto acid dehydrogenase (BCKD)(Premaratne *et al.,* 1991). It is the goal of my thesis to understand the mechanism of how scavenged lipoate modulates the nutritional requirements of *L. monocytogenes* while growing in the host cytosol. To understand how lipoate influences *L. monocytogenes* pathogenesis, we must first review exactly when lipoate is required during biosynthesis and what nutrients this cofactor helps to generate.

Table 1.1 Composition of *L. monocytogenes* improved minimal medium

COMPONENT	G L^{-1}
Ferric citrate	0.088
Glucose	10
L-Glutamine	0.6
L-Leucine	0.1
DL-Isoleucine	0.1
DL-Valine	0.1
DL-Methionine	0.1
L-Arginine	0.1
L-Cysteine	0.1
L-Histidine	0.1
L-Tryptophan	0.1
L-Phenylalanine	0.1
Adenine	2.5
Biotin (mg)	0.5
Riboflavin (mg)	5
Thiamine (mg)	1
Pyridoxate (mg)	1
Para-aminobenzoic acid (mg)	1
Calcium panthenate (mg)	1
Nicotinamide (mg)	1

Lipoic Acid (µg)	5
Table adapted from (Phan-Thanh and Gormon, 1997)	

PENTOSE PHOSPHATE PATHWAY AND GLYCOLYSIS

The transformation of carbon into acetyl-CoA with metabolic enzyme complexes can require lipoate. *In vitro, L. monocytogenes* is capable of growing with a variety of different carbon sources including glucose, fructose, trehalose, cellobiose, maltose, glycerol, and mannose (Phan-Thanh and Gormon, 1997; Tsai and Hodgson, 2003). While glucose catabolism can occur through both glycolysis and the pentose phosphate pathways, products of the pentose phosphate pathway are fed into glycolysis, where they are eventually converted into pyruvate. Pyruvate can then act as a substrate for pyruvate dehydrogenase (PDH) during the biosynthesis of acetyl-CoA (Fig. 1.2) (Eisenreich *et al.,* 2006; Joseph *et al.,* 2006). PDH, a large complex containing many copies of the three subunits E1, E2, and E3, is lipoylated on the E2 subunit. The generation of acetyl-CoA is catalyzed when pyruvate reacts with the E1 subunit, where the product is then oxidized to acetate with a lipoyl-group on the E2 subunit. The protruding lipoyl-group on the E2 subunit can then spatially swing the acyl moiety to other regions of the E2 subunit, where acyl is finally transferred to CoA (Perham, 2000). The attachment of lipoate to the E2 subunit is therefore critical for the generation of acetyl-CoA through PDH. Acetyl-CoA is then used as a

substrate for the Tricarboxylic Acid Cycle (TCA) in the generation of many essential nutrients.

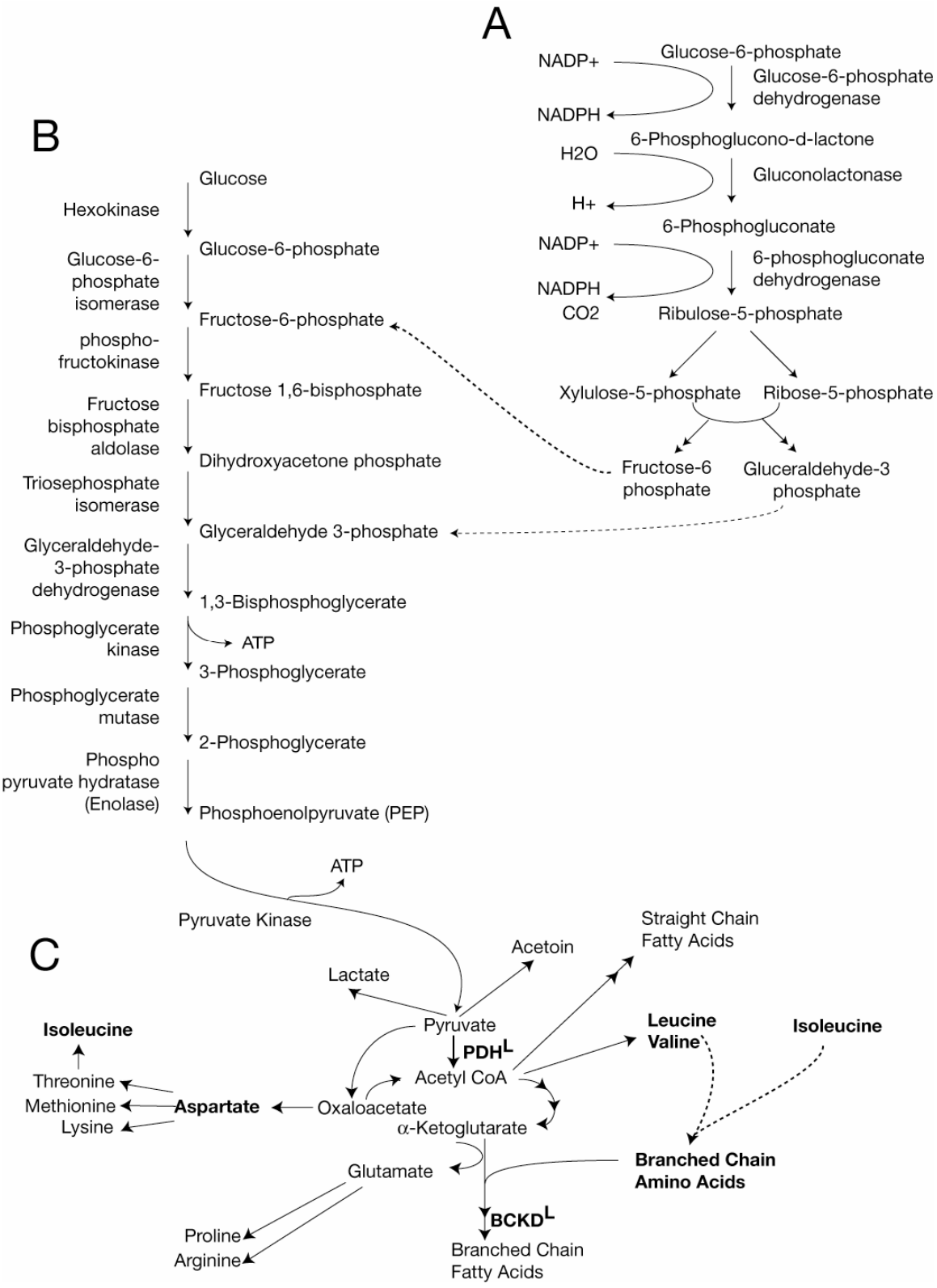


Figure 1.2 Predicted metabolite production in *Listeria*

Based upon genomic analysis, glycolysis and the pentose phosphate pathway is intact in *L. monocytogenes,* while the tricarboxylic acid cycle is incomplete {Glaser, 2001, p03415}. (A) This portion of the figure represents the pentose phosphate pathway. When *L. monocytogenes* was is grown in the cytosol of host cells rather than BHI, genes encoding the pentose phosphate pathway were are upregulated {Joseph, 2006, p05246}. In the pentose phosphate pathway, glucose-6-phosphate is oxidatively decarboxylated to form 6-phosphoglucono-d-lactone, hydrated, and then oxidatively decarboxylated to generate ribulose-5-phosphate. Ribulose-5-phosphate is then isomerized or epimerized into either ribose-6-phosphate or xylulose-5-phosphate. After conversion of these products into glyceraldehyde-3-phosphate and fructose-6-phosphate, the products of the pentose phosphate pathway are fed into glycolysis. (B) Glycolysis is diagrammed in this section of the figure. When *L. monocytogenes* was grown in BHI instead of the host cytosol, genes encoding glycolysis were upregulated {Joseph, 2006, p05246}. In glycolysis, glucose is phosphorylated and then rearranged into fructose-6-phosphate, which is then converted into fructose 1,6-bisphosphate. The hexose ring can then be split by an aldolase into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The dihydroxyacetone phosphate is then isomerized into glyceraldehyde 3-phosphate and then dehydrogenated and dephosphorylated to form 3-phosphoglycerate and ATP. The 3-phosphoglycerate is then converted into 2-phosphoglycerate by a mutase; the product then acts as a precursor for phosphoenolpyruvate, which is then dephosphorylated to form pyruvate and ATP with pyruvate kinase. (C) Amino and fatty acid biosynthesis is diagrammed in this section of the figure. During intracellular growth, pyruvate in *L. monocytogenes* can be converted into valine, leucine, oxaloacetate, or acetyl-CoA with lipoylated pyruvate dehydrogenase (PDH^L). These biosynthetic precursors then generate many other amino acids. **PDH^L and the amino acids in bold typeface have been either directly or indirectly implicated in *L. monocytogenes* intracellular growth {MO'Riordan, 2003, p00143; Eylert, 2008, p08180}.**

TRICARBOXYLIC ACID CYCLE

When compared to the well characterized TCA cycle in *E. coli,* the *L. monocytogenes* TCA cycle is incomplete as it is missing enzyme activity and the genes for α-ketoglutarate dehydrogenase, succinyl coenzyme A synthetase, succinate dehydrogenase, and malate dehydrogenase (Eisenreich *et al.,* 2006; Glaser *et al.,* 2001b; Kim *et al.,* 2006; Trivett and Meyer, 1971). Therefore, α-ketoglutarate cannot be oxidatively decarboxylated into oxaloacetate by α-ketoglutarate dehydrogenase (Fig. 1.3). However, oxaloacetate can still be

produced by *L. monocytogenes* directly through carboxylation of pyruvate, bypassing the requirement for lipoate in the reductive branch of the TCA cycle (Eylert *et al.,* 2008). Many of the products generated by both the lipoate dependent and lipoate independent branches of the *L. monocytogenes* TCA cycle are precursors for essential metabolites, such as amino acids, fatty acids, and nucleotides.

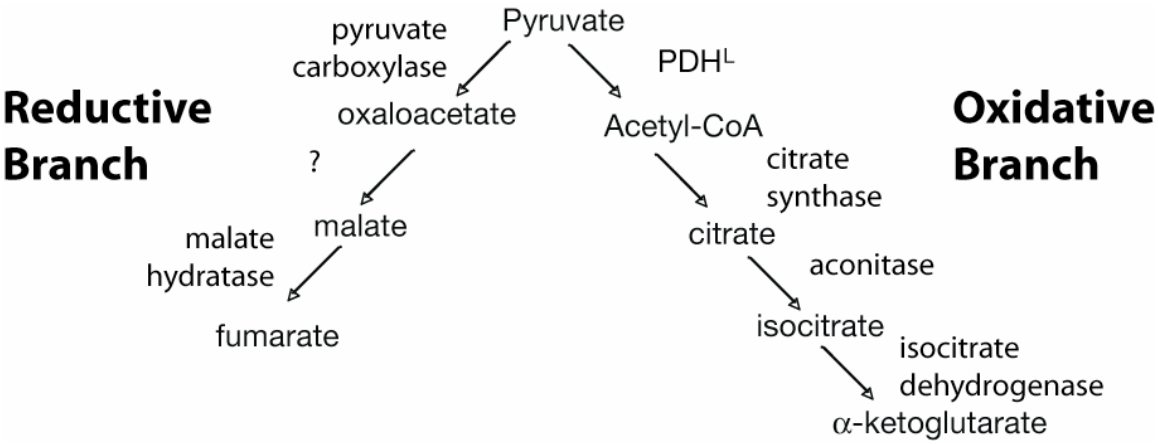


Figure 1.3 Predicted Tricarboxylic Acid Cycle in *L. monocytogenes*

Pyruvate is oxidatively decarboxylated by lipoylated pyruvate dehydrogenase to form acetyl-CoA. Through aldol condensation of oxaloacetate and acetyl-CoA with citrate synthase, citrate is formed. Citrate then is dehydrated and hydrated by aconitase, generating isocitrate. Isocitrate is then oxidatively decarboxylated by isocitrate dehydrogenase to form α-ketoglutarate and NADH. On the reductive branch, pyruvate is directly carboxylated into oxaloacetate by pyruvate carboxylase. The enzyme involved in malate biosynthesis is currently unidentified, but malate is then converted in *L. monocytogenes* to fumarate with malate hydratase {Eisenreich, 2006, p02290; Glaser, 2001, p03415}.

AMINO ACID BIOSYNTHESIS

In *E. coli* and in other organisms used as model systems for characterization of metabolic pathways, TCA intermediates are used to generate amino acids

(Kanehisa *et al.,* 2002) (Fig. 1.2C). α-ketoglutarate generated by the TCA cycle is transanimated into glutamate, which is a precursor for the amino acids proline and arginine. In *E. coli,* aspartate, lysine, methionine, and threonine are all biosynthetically generated from oxaloacetate (Kanehisa *et al.,* 2002) (Fig. 1.2C). The generation of the branched chain amino acids valine, leucine, and isoleucine (BCAAs) then occurs by transanimation of pyruvate and threonine (Kanehisa *et al.,* 2002) (Fig. 1.2C). While these pathways have been well characterized in *E. coli*, in *L. monocytogenes* they have not yet been confirmed to be active or functional when the bacterium is growing in diverse nutrient environments.

FATTY ACID BIOSYNTHESIS

L. monocytogenes contains a mixture of straight-chain fatty acids (SCFA) and branched chain fatty acids (BCFA) in its membrane (Zhu *et al.,* 2005a). In bacteria and plants, *de novo* synthesis of SCFAs occurs using acetyl-CoA as the primer and malonyl-CoA as a chain extender of the primer, producing SCFAs such as palmitic, stearic, hexadecenoic, octadecenoic, cyclopropanic, 10-methylhexadecanoic, and 2- or 3-hydroxyl fatty acids (Kaneda, 1991) (Fig. 1.4). Unlike SCFAs, BCFAs, such as iso-, anteiso-, and ω-alicyclic fatty acids, are neither present nor synthesized in all bacteria (Kaneda, 1991). However, it is thought that the biosynthesis pathway to generate BCFAs is the same as that for synthesis of SCFAs, where the only difference is the primer source (Fig. 1.4**)**. BCFA biosynthesis uses a primer with a branched carbon rather than acetyl-CoA (Kaneda, 1991) (Fig. 1.4). In bacteria, there are three types of branched primer

sources; branched-chain α-keto acids, branched short-chain carboxylic acids, and cyclic carboxylic acids (Kaneda, 1991).

To use branched short-chain carboxylic acids as primers, they first need to be transformed into an acyl-CoA ester (Fig. 1.4). The enzyme used for this process is the lipoylated branched-chain α-keto acid dehydrogenase complex (BCKD) (Kaneda, 1991) (Fig. 1.2, 1.4). These acyl-CoA esters are not efficiently used by branched-chain fatty acid synthetase, and are converted first to their acyl-carrier protein (ACP) derivative prior to condensation with malonyl-CoA to form a BCFA (Kaneda, 1991) (Fig. 1.4). When bacteria, such as *B. subtilis,* use α-keto acids as primer sources, the process of BCFA production is slightly different. While it was initially thought BCKD was essential for this process, CoA and NAD, cofactors for BCKD, inhibited rather than enhanced synthesis of BCFA from α-keto acid substrates (Kaneda, 1973). Instead, a branched chain α-keto acid decarboxylase was determined to be essential for BCFA biosynthesis from branched chain α-keto acid substrates (Oku and Kaneda, 1988). The decarboxylated primer then is thought to condense with malonyl-ACP to form BCFAs (Kaneda, 1991).

The role of BCKD in production of BCFAs is the only mechanism experimentally confirmed in *L. monocytogenes* BCFA biosynthesis (Zhu *et al.,* 2005a). When two subunits of *L. monocytogenes* BCKD were are disrupted by transposon insertion, BCFA content was is decreased from 90% of total fatty acid to less

than 40% of total fatty acid. However, when branched short-chain carboxylic acids such as 2-methylbutyrate were are added to the mutant strains, BCFA production was is restored, indicating an alternate mechanism in *L. monocytogenes* for generation of BCFA other than BCKD (Zhu *et al.,* 2005a) (Fig. 1.4). Unfortunately, there is not a clear picture of how SCFA or BCFAs are produced in *L. monocytogenes* as little work has been done to clarify what enzymes are involved. However, the lipoylated BCKD complex does appear to influence fatty acid composition in this bacterium.

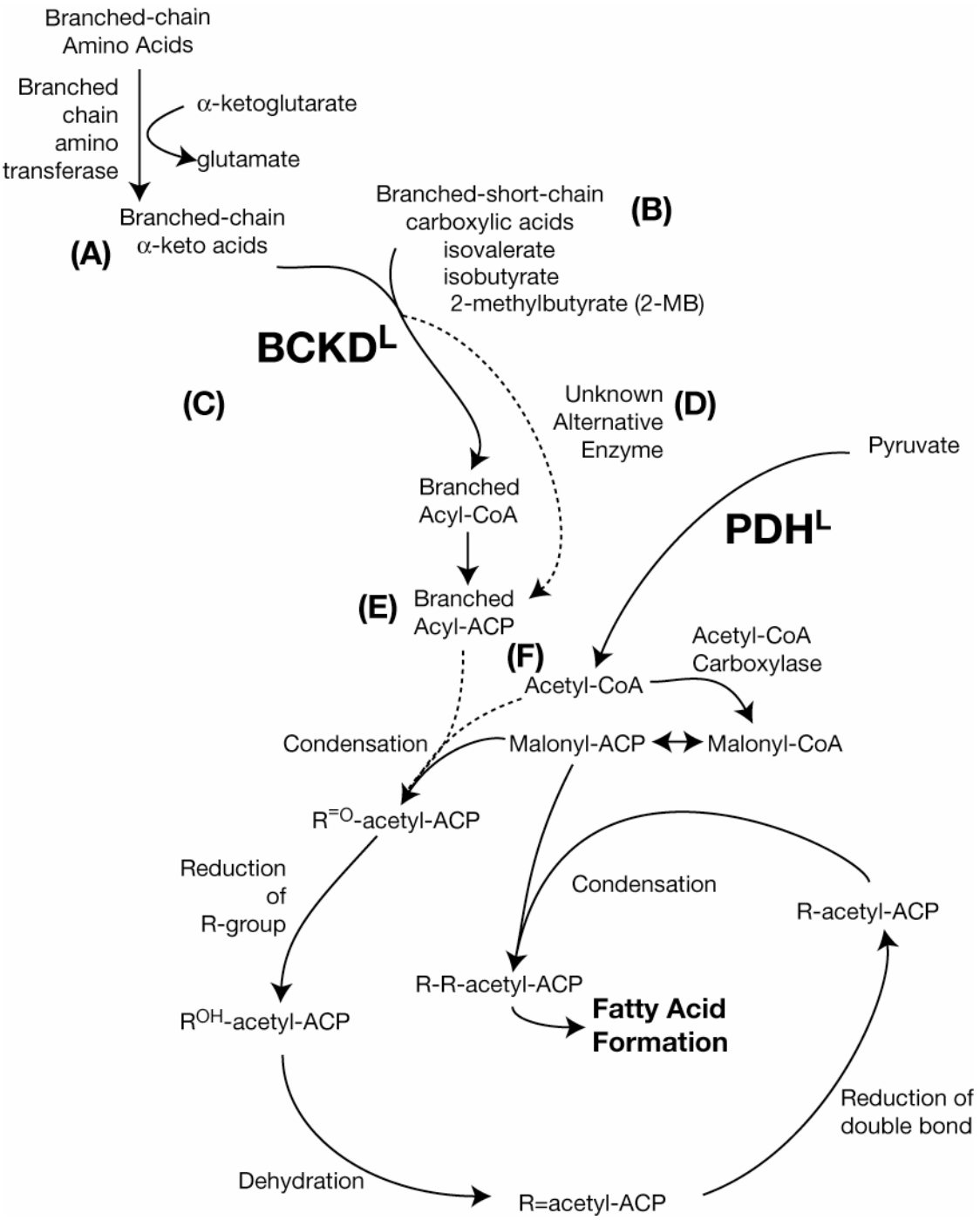


Figure 1.4 Model of fatty acid biosynthesis in *L. monocytogenes*

Generation of branched and straight chain fatty acids in *L. monocytogenes* is largely uncharacterized. This figure was generated by incorporating data reviewed in (Kaneda, 1991). To generate straight chain fatty acids, acetyl-CoA is thought to condense with malonyl-ACP. The acetyl R group (F) then is reduced, dehydrated, and reduced again before condensing with another malonyl-ACP moiety to form an elongated fatty acid. This final condensation repeats multiple times to generate different chain length straight

chain fatty acids. In the generation of branched chain fatty acids, the R group is instead donated from branched acyl-ACPs (E). Branched acyl-ACPs can be generated in *Bacillus subtilis* from transanimated branched chain amino acids (A), as well as directly from branched short chain carboxylic acids, such as isovaleric, isobutyric, and 2-methylbutyric acid (B) (Kaneda, 1991). These branched carbon donors can be converted to branched acyl-CoAs with lipoylated branched-chain α-keto acid dehydrogenase (BCKD, C), and then converted to their corresponding ACP derivatives. Alternatively, an unknown enzyme can also generate branched acyl-ACPs from branched-short-chain carboxylic acids and possibly also branched-chain α-keto acids (D), as mutants for BCKD can still generate branched chain fatty acids, albeit at reduced levels (Zhu *et al.,* 2005a). **In all of these pathways, lipoylated metabolic enzyme complexes play a critical role in the generation of intermediates during the biosynthesis of fatty acids.**

NUTRIENT REQUIREMENTS OF INTRACELLULAR *L. monocytogenes*

The nutrient environment surrounding *L. monocytogenes* in the cytosolic compartment may dictate its biosynthesis of metabolites that may be lacking in this compartment. At the start of this thesis, it was known that PrfA was required for the transcription of a hexose phosphate transporter (*hpt*) (Chico-Calero *et al.,* 2002). When this transporter was deleted from the genome, the bacterium could still escape into the cytosol, but it was modestly impaired for optimal intracellular replication (Goetz *et al.,* 2001). Furthermore, *hpt* mutant bacteria were approximately 10-fold less virulent than wildtype bacteria in a mouse model of infection (Chico-Calero *et al.,* 2002). These data suggested that when growing in the cytosol of mammalian host tissues, *L. monocytogenes* has access to, and uses, hexose phosphates as a carbon source. Further studies with ^{13}C labeled glucose and mutants of *L. monocytogenes* defective in the uptake of glucose 6-phosphate, glycerol catabolism, and dihydroxyacetone phosphorylation confirmed glucose 6-phosphate was a major carbon source for the bacterium

when growing in the cytosol of J774A.1 cells (Eylert *et al.,* 2008). *L. monocytogenes* may also transport oligopeptides from the host, as a deletion mutant for *oppA*, which encodes oligopeptide binding protein, was slightly impaired in cytosolic replication in macrophages (Borezee *et al.,* 2000). These data suggest *L. monocytogenes* encounters and utilizes glucose-6-phosphate and oligopeptides when growing in the cytosol. As reviewed above, the conversion of these carbon sources into critical metabolites, such as amino acids and fatty acids, requires the activity of the nutrient lipoate. However, it is possible that during intracellular growth, *L. monocytogenes* encounters and scavenges amino acids and fatty acids directly from the host, bypassing the need for lipoate. To fully appreciate the relevance of lipoic acid during intracellular growth, it is necessary first to examine the availability and influence amino acids and fatty acids have upon *L. monocytogenes* and other medically relevant bacteria in the intracellular environment.

AMINO ACID SCAVENGING OF INTRACELLULAR *L. monocytogenes*

It is currently unclear what amino acids are available to *L. monocytogenes* for scavenging while within the host, but when the bacterium was grown in the host cytosol rather than defined minimal medium, some single amino acid auxotrophs were no longer attenuated for growth (Marquis *et al.,* 1993; Stritzker *et al.,* 2004). These results suggested *L. monocytogenes* could acquire host-derived amino acids in this environment. However, this same study revealed efficient intracellular growth was impaired when *de novo* synthesis of adenine and the

aromatic amino acids tyrosine, tryptophan, phenylalanine, was disrupted. These results demonstrated not all amino acids can be scavenged by *L. monocytogenes* from the host cytosol (Marquis *et al.,* 1993).

Further studies confirmed genes involved in aromatic amino acid biosynthesis (*aroA, aroB, aroE)* were important during intracellular growth of *L. monocytogenes*; deletion of any one of these genes resulted in a 10^4 fold decrease in virulence (Stritzker *et al.,* 2004). Most recently, work exploring the intracellular metabolism of *L. monocytogenes* using ^{13}C labeling of glucose indicated that alanine, aspartate, and glutamate were synthesized by *L. monocytogenes* when the bacterium was growing in J774A.1 macrophage host cells, demonstrating again that not all amino acids are scavenged from the host environment (Eylert *et al.,* 2008). The same study demonstrated the BCAA isoleucine was not synthesized by *L. monocytogenes* when it grew in the cytosol of J774A.1 macrophages, indicating host cells must provide this amino acid for scavenging (Eylert *et al.,* 2008). All of these studies revealed that when *L. monocytogenes* was growing in the host cytosol, it was capable of both scavenging amino acids and generating amino acids through biosynthesis. Despite the presence of all the genes necessary for biosynthesis of these amino acids, the nutrient environment of the host cytosol appears to dictate bacterial biosynthesis. It is currently not known if the bacterium requires lipoate-mediated metabolism for biosynthesis of all of these important metabolites.

L. monocytogenes AMINO ACID BIOSYNTHESIS IS MODULATED BY GROWTH ENVIRONMENT

Amino acid biosynthesis by *L. monocytogenes* appears to be controlled by the growth environment. When the bacterium was grown in minimal medium, *de novo* biosynthesis of arginine was undetectable, indicating this amino acid may not be synthesized by *L. monocytogenes* (Eisenreich *et al.,* 2006). However, genes involved in the biosynthesis of arginine were induced 100-fold when *L. monocytogenes* was growing in the cytosol of macrophages, suggestive that arginine biosynthesis may be regulated by the growth environment (Klarsfeld *et al.,* 1994). Biosynthesis of proline, another product from glutamate, was induced when the intracellular virulence regulator PrfA was constitutively activated (Eisenreich *et al.,* 2006). However, further studies indicated when *L. monocytogenes* was grown in the host cytosol of J774A.1 macrophages, the bacteria did not synthesize proline (Eylert *et al.,* 2008). Even though *L. monocytogenes* encodes all genes predicted to be necessary for arginine and proline biosynthesis, the data suggest the bacterium regulates the production of these amino acids based on the growth environment (Glaser *et al.,* 2001a).

The production of the BCAAs isoleucine, leucine and valine also appear to be regulated by the nutritional environment of *L. monocytogenes.* When *L. monocytogenes* was growing in the host cytosol, no bacterially synthesized isoleucine or leucine was detected, but in broth culture all three BCAAs were detected as being generated by the bacterium when PrfA was constitutively

activated (Eisenreich *et al.,* 2006; Eylert *et al.,* 2008). When the bacteria were supplemented with BCAAs in glucose based minimal medium, growth was enhanced. These data suggested that even if the bacterium was capable of biosynthetically producing these amino acids, optimal growth required the bacterium to scavenge amino acids from its growth environment (Tsai and Hodgson, 2003). When *L. monocytogenes* was growing in depleted minimal medium, it absolutely required supplementation with isoleucine, possibly because it can be catabolized to form acetyl-CoA (Eisenreich *et al.,* 2006). BCAAs are also precursors for α-keto acids, which are essential in branched chain fatty acid biosynthesis (Kaneda, 1991) (Fig. 1.2). These observations suggest *L. monocytogenes* generates different amino acids and downstream nutrients when growing in diverse environments.

FATTY ACID REQUIREMENTS OF *L. monocytogenes* AND OTHER MEDICALLY RELEVANT PATHOGENS

Fatty acids are also produced with the assistance of lipoate dependent metabolic enzymes. The availability of fatty acids can influence the ability of a microbe to adapt and invade its host. In the experimental system of During *S.* Typhimurium infection, it was found that the short chain fatty acid formate could can act as a diffusible signal and induce bacterial penetration of the intestinal epithelium, a necessary first step before intracellular invasion (Huang *et al.,* 2008). Short chain fatty acids also negatively influenced influences parasite infectivity in the colon. A study of *Entamoeba invadens* parasites revealed they multipliedy as trophozoites and differentiated into cysts within the mucus layer of the colonic

epithelium. ; aAddition of short-chain fatty acids to this environment inhibited inhibits cyst formation, effectively preventing formation of the infectious form of the parasite (Byers *et al.,* 2005). It is unknown what effect fatty acids have upon *L. monocytogenes* invasion, but these nutrients do influence the invasion of other pathogens.

In addition to fatty acids modulating invasion in some pathogens, the bacterial fatty acid profile can also be associated with the ability of an organism to adapt to adverse environments. When *Streptococcus mutans* was are treated with a fatty acid biosynthesis inhibitor, it could no longer alters its membrane fatty acid profile and was is also unable to survive severe acid stress (Fozo and Quivey, 2004) Additionally, *S.* Typhimurium cyclopropane fatty acid biosynthesis was is essential for resistance to low pH conditions (Kim *et al.,* 2005a). Finally, in *Staphylococcus aureus,* insertional inactivation of branched-chain α-keto acid dehydrogenase resulted results in a loss in membrane branched chain fatty acids, decreased adherence to eukaryotic cells, and reduced pathogen survival inside a murine host (Singh *et al.,* 2008). Together, these data indicate Accordingly, fatty acid biosynthesis may play a significant role in pathogen resistance to environmental and host stress.

The role bacterial fatty acid biosynthesis has upon during intracellular growth is unknown. It is known that fFatty acid composition may play a role in *L. monocytogenes* resistance to environmental stress. Reduced growth of *L.*

monocytogenes in acidic conditions was is partially rescued by addition of the anteiso-branched chain fatty acid precursor 2-methylbutyrate (2-MBA) (Giotis *et al.,* 2007). The fatty acid composition of *L. monocytogenes* also affected affects its ability to adhere to abiotic surfaces and form biofilms, possibly by altering hydrophobic bonding to these surfaces (Gianotti *et al.,* 2008). Finally, the branched chain fatty acid anteiso-C15:0 also played plays a critical role in *L. monocytogenes* growth at low temperatures. While 2-MBA can act as a precursor for this fatty acid, isoleucine can also act as a precursor. Supplementation of minimal medium with isoleucine enabled *L. monocytogenes* growth at cold temperatures, and also increased its anteiso-C15:0 fatty acid ratio (Zhu *et al.,* 2005b). As reviewed above, fatty acids, which appear to influence virulence of other pathogens as well as the ability of *L. monocytogenes* to adapt to stress, are made with the assistance of lipoylated metabolic enzyme complexes. The influence lipoate has on microbial pathogens is still incompletely understood, but it will be interesting to determine what role it plays during intracellular replication of *L. monocytogenes*.

KNOWN ROLE OF LIPOATE IN PATHOGENESIS

LIPOATE SCAVENGING AND LIPOATE LIGASE FUNCTION

L. monocytogenes, as well as other pathogens such as *Enterococcus faecalis,* are auxotrophs for lipoate when growing in defined minimal medium, meaning

the bacteria must scavenge lipoate to support replication in certain environments (Phan-Thanh and Gormon, 1997; Tsai and Hodgson, 2003). Even with the ability to synthesize lipoate, the pathogens *Toxoplasma gondii* and *Plasmodium falciparum* behave as lipoate auxotrophs (Crawford *et al.,* 2006). These data indicate lipoate scavenging may be important for many diverse intracellular pathogens, even those that have the ability to synthesize lipoate.

In *E. coli*, scavenged lipoic acid is ligated to the E2 subunit with a lipoate protein ligase (LplA) (Morris *et al.,* 1994, 1995; Reed and Cronan, 1993). In this organism, lipoate-protein ligase A (LplA) activates exogenously supplied free lipoic acid with ATP to generate lipoyl-AMP, which is then sequestered in the ligase active site. The lipoyl-AMP is then attached to the E2 target in a mechanism similar to biotinyl protein ligase (BPL), where the nucleophilic epsilon-amino group of a target lysine residue attacks the mixed anhydride of the 5'-AMP bound within the ligase active site, generating a stable amide bond between the substrate and target (Chapman-Smith and Cronan, 1999).

LplA is not the only lipoate ligase in *E. coli.* The bacterium can also generate its own lipoate through *de novo* fatty acid biosynthesis, where lipoate is ligated to target enzymes using the LipA and LipB enzymes. LipA catalyzes the conversion of octanoyl-ACP into lipoyl-ACP; the lipoate moiety is then transferred to the target by LipB (Morris *et al.,* 1995). Therefore, *E. coli* has two distinct mechanisms to attach lipoate to metabolic enzyme complexes, where one

system, LplA, utilizes scavenged lipoate, while the other, LipA and LipB, utilizes *de novo* synthesized lipoate. Unlike *E. coli*, *L. monocytogenes* only has a scavenging pathway, as it is a lipoate auxotroph and also does not contain any genes with homology to LipA or LipB (Glaser *et al.,* 2001b) (O'Riordan *et al.,* 2003). *L. monocytogenes* does encode two putative lipoate ligases, LplA1 and LplA2, which share 54% and 49% similarity, respectively, with LplA of *E. coli.*

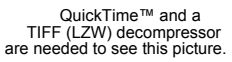


Figure 1.5 Lipoate ligases in *E. coli*

From (Morris *et al.,* 1995). In *E. coli,* lipoate is either scavenged and ligated to target enzymes with the lipoate ligase LplA, or it is synthesized by *de novo* synthesis and ligated to target enzymes with LipA and LipB. In the scavenging pathway, lipoate is activated with ATP to a lipoyl-AMP derivative prior to ligation. In the *de novo* synthesis pathway, octanoyl-ACP is converted to lipoyl-ACP with LipA, and then the lipoate moiety is transferred to the target with LipB.

IDENTIFICATION AND INITIAL CHARACTERIZATION OF LPLA1

When growing in the host cytosol, the *lplA1* null mutant of *L. monocytogenes* exhibited a 250-fold decrease in virulence in a mouse model of infection, indicating lipoate ligases are important in *L. monocytogenes* pathogenesis

(O'Riordan *et al.,* 2003). The role of LplA1 in virulence was initially identified through a genetic selection designed to isolate *L. monocytogenes* mutants defective in intracellular growth (O'Riordan *et al.,* 2003). An in-frame deletion of a candidate gene identified through this selection confirmed *lplA1* was responsible for a growth defect in both J774 macrophages and growth and spread in a plaque assay of L2 fibroblasts. Additionally, when the *lplA1* mutant was grown in macrophages, the E2-subunit of bacterial PDH was no longer lipoylated (O'Riordan *et al.,* 2003). These data suggest *lplA1* encodes a lipoate ligase with similar function to the well-characterized *E. coli* LplA that ligates scavenged lipoate to the E2 subunit of bacterial metabolic enzymes.

GOALS FOR THESIS RESEARCH

DETERMINE WHY LPLA1 IS REQUIRED DURING INTRACELLULAR GROWTH

At the start of this thesis, it was unknown what the relative contribution LplA1 and LplA2 had towards growth of *L. monocytogenes* outside and inside the cell. Despite the importance of *lplA1* during intracellular growth, there was is no defect in growth and lipoylation of bacterial PDH when the *lplA1* mutant was grown in BHI (O'Riordan *et al.,* 2003). This prompted us to hypothesize that LplA1 is specifically important during intracellular growth because it utilizes a host-derived source of lipoate that LplA2 cannot use (Chapter 2). Additionally, we postulated that that the unique topology of LplA1 and LplA2 may play a role in differentiating between two unique sources of lipoate, host-derived lipoate and free lipoate (Chapter 4). Identification of the host-specific lipoate substrates that LplA1 and

LplA2 utilize to support bacterial replication, as well as characterizing structural reasons for differences in substrate preference, will provide a deeper understanding of how bacterial pathogens exploit different environmental replicative niches.

DETERMINE WHY LIPOATE DEPENDENT METABOLISM ENHANCES CYTOSOLIC REPLICATION

The importance of the lipoate ligase LplA1 in intracellular growth of *L. monocytogenes* has been demonstrated, yet why its function enables intracellular growth is uncertain (O'Riordan *et al.,* 2003). Some of the known functions of lipoyl-E2 may provide clues as to why LplA1 is important for growth *in vivo.* As previously mentioned, lipoate ligases activate enzymes by adding the essential co-factor lipoate to the E2 subunit of a variety of metabolic enzyme complexes. In *L. monocytogenes*, the activity of lipoyl-E2 enables the PDH complex to generate acetyl-CoA and many biosynthetic intermediates for amino acid and SCFA biosynthesis. Additionally, the lipoyl-E2 complex of BCKD enables the generation of BCFAs (Zhu *et al.,* 2005a). We hypothesize *L. monocytogenes* lipoate dependent metabolism enhances intracellular growth because the bacterium must synthesize specific metabolites to rapidly replicate in the cytosol (Chapter 3).

SUMMARY

Like many other medically relevant bacteria, *L. monocytogenes* replicates in the intracellular environment of mammalian hosts. The ability of *L. monocytogenes*

to adapt its metabolism enables this bacterium to replicate in diverse environments, including the host cytosol. While *L. monocytogenes* can scavenge many nutrients in the cytosol, it also generates essential metabolites through bacterial mediated biosynthesis, including amino acids and branched chain fatty acids. *L. monocytogenes* requires the lipoate protein ligase LplA1 for this intracellular replication, indicating lipoate dependent metabolism could play an important role in intracellular growth of *L. monocytogenes.* **It is the aim of my doctoral work to define the specific contributions of lipoate, an essential vitamin, to the intracellular growth and metabolism of *L. monocytogenes*.** Further characterization of the intracellular metabolism of *L. monocytogenes* is critical for understanding how this pathogen adapts to exploit the cytosol of its mammalian host. This work has broad significance, as it will lend insight into the role metabolic adaptation plays during pathogenesis, and specifically clarify how the nutritional environment influences growth of the environmentally ubiquitous *L. monocytogenes* as well as other pathogens.

Chapter 2

LPLA1-DEPENDENT UTILIZATION OF HOST LIPOYL-PEPTIDES ENABLES *LISTERIA* CYTOSOLIC GROWTH AND VIRULENCE

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 co-factor, 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 *ΔlplA1* 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 (Goetz *et al.,* 2001; Portnoy *et al.,* 1992). 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 9 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 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 wildtype bacteria in a mouse model of infection. Genes involved in aromatic amino acid biosynthesis (*aroA, aroB, 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 extracellular growth (O'Riordan *et al.,* 2003). The LD_{50} of LplA1-deficient *L. monocytogenes* is 250-fold greater than the wildtype parental strain in C57BL/6 mice demonstrating the importance of LplA1 for virulence.

The function of lipoate ligases have been extensively characterized in the model organism *Escherichia coli* (Fujiwara *et al.,* 2005; Morris *et al.,* 1994, 1995). Lipoate, a thiol containing co-factor, is essential for the oxidative decarboxylation reactions of aerobic metabolism, and also acts as an anti-oxidant (Bast and

Haenen, 2003; Jordan and Cronan, 1997). Lipoate ligases catalyze formation of an amide linkage between lipoate and a conserved lysine residue within target apoenzymes that include components of the pyruvate dehydrogenase (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 (Glaser *et al.,* 2001b; Welshimer, 1963). 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.,* 2001b). 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). Since 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.* 2001). LplA1 shares significant amino acid identity and similarity with LplA2 (*lmo0764*) and *E. coli* LplA (Fig. A2.1)(Glaser *et al.,* 2001b). 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. 2.1A). The *ΔlplA1* and *ΔlplA2* mutant strains exhibited lipoyl modification of a 75kD 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::Tn917ΔlplA2*) showed no detectable lipoylation. Both the *ΔlplA1* and *ΔlplA2* mutant strains grew similarly to the parental wildtype *L. monocytogenes* strain in BHI, but the *lplA1::Tn917ΔlplA2* 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. 2.1A). *Lm*PDH lipoylation was decreased in the single mutants compared with the

wildtype strain. However, the single mutants grew as well as wildtype *L. monocytogenes* in IMM, demonstrating functional redundancy of LplA1 and LplA2 in medium containing free lipoic acid (Fig. 2.1B). While we could observe limited growth of the double mutant in BHI, this strain did not grow in IMM, thus the *lplA1::Tn917ΔlplA2* 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

Since 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 with wildtype and mutant *L. monocytogenes*, and colony-forming units (CFU) from spleen and liver were enumerated at 72 hrsh (Fig. 2.2A). The bacterial burden in mice infected with wildtype or *ΔlplA2* bacteria was several orders of magnitude higher than mice infected with the *ΔlplA1* mutant strain (Fig. 2.2A). We also assessed the ability of *ΔlplA1* and *ΔlplA2* mutants to compete against wildtype *L. monocytogenes* by performing a competitive index (CI) analysis. Mice were co-infected with wildtype and mutant *L. monocytogenes* and CFU were enumerated at 72 hrsh (Fig. 2.2B). The competitive index was calculated by dividing the number of wildtype CFU (antibiotic sensitive) by the mutant CFU (antibiotic resistant). While the CI of the *ΔlplA1* strain was approximately 32 and 603 in liver and spleen respectively, the CI of the *ΔlplA2*

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 *ΔlplA1* 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 wildtype, *ΔlplA1*, and *ΔlplA2* strains in IMM without lipoic acid prior to intracellular infection. LmPDH 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. 2.3A). 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. Wildtype and LplA2-deficient L. monocytogenes exhibited similar intracellular growth whether or not the bacteria were lipoate starved (Fig 2.3B). However, lipoate starved *ΔlplA1* 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 *ΔlplA1* 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 *ΔlplA1* mutant strain was due to lack of intracellular lplA2 expression, we measured levels of *lplA1* and *lplA2* transcript by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in wildtype L. monocytogenes isolated from J774 macrophages at 6 hrsh p.i. LplA2 transcripts were present in intracellular bacteria, albeit at lower levels than lplA1 (Fig 2.3C). Since 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 *ΔlplA1* 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. 2.3D). DHLA supplementation did not further enhance the intracellular growth of wildtype *L. monocytogenes* but did rescue growth of the *ΔlplA1* mutant. Taken together, these results suggest LplA2 is competent to enable intracellular bacterial growth only if free lipoate is added exogenously. Since 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 predominant 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 analyzed by SDS-PAGE and immunoblot using an anti-lipoic acid antibody (Fig. 2.4A). 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 since 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.

Since 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 2.4B). However, it was possible *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. 2.4B). 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 tri-peptides (~520 Da), supported growth of WT but not *ΔlplA1* (Fig. 2.4B 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 dialyzed ProK digested PDH using a membrane with a 500 Da molecular weight cutoff (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 wildtype bacteria to the same extent (Fig. 2.4D). However, the *ΔlplA1* 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 wildtype 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^LA

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. 2.5A). 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^LA), the smallest lipoyl peptide predicted from ProK digestion of porcine PDH (Fig. 2.5A and A2.2AB). At 5 μg/l, DK^LA supported growth of WT but not *⊿lplA1*, demonstrating the specific contribution of LplA1 in utilizing lipoylated peptides for proliferation (Fig. 2.5B). To test whether replication required the lipoyl moiety, we also supplemented IMM with non-lipoylated tri-peptide (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^LA to lipoate starved bacterial cultures (Fig. 2.5C). Lower concentrations of lipoyl tri-peptide only supported replication of wildtype bacteria, while high concentrations supported growth of both the wildtype and *⊿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 tri-peptides, we digested lipoylated $DK^{L}A$ with the protease aminopeptidase M (Fig. A2.2C). Unlike the preferential growth of wildtype bacteria mediated by undigested lipoyl $DK^{L}A$, peptide digestion products allowed growth of both the wildtype and *ΔlplA1* strains, suggesting that lipoyl-dipeptide or lipoyl-lysine does not dictate LplA1-dependent bacterial growth (Fig. 2.5D). 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 *ΔlplA1* mutant strain as compared to wildtype bacteria at suboptimal lipoamide concentrations, both wildtype and *ΔlplA1* replicated in lipoamide-containing medium, demonstrating that lipoamide did not define the host specific requirement for LplA1 (Fig. 2.5E). Overall, these data indicate 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). Since 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* (Allary *et al.,* 2007; Wada *et al.,* 2001). 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. 2.6A), but only *E. coli lplA* and *L. monocytogenes lplA1* complemented growth of TM131 when acetate and succinate were removed (Fig. 2.6B 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 wildtype *E. coli* strain, thus it is still unknown whether LplA2 can act directly as a functional lipoate ligase (Fig. A2.3A, 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. A2.3B). 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 modeled 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 apo-domain was conserved between the *E. coli* and *L. monocytogenes* enzymes (Fig. A2.1) (Kim *et al.,* 2005b). 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. 2.7). 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 *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 *⊿lplA1* strain was substantially less able to compete with the wildtype strain in spleen when compared to 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 defense, which might also contribute to decreased fitness of the *⊿lplA1* mutant. Lipoic acid is a potent anti-oxidant 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, since the parasite biosynthetic pathways supply only the apicoplast and not the mitochondrion (Allary *et al.,* 2007; Crawford *et al.,* 2006; Wrenger and Müller, 2004). 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 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 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. A2.1). 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 since 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 (Ma *et al.,* 2006; Zhao *et al.,* 2003). 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 describes how LplA1 contributes to *L. monocytogenes* pathogenesis.

EXPERIMENTAL PROCEDURES

Bacterial culture

Strains used in this study are described in Table 2.1. For intracellular growth curves, all *L. monocytogenes* strains were grown to mid-log (OD_{600} 0.2-0.6) in improved minimal medium (IMM) at 37°C unless otherwise specified. Improved minimal medium 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 5μg/L except when otherwise specified in the figure legends, or substituted where indicated with various concentrations of lipoamide (0-5μg/L), $DK^{L}A$ (0-25μg/L), digested lipoyl-PDH (5mg/L) or digested lipoyl-KGDH (5mg/L). For growth curves in IMM, single colonies from freshly streaked BHI plates were inoculated into IMM without lipoic acid (IMM^{-L}) for 10-14 hours 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-polymerase chain reaction (SOE-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::917⊿lplA2*;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 competitive index 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 $2x10^5$ bacteria in 200 ml were injected i.p. into 4-6 wk old C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine). At 72 hrsh p.i., the animals were sacrificed, and livers and spleens homogenized in 0.1% NP-40, serially diluted, and plated onto LB agar. For the competitive index infections, the homogenization buffer also contained 0.1μg/ml erythromycin

(erm), and the lysate was plated on LB agar with or without 1μg/ml erm. The competitive index was calculated by dividing the number of wildtype 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 of 1:1 for 6 hrsh, host cells were lysed with 50mM Tris, pH 8, 150mM NaCl, and 0.05% NP-40 for 10 minutes on ice. After vortexing and pelleting the host nuclei at 3000*xg* for 30 seconds, 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 mini-columns. cDNA was generated from 2.5 μg RNA using random primers and M-MLV reverse transcriptase (Invitrogen). Primers for *lplA1*, *lplA2,* and *rpoA* (Table A2.1) 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 4hrsh in 0.02% Tween-20 and 200mM ammonium bicarbonate (pH 8.9). The proteins were digested with a ratio of 1 mg protein to 8 μg proteomics grade trypsin in a total volume of 500μl; the reaction was stopped with 0.0003% trifluoroacetic acid. ProK digestion was performed at a ratio of 5 mg protein to 20 μg enzyme (Sigma P-4850) in 20mM Tris-HCl, 1mM

$CaCl_2$ at pH 7.4 at 25°C for 4hrsh and heat inactivated for 10 minutes 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 dialyzed against 20mM Tris-HCl, 1mM $CaCl_2$ at pH 7.4. Synthetic lipoyl tripeptide DK^LA was synthesized by Anaspec using a previously published protocol (Fig. A2.2AB) (Konishi *et al.,* 1996). Digestion of DK^LA with immobilized aminopeptidase M was performed at a ratio of 0.5U enzyme to 250 µg peptide for 18hrsh 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 analyzed in positive ion mode (Fig. A2.2C). 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 $1x10^7$ macrophages in 10mM HEPES pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, and 0.2mM PMSF, incubation on ice for 10 min and vortexing for 1 min. After lysis, salts were added to a final concentration of 30mM HEPES (pH 7.9), 140mM KCl, and 3mM $MgCl_2$. After removing nuclei by centrifuging at 3000*xg* for 30 seconds, and washing 3 times, mitochondria were spun at 80,000*xg* for 4hr 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 analyzed 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 seconds at a setting of 6.0 in Lysing Matrix B. For cytosolic and mitochondrial peptide analysis by immunoblot, 0.75M 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} prestarved bacteria were used at an MOI of 3, resulting in ~10% macrophage infection. In dihydrolipoic acid (DHLA, Sigma-Aldrich T-8260) supplementation experiments, 50μM 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 nonspecific 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 wildtype 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, 5mM sodium acetate, 5mM sodium succinate, and 100μg/ml ampicillin. Primers used to amplify the lipoate ligase genes with Pfx platinum polymerase are listed on Table S1. TM131 transformed strains were also grown in the presence of 50μg/ml kanamycin and 125μg/ml tetracycline. Transformants were restreaked onto M9 minimal medium plates containing 0.4% glucose, 1mM IPTG, thiamine (10μg/ml), 2mM $MgSO_4$, 0.1mM $CaCl_2$, and the appropriate antibiotics with or without 5mM sodium acetate and 5mM sodium succinate as indicated. Lysates for western blotting were grown in LB containing 0.4% glucose, 5mM sodium acetate, 5mM sodium succinate, 1mM IPTG, and the appropriate antibiotics.

Structural modeling of LplA1 & LplA2

Due to its high homology with LplA1 (65.8 %) and LplA2 (66.6 %), the structure of LplA from *Streptococcus pneumoniae* (PDB ID: 1VQZ; Joint Center for Structural Genomics) was used as the foundation for modeling 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 (Fujiwara *et al.,* 2005; Jones *et al.,* 1991). 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 CNS (Brünger *et*

al., 1998). The lipoate structure from 1X2H was attached to the NZ atom of a modeled 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/cgibin/gerard/hicup_server.pl). The tripeptide ($DK^{L}A$) was then created via CNS and modeled 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.

TABLES AND FIGURES

Table 2.1 Strains used In Chapter 2

Strains	Genotype/description	Reference
10403S	*L. monocytogenes* serotype 1/2a, parental strain used as wildtype	(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, wildtype 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

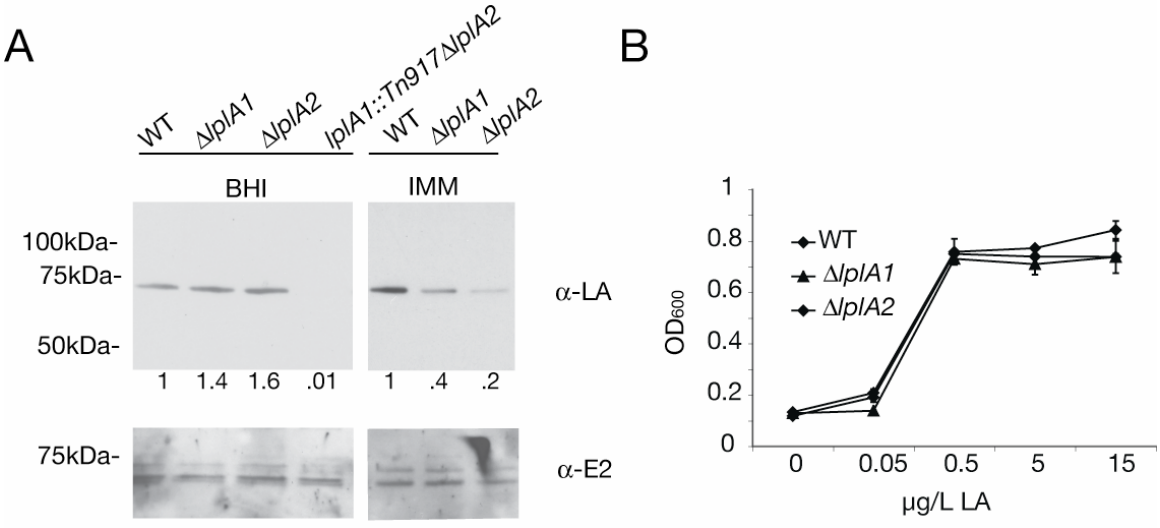


Figure 2.1 *L. monocytogenes* has two functional lipoate ligases.

(A) Equivalent numbers of stationary phase wildtype, *ΔlplA1, lplA1::Tn917, ΔlplA2,* and *lplA1::Tn917ΔlplA2 L. monocytogenes*, based on OD_{600}, were pelleted and protein harvested using the FastProtein™ Blue Matrix (MP Biomedicals). Bacterial lysates were analyzed by SDS-PAGE, followed by immunoblot with an anti-lipoic acid antibody. The band slightly below 75kDa has previously been identified by Mass-Spectrometry as lipoyl-E2-PDH (O'Riordan *et al.,* 2003). Relative intensities of the bands to wildtype 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::Tn917ΔlplA2* was only studied in BHI as it exhibited impaired growth in IMM. **(B)** Wildtype *L. monocytogenes* and the *ΔlplA1* and *ΔlplA2* mutant strains were grown in IMM containing different concentrations of lipoic acid. The OD_{600} was measured after bacteria had reached stationary phase (30 hrsh) and plotted against lipoic acid concentration. The OD_{600} was measured in a Bioscreen Growth Curve Analyzer.

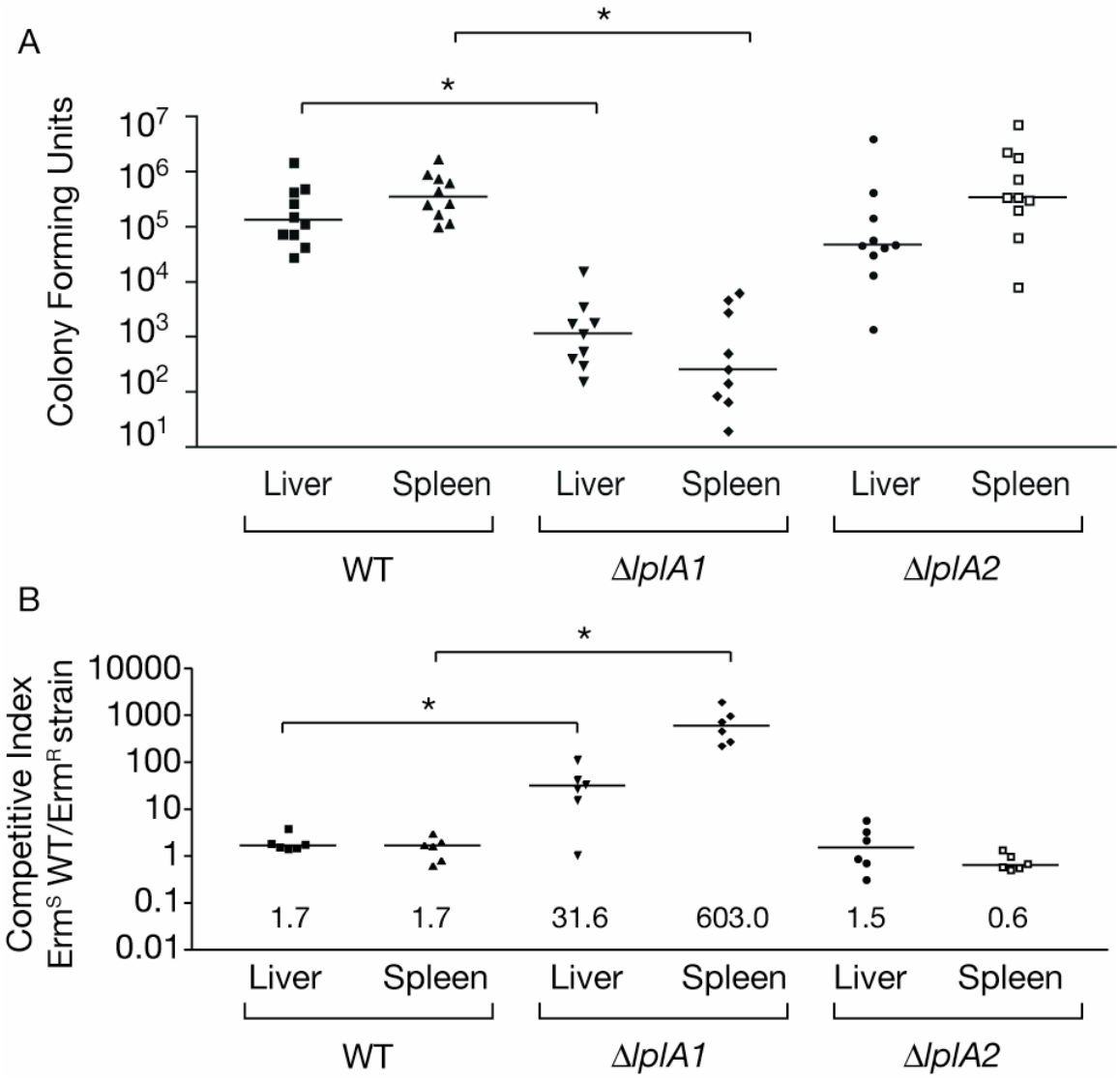


Figure 2.2 Bacterial virulence *in vivo* requires LplA1, but not LplA2.

(A) $2x10^4$ total CFU of exponentially growing cultures of wildtype and mutant *L. monocytogenes* were injected i.p. into six 5-7 week old male C57BL/6 mice. After 72 hrsh, spleens and livers were harvested, homogenized, and plated onto LB. (B) Exponentially growing cultures of wildtype and mutant *L. monocytogenes* were mixed at a 1:10 (WT:*ΔlplA1*) or 1:1 ratio (WT:*ΔlplA2*) and 2x104 total CFU injected i.p. into six 5-7 week old male C57BL/6 mice. After 72 hrsh, spleens and livers were harvested, homogenized, and plated onto LB with or without 1µg/ml erm. The competitive index was calculated by dividing the number of wildtype 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.

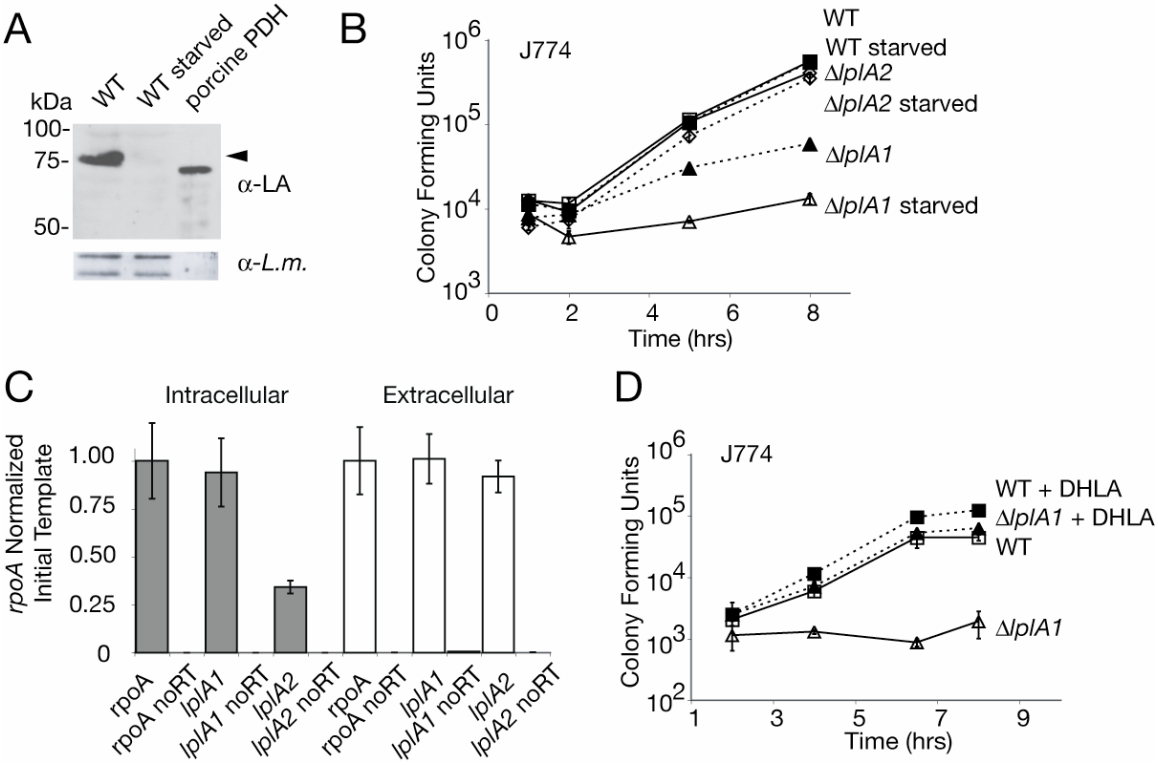


Figure 2.3 LplA1, but not LplA2, is essential for intracellular growth.

(A) A culture of wildtype *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 analyzed 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) Wildtype, *ΔlplA1,* and *ΔlplA2* bacterial strains were grown in IMM in the absence (dashed lines) or presence (solid lines) of lipoic acid overnight at 37°C, and used to infect J774 cell cultures. Intracellular growth was quantified by enumerating CFU. (C) Wildtype bacteria were grown in BHI overnight at 37°C and used to infect J774 cells. At 6 h.p.i., 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 (labeled "no RT") and analyzed by QRT-PCR. (D) J774 cells were infected as previously described in (B) 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 timepoint (*n=3*).

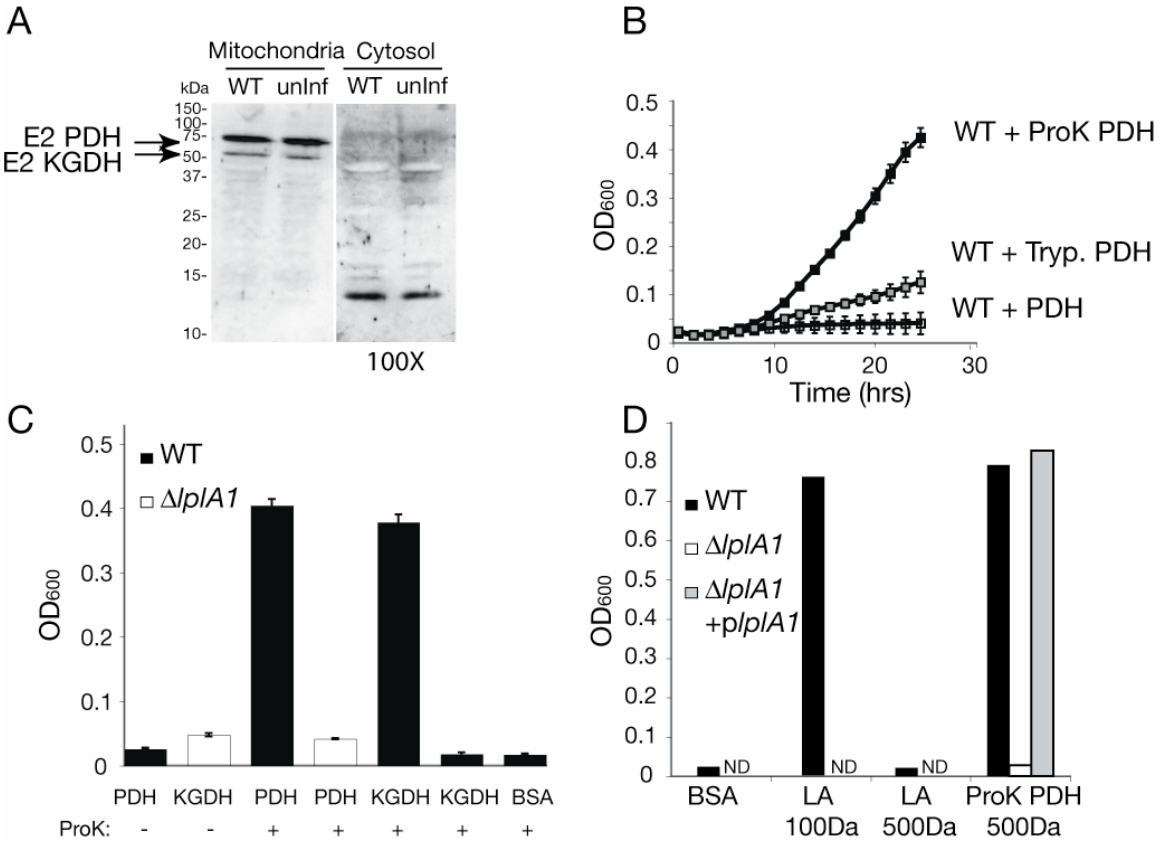


Figure 2.4 LplA1 enables utilization of degraded host derived PDH for bacterial growth.

(A) J774 cell lysate was separated into cytosolic and mitochondrial fractions as described in Materials and Methods. Mitochondrial fractions and concentrated cytosolic fractions (100X) were analyzed 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) Wildtype *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 timepoint (*n=3)*. (C) Wildtype and *ΔlplA1 L. monocytogenes* were grown in IMM containing undigested or ProK digested porcine PDH or KGDH at 5mg/l. After 35 hrsh 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 5mg/l was also digested with ProK as a negative control. Mean values ± SD were calculated for each timepoint (*n=3)*. (D) Wildtype, *ΔlplA1,* and *ΔlplA1* complemented with a plasmid expressing LplA1 were grown in IMM containing the lipoate sources indicated; ProK digested BSA at 5 mg/l was used as a negative control. Free lipoic acid (206 Da) (5µg/L) and ProK-digested porcine PDH (5mg/L) were dialyzed against a 100 Dalton or a 500 Dalton MWCO membrane, and the retentate was used to supplement IMM. After 19.5 hrsh 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 *ΔlplA1* and *ΔlplA1* complemented with a plasmid expressing LplA1.

Figure 5

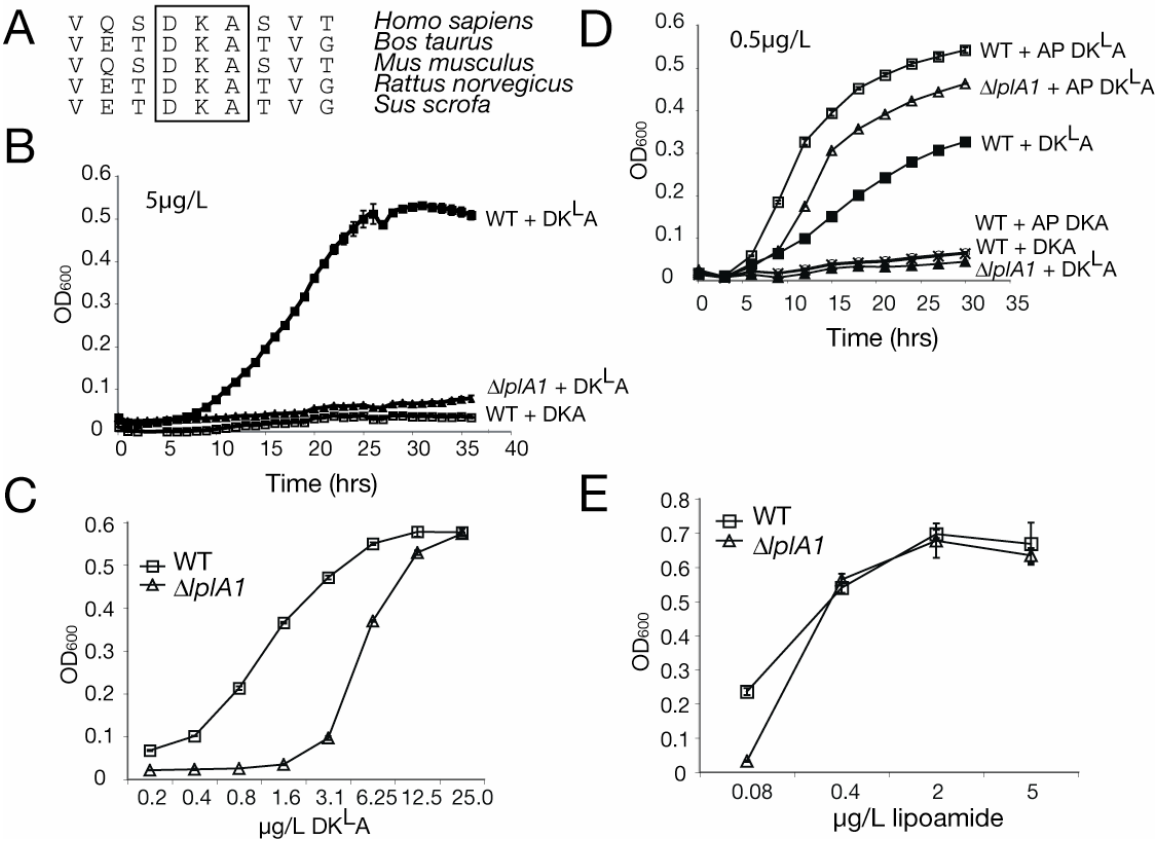


Figure 2.5 LplA1 is required for optimal growth on small lipoyl-peptides.

(A) The amino acid sequences of the dihydrolipoyl transacylase lipoyl domain from *Homo sapiens* (Accession #AAA64512), dihydrolipoamide S-acetyltransferase lipoyl domain of *Bos taurus* (Accession #XP_588501), dihydrolipoamide branched chain transacylase E2 lipoyl domain of *Mus musculus* (Accession #NP_034152), dihydrolipoamide S-acetyltransferase lipoyl domain of *Rattus norvegicus* (Accession #AAI07441), and the dihydrolipoamide acetyltransferase lipoyl domain of *Sus scrofa* (Accession #NP_999159) aligned. (B) Wildtype *L. monocytogenes* and the *ΔlplA1* mutant strain were grown in IMM containing 5 µg/l DKLA, or 5 µg/l non-lipoylated DKA. The OD_{600} was measured over time in a Bioscreen Growth Curve Analyzer, and plotted as a function of time. (C) Wildtype *L. monocytogenes* and the *ΔlplA1* mutant strain were grown as in (B), but in IMM containing different concentrations of DKLA. The OD_{600} was measured after bacteria had reached stationary phase (25 hrsh) and plotted against lipoamide concentration. (D) Bacterial growth curves were performed as in (B), but 0.5 µg/L tri-peptide (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 hrsh of growth, bacteria had reached stationary phase, and OD_{600} values were

plotted against lipoamide concentration. The mean value ± SD was calculated for each timepoint (*n=3)* in (B-E).

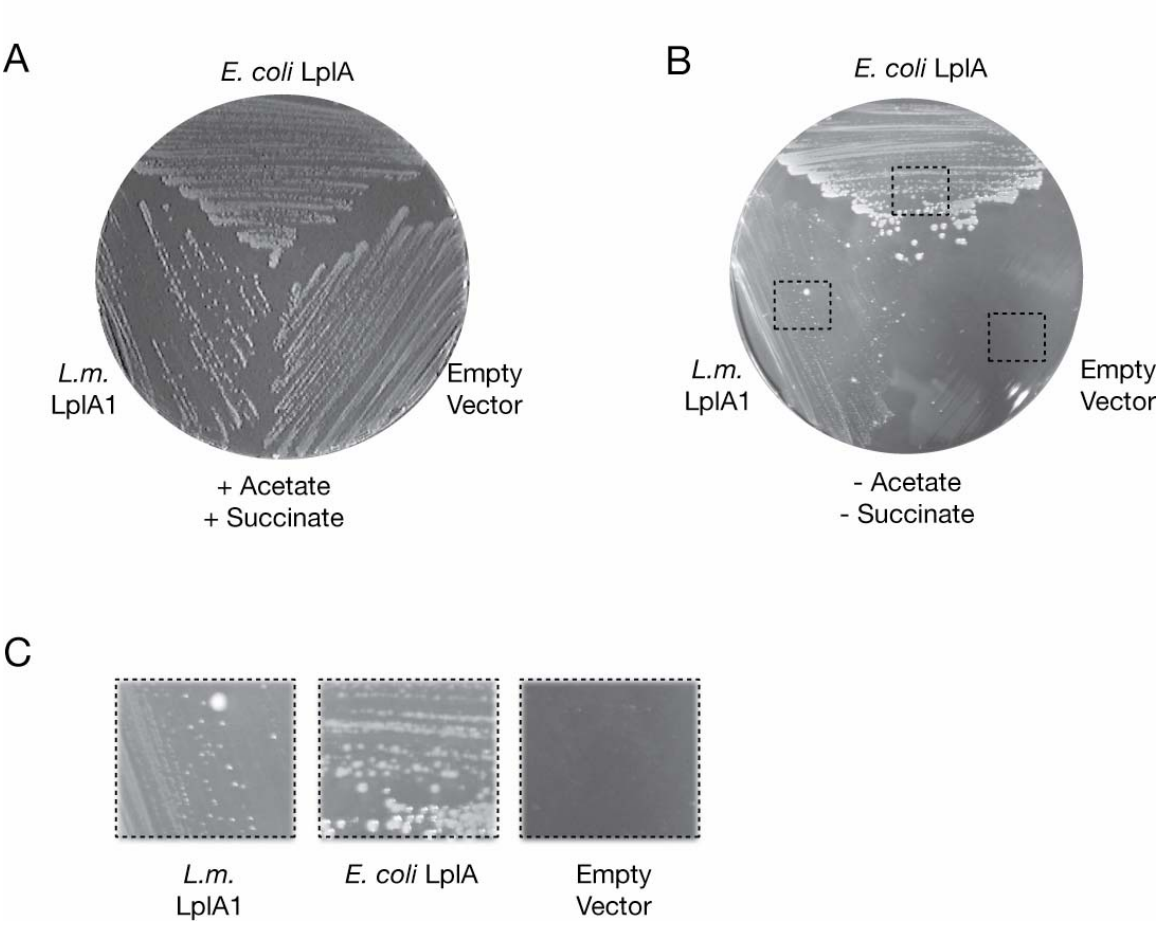


Figure 2.6 Complementation of an *E. coli* strain deficient in lipoate utilization by *L. monocytogenes* LplA1.

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).

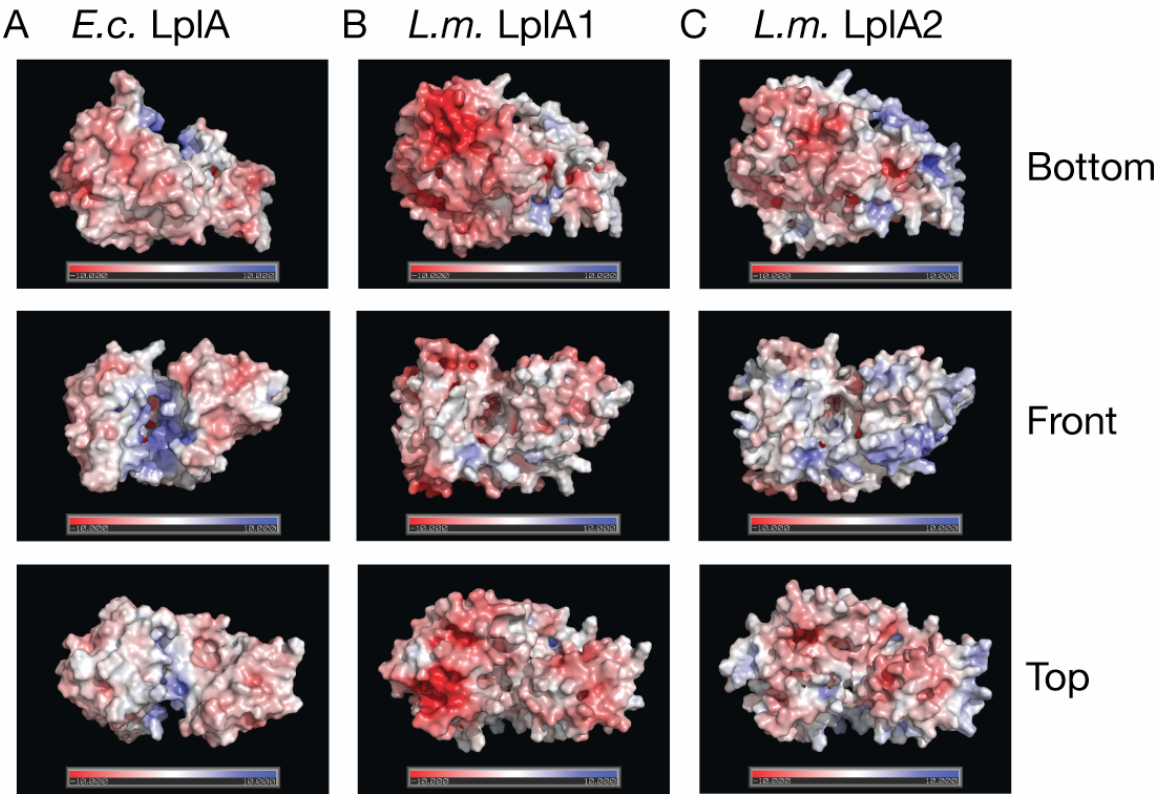
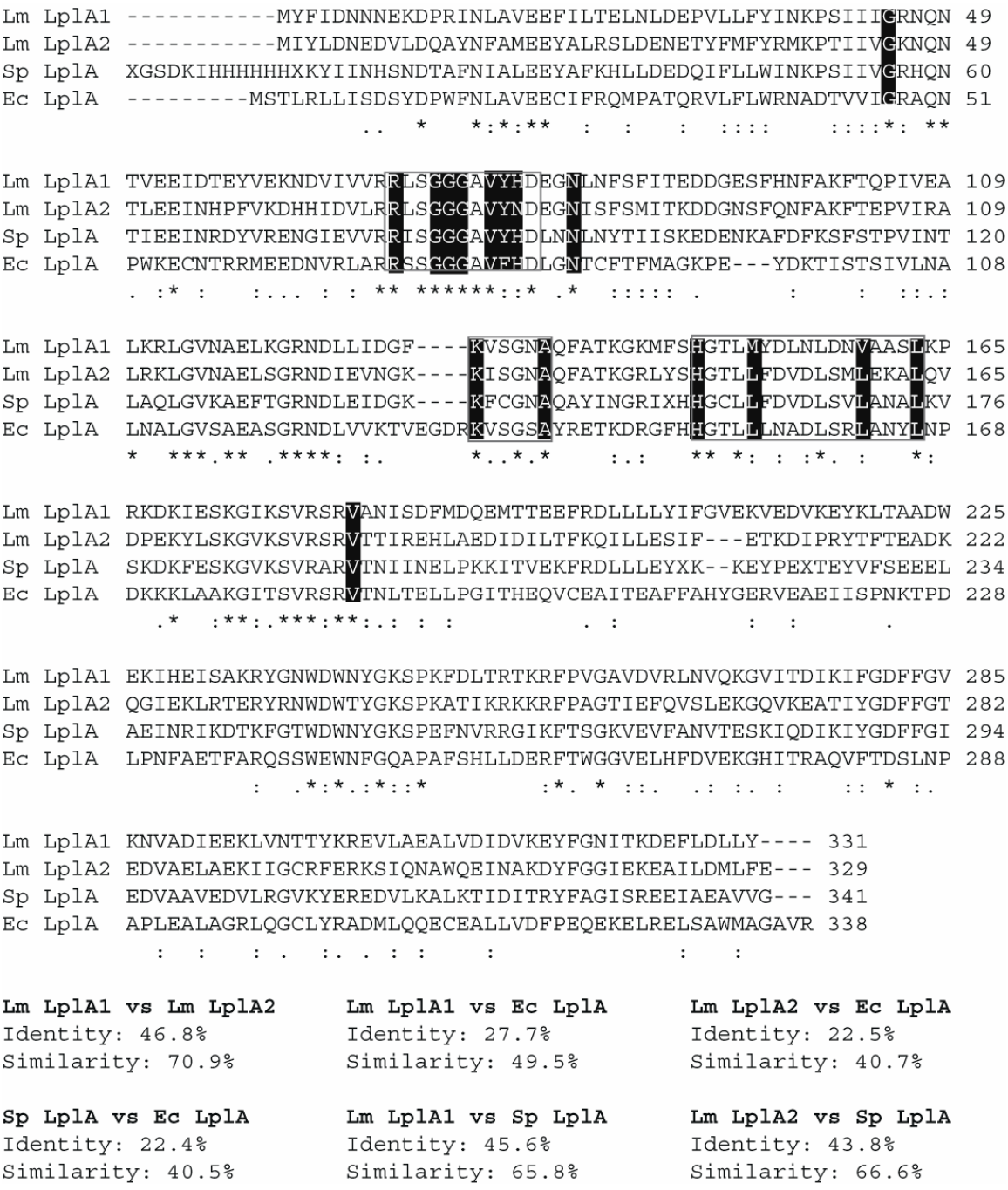


Figure 2.7 Structural modeling of *L. monocytogenes* LplA1 and LplA2.

Electrostatic surface potentials for the crystallographic structure *E. coli* LplA (PDB ID 1X2H) and the modeled 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) are shown in red, positive potentials (10 kT/e) 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.

CHAPTER 2 APPENDIX

Table 2A.1 Primers used in Chapter 2	
SOE	
5'F1	5'-TATACTTGGTGCGGTTGCTGCT-3'
5'R1-SOE	5'-TCTTGCCAAGCACGCAGCGCATATTCTTCCATTGC-3'
3'R1	5'-ATTCGCAAGCATCGTCACTTGGTC-3'
3'F1-SOE	5'-ATATGCGCTGCGTGCTTGGCAAGAAATAAATGCGAAAGAC-3'
QRT-PCR	
RpoA F	5'-TGCTTCTTCTGGGCTGATACTTCC-3'
RpoA R	5'-TGCAAGGTCCTGGTGTAGTAACTG'3'
LplA1 F	5'-TGTCATCGTTGTGCGCAGACTTTC-3'
LplA1 R	5'-ACGCCTAAACGTTTCAGAGCTTCC-3'
LplA2 F	5'-ATCGACGTTCTCCGCCGTTTATCT-3'
LplA2 R	5'-AGCATTTACACCAAGCTTGCGGAG-3'
Complementation	
EcLplA F	5'-GCCCATGGCATCCACATTACGCCTGCTCA-3'
EcLplA R	5'-CCCGTCGACTCAGTGGTGGTGGTGGTGGTGCCTTACAGCCCCCGC-3'
LmLplA1 F	5'-GCCCATGGCATATTTTATAGATAACAATAATGAGAAAGATCCA-3'
LmLplA1 R	5'-CCCGTCGACTCAGTGGTGGTGGTGGTGGTGATAAAGTAAATCTAAAAATTCATCTTTAGTAAT-3'
LmLplA2 F	5'-GCTCATGACAATTTATTTAGATAACGAAGATGTACTTGAT-3'
LmLplA2 R	5'-CCCCTCGAGTCAGTGGTGGTGGTGGTGGTGTTCAAATAACATATCTAAAATTGCTTCTTT-3'



Lm LplA1 vs Lm LplA2	**Lm LplA1 vs Ec LplA**	**Lm LplA2 vs Ec LplA**
Identity: 46.8%	Identity: 27.7%	Identity: 22.5%
Similarity: 70.9%	Similarity: 49.5%	Similarity: 40.7%
Sp LplA vs Ec LplA	**Lm LplA1 vs Sp LplA**	**Lm LplA2 vs Sp LplA**
Identity: 22.4%	Identity: 45.6%	Identity: 43.8%
Similarity: 40.5%	Similarity: 65.8%	Similarity: 66.6%

Figure 2A.1 Amino Acid Alignment of Lipoate Ligases.

The predicted protein sequences of LplA1 and LplA2 align with 47% identity and 72% similarity using the Needleman-Wunsch global alignment (EMBOSS). For comparison, LplA from *E. coli* (Accession NP_418803) and *Streptococcus pneumoniae* TIGR4 (accession Np_345629.1) are aligned as well. Stars indicate identity, while two dots indicate greater similarity than one dot. Microbial and human lipoyl transferases share three signature sequence motifs, identified by grey outline boxes: **RR***XX***GGG***X***V**(F/Y)**HD** (residues 68- 79), **K**h***X*****G*****X*****A** (residues 131-136), and **H***XX*(L/M)**L***XXX*(B/N)L***XX*****L***XX*h**L** (residues 147-163) (Kim *et al.,* 2005b). Highly conserved residues that line the surface

of the lipoyl-AMP binding pocket in *Thermoplasma acidophilum* LplA (SWISS-PROT accession code: Q9HKT1) are identified by dark boxes behind each amino acid (Kim *et al.*, 2005b).

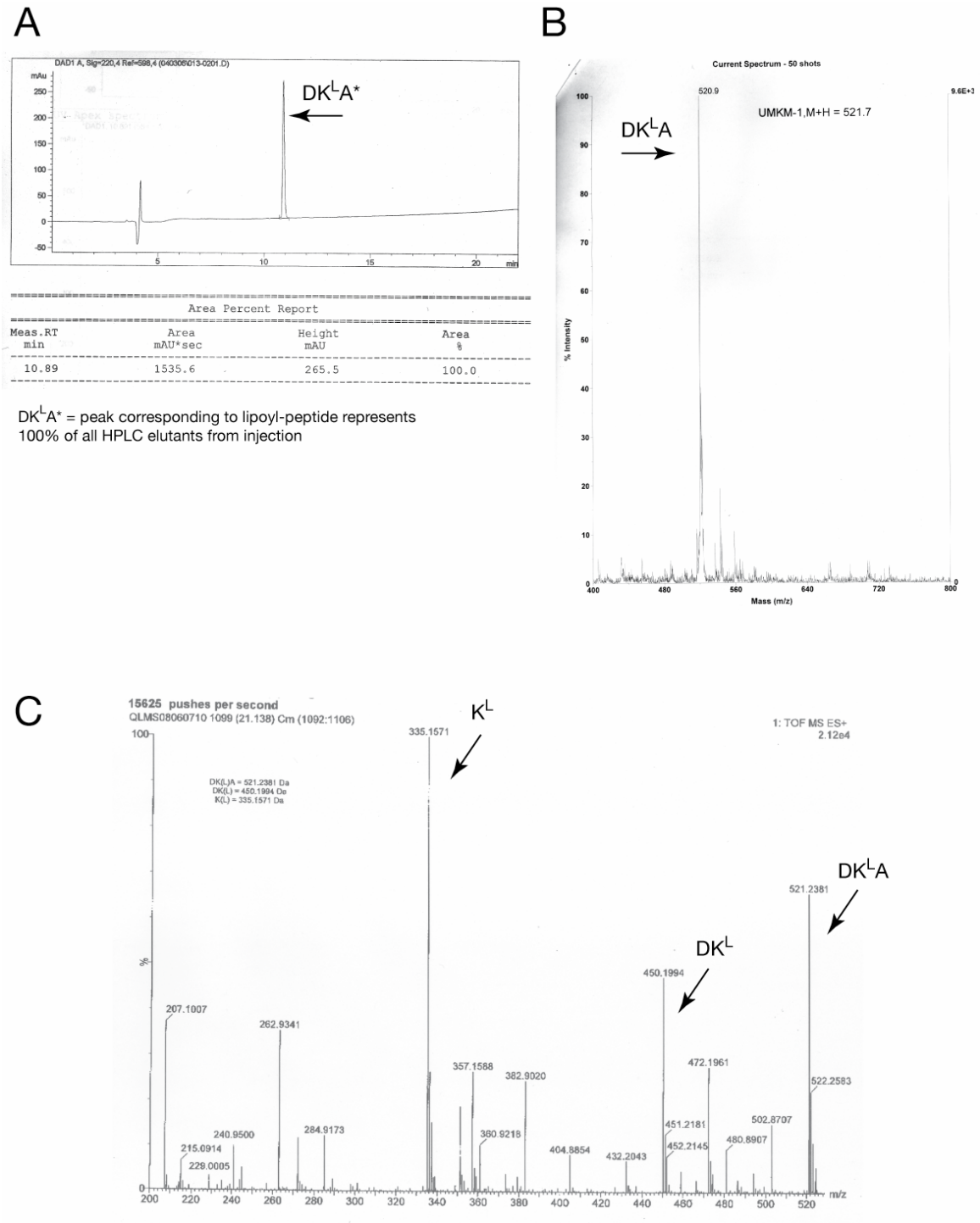


Figure 2A.2 Analysis of synthetic DK^{L}A and digestion products.

(A) HPLC chromatogram of synthetic lipoyl tripeptide DK^LA eluting from a C18 column on a HP1090 instrument (Konishi *et al.,* 1996). The only peak eluted at 10.89 min and contained 100% of the area, indicating there were no byproducts from synthesis that were not pure DK^LA. **(B)** LC-MS mass spectrum of Anaspec synthesized DK^LA injected onto a C18 column. DK^LA exhibited a peak at 521.7 Da **(C)** LC-MS mass spectrum of aminopeptidase M digested DK^LA injected onto a C18 column and analyzed in positive ion mode on a nanoAcuity/Qtof premier instrument. DK^LA exhibited a peak at 521.2381 Da, DK^L exhibited a peak at 450.1994 Da, and K^L exhibited a peak at 335.1571 Da.

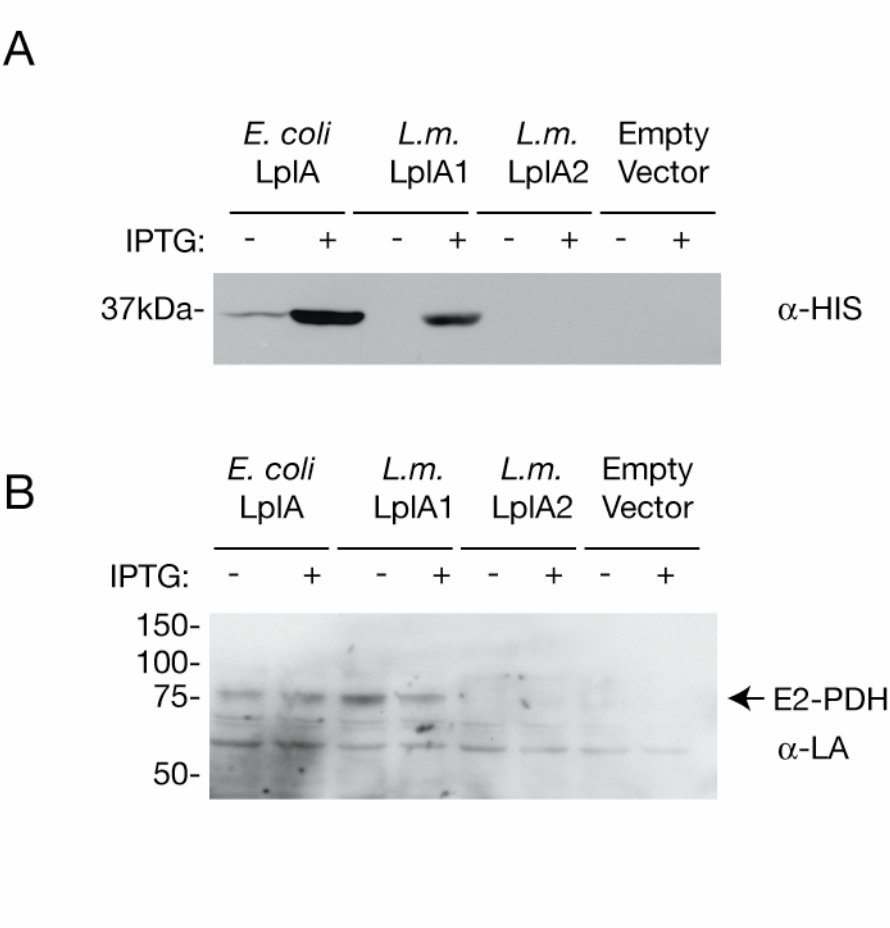


Figure 2A.3 Heterologous expression of *L. monocytogenes* lipoate ligases in *E. coli*.

E. coli TM131 transformed with an empty IPTG inducible vector or vector containing a C-terminal histidine-tagged *E. coli* LplA, *L. monocytogenes* LplA1 or LplA2. Strains were grown in LB with 1mM IPTG. Bacterial pellets were lysed with SDS-PAGE buffer, boiled and analyzed by SDS-PAGE, followed by immunoblot with **A)** an anti-his antibody and **B)** an anti-lipoic acid antibody. The bands appearing at 37kDa on the anti-histidine blot correspond to the predicted size of *E. coli* LplA and *L. monocytogenes* LplA1, and the 75kDa band in the anti-lipoic acid blot corresponds with lipoylated E2-PDH from *E. coli*.

Chapter 3

GENERATION OF BRANCHED CHAIN FATTY ACIDS THROUGH LIPOATE DEPENDENT METABOLISM FACILITATES INTRACELLULAR GROWTH OF *Listeria monocytogenes*

SUMMARY

The Gram-positive bacterial pathogen *Listeria monocytogenes* has evolved mechanisms to rapidly replicate in the host cytosol, implying efficient utilization of host-derived nutrients. However, the contribution of host nutrient scavenging versus bacterial biosynthesis toward rapid intracellular growth remains unclear. Nutrients that contribute to growth of *L. monocytogenes* include branched chain fatty acids, amino acids, and other metabolic intermediates generated from acyl-CoAs, which are synthesized using lipoylated metabolic enzyme complexes. To characterize which biosynthetic pathways support replication of *L. monocytogenes* inside the host cytosol, we impaired lipoate dependent metabolism (LDM) by disrupting two lipoate ligase genes that are responsible for bacterial protein lipoylation. Interrupting LDM lipoate dependent metabolism modestly impaired replication in rich broth medium, but strongly inhibited growth in minimal medium and host cells, and impaired the generation of amino acids and branched chain fatty acids (BCFAs). Amino acid supplementation substantially respored the BCFA profile, even in the absence of lipoate

dependent metabolism, indicating BCFAs must also be generated by a BCKD-independent mechanism. Addition of short short branched chain fatty acids (BCFA) and amino acids restored growth of the A1A2^{-} mutant in minimal medium, and BCFA alone rescued intracellular growth and spread in L2 fibroblasts. Lipoate dependent metabolism LDM was also required *in vivo,* as a wild-type strain robustly out-competed the lipoylation deficient mutant in a murine model of Listeriosis. Thus, lipoate dependent metabolism LDM influences branched chain fatty acid composition, which is critical for intracellular growth and virulence of *L. monocytogenes*.

INTRODUCTION

The Gram-positive foodborne pathogen, *L. monocytogenes,* replicates in diverse settings ranging from soil to the intracellular environment of mammalian host cells (Swaminathan and Gerner-Smidt, 2007; WELSHIMER, 1960, 1968). The ability of *L. monocytogenes* to thrive in soil and food indicates the bacterium has the capacity to adapt to different nutritional environments. When carbon source availability changes, *L. monocytogenes* alters activity of the global virulence regulator, PrfA (Gilbreth *et al.,* 2004; Joseph *et al.,* 2008; Milenbachs *et al.,* 1997). While the nutritional milieu surrounding *L. monocytogenes* affects virulence gene expression by altering PrfA function, deletion of *prfA* also appears to decrease the metabolite pool of BCAAs (Eisenreich *et al.,* 2006; Kim *et al.,* 2006; Trivett and Meyer, 1971). It is not fully appreciated why PrfA, a regulator that enables virulence, would influence biosynthesis. Although it is known that biosynthesis of aromatic amino acids is important for *L. monocytogenes* virulence, it is of interest that some single amino acid auxotrophs are not attenuated for growth in the cytosol, implying that the bacterium can acquire host derived amino acids to support intracellular growth (Marquis *et al.,* 1993; Stritzker *et al.,* 2004). While the data indicate amino acids are central to *L. monocytogenes* pathogenesis, it remains unclear what metabolic precursors the bacterium must synthesize, and what are scavenged from the host environment.

Strategies for increasing biosynthesis, or obtaining key nutrients from the host, are essential aspects of pathogenesis (Brown *et al.,* 2008). Intracellular pathogens such as *S.* Typhimurium and *Mycobacterium tuberculosis* can adapt to the mammalian host environment by differentially regulating nutrient transporters and metabolic biosynthesis pathways (Heithoff *et al.,* 1997; Schnappinger *et al.,* 2003). *L. monocytogenes* may share these adaptive strategies when transitioning from the extracellular environment to the host cytosol, as it upregulates genes that enable the biosynthesis of BCAAs, as well as genes encoding glycerol-3-phosphate dehydrogenase and enzymes of the pentose phosphate pathway (Chatterjee *et al.,* 2006; Eisenreich *et al.,* 2006; Joseph *et al.,* 2006). *L. monocytogenes* also exploits the cytosolic nutrient environment by expressing a hexose phosphate transporter, and induces sugar transporter gene expression when replication is shifted from the vacuole to the cytosol (Chatterjee *et al.,* 2006; Chico-Calero *et al.,* 2002; Joseph *et al.,* 2008). Thus, *L. monocytogenes* can respond to nutritional changes by synthesizing or transporting metabolic intermediates to support virulence.

One nutrient *L. monocytogenes* cannot synthesize is lipoate, an essential cofactor for metabolic enzyme complexes such as pyruvate dehydrogenase (PDH) and branched chain α-keto acid dehydrogenase (BCKD)(Premaratne *et al.,* 1991). We previously demonstrated that during growth in nutrient limiting conditions, *L. monocytogenes* requires the lipoate ligase LplA1 for utilization of physiological concentrations of host-derived lipoyl-peptides. Despite the

presence of another functional lipoate ligase, LplA2, LplA1 is essential for bacterial pathogenesis of *L. monocytogenes* (Keeney *et al.,* 2007). It is not yet fully understood why lipoate dependent metabolism (LDM) is important for bacterial virulence. Studies of microbial physiology in model organisms such as the Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis* have demonstrated that lipoylated PDH generates acetyl-CoA from pyruvate, while lipoylated BCKD makes branched acyl-CoAs from BCAA catabolism (Kaneda, 1991). Unlike *E. coli*, generation of tricarboxylic acids in *L. monocytogenes* is non-cyclical and is unlikely to generate energy in the form of ATP, as it is missing α-ketoglutarate dehydrogenase activity (Eisenreich *et al.,* 2006). Thus, the lipoylated metabolic enzymes complexes of *Listeria* may only play a critical role in the biosynthesis of amino acids and branched chain fatty acids (BCFAs), key components of the bacterial membrane of *L. monocytogenes* (Zhu *et al.,* 2005a). BCFAs enhance survival of *L. monocytogenes* during adverse pH conditions and cold stress, although it is unknown what role they play during pathogenesis (Giotis *et al.,* 2007; Zhu *et al.,* 2005a). While amino acids and peptides are scavenged by *L. monocytogenes* in the host cytosol, the bioavailability of other key metabolites, such as BCFAs, has not been determined (Eylert *et al.,* 2008).

Neither PDH nor BCKD are predicted to be functional in the absence of lipoate, and mutations that render *E. coli* unable to ligate lipoate or lipoate precursors to metabolic enzymes are lethal in the absence of nutritional supplements to bypass the requirement for the Tricarboxylic Acid Cycle (Morris *et al.,* 1995). To

determine what nutrients must be synthesized by *L. monocytogenes* to survive in the intracellular environment, we assessed the ability of a *L. monocytogenes* mutant deficient in the two known lipoate ligases to replicate *in vitro* and *in vivo.* Unlike the previously characterized LplA1 lipoate ligase mutant, which can lipoylate bacterial proteins when *L. monocytogenes* is in the extracellular environment, this lipoate dependent metabolism mutant is deficient in both LplA1 and LplA2 (*lplA1::Tn917⊿lplA2,* A1A2$^-$) and does not detectably lipoylate any bacterial target proteins (Keeney *et al.,* 2007). In this study, we demonstrate that lipoate dependent metabolism LDM in *Listeria monocytogenes* controls amino acid and anteiso-BCFA composition. Lipoate dependent metabolism was not essential for *L. monocytogenes* growth in rich medium, but was required for cytosolic replication and in nutrient limiting conditions. Growth of the A1A2$^-$ mutant in defined medium could be rescued by enriching with amino acids and BCFA precursors, but only the BCFA precursors stimulated intracellular growth. These data suggest that intracellular growth of *L. monocytogenes* requires bacterial biosynthesis of BCFAs because the cytosolic environment may be limiting for BCFAs.

RESULTS

L. monocytogenes growth in the absence of lipoate ligases in rich nutrient broth

To determine the role of lipoate dependent metabolism LDM in *L. monocytogenes* extracellular growth, we characterized a previously constructed bacterial mutant deficient in both LplA1 and LplA2, lacking all detectable bacterial lipoylation (Keeney *et al.,* 2007). We first tried to generate this mutant by constructing an in-frame double deletion mutant without insertion of a drug selection cassette, but we were unable to recover mutants. We hypothesized this was due to a replication defect in the mutant. We therefore transduced the previously characterized null transposon insertion allele with drug selection and were able to recover a double disruption mutant (*lplA1::Tn917⊿lplA2*, designated A1A2^{-}) (Keeney *et al.,* 2007; O'Riordan *et al.,* 2003). In the absence of these two ligases, we were unable to detect lipoylated bacterial target proteins as observed by immunoblot with an antibody against lipoic acid (Fig. 3.1A) (Keeney *et al.,* 2007). The 75 kD lipoylated protein species in WT was previously identified as the E2 subunit of PDH, and the migration of the 50 kD protein species is consistent with the predicted size for the lipoylated E2 subunit of BCKD (O'Riordan *et al.,* 2003). *L. monocytogenes* encodes a functional BCKD, as when the genes encoding subunits of BCKD are disrupted, branched chain fatty acid biosynthesis is perturbed (Zhu *et al.,* 2005a). Another lipoylated enzyme found in

some bacteria, such as *E. coli,* is α-ketoglutarate dehydrogenase (KGDH). However, the gene encoding KGDH is missing in *L. monocytogenes*, and α-ketoglutarate dehydrogenase activity is also absent in bacterial extracts (Eisenreich *et al.,* 2006). Thus, the two dominant lipoylated bands in WT *L. monocytogenes*, but absent in the A1A2$^-$ mutant, likely correspond to bacterial PDH and BCKD. We therefore concluded the lipoate ligases LplA1 and LplA2 are responsible for all detectable protein lipoylation in *L. monocytogenes* (Keeney *et al.,* 2007).

Without lipoylation, neither PDH nor BCKD can function to generate biosynthetic intermediates (Perham, 2000). We considered the possibility that complete loss of lipoylation would result in a replication defect. Analysis of bacterial growth in BHI showed that the doubling times of the WT and AlA2$^-$ mutant during exponential growth were similar (63-66 minutes), although the AlA2$^-$ mutant entered stationary phase earlier than the wildtype strain (Fig. 3.1BC). This trend was confirmed by measuring colony-forming units in addition to the reported OD_{600} values found in Figure 1 (data not shown). We also observed the A1A2- mutant had a slight defect in acidification of its growth medium when compared with WT (Fig. B.3). Thus, *L. monocytogenes* is able to grow in rich medium even in the absence of lipoyl ligases.

Metabolic alterations in the absence of lipoylation

To determine if we were altering known lipoate-dependent metabolic pathways, we grew WT and the AlA2$^-$ mutant in BHI, and quantified lactate, acetoin, fatty

acid content, and also measured RNA transcript levels of enzymes downstream from PDH in the oxidative branch of *L. monocytogenes* TCA cycle (Fig. 3.2, Fig. B.2). Notably, RNA transcript levels for *citB,* which encodes aconitase, were repressed in the A1A2$^-$ mutant, suggesting the bacterium can detect a loss in lipoylation of PDH and regulate expression of downstream enzymes (Fig. B.2). Without lipoylated PDH, pyruvate generated from glycolysis would likely be converted into alternate metabolites such as lactate and acetoin through lactate dehydrogenase and the 2-3 butanediol pathway (Fig. 3.2A). While lactate levels were relatively similar, the amount of secreted acetoin was 4 fold higher in the AlA2$^-$ mutant as compared to WT (Fig. 3B and C). We confirmed acetoin secretion was optimal during aerobic growth (Fig. B.1). Notably, acetoin secretion seemed to be correlated with lipoylation of the bacterium, ; when WT was grown in IMM without lipoate, acetoin secretion was increased as well (Fig. B.1). Quantification of total amino acid content showed an eight percent drop in the levels of glutamate and glutamine (glx) in AlA2$^-$ mutant, as well as a decrease in homocysteine levels, but there were no other striking differences in total amino acid levels between WT and the A1A2$^-$ mutant (Table 3.2). These results were not surprising, as *L. monocytogenes* can scavenge amino acids from its growth environment, and BHI is rich in amino acids (Marquis *et al.,* 1993).

In contrast to the amino acid results, we expected fatty acid content to be altered when lipoylation of BCKD was disrupted, as BCKD-deficient mutants have a decreased capacity to generate branched chain fatty acids during growth in BHI

(Zhu *et al.,* 2005a). To measure fatty acid composition, we grew and collected bacterial cell pellets and submitted them for fatty acid analysis. Straight chain fatty acids predominated in the AlA2$^-$ mutant, while BCFAs were the predominant fatty acid species in WT (Fig. 3.2D), confirming that lipoylation is essential for *L. monocytogenes* BCFA biosynthesis even in rich medium. We therefore conclude that despite the ability of *L. monocytogenes* to grow in BHI without lipoylation, fundamental metabolic pathways that generate acetoin and branched chain fatty acids were are altered in the absence of lipoylation.

L. monocytogenes pathogenesis requires LDMlipoate dependent metabolism

As the AlA2$^-$ mutant could grow in BHI, we tested the ability of the AlA2$^-$ mutant to grow in a murine model of *L. monocytogenes* infection. Erythromycin sensitive (Erm^s) WT *L. monocytogenes* was mixed with erythromycin resistant (Erm^r) AlA2$^-$ mutant and co-injected into female C57Bl/6 mice. After 24h, livers and spleens were harvested and colony forming units (CFU) were enumerated. The competitive index of infection (CI) was calculated by dividing the recovered Erm^s WT by Erm^r AlA2$^-$ mutant bacteria. We previously demonstrated that the CI for the *ΔlplA1* single mutation is 31.6 and 603.0 in the liver and spleen of C57Bl/6 mice, indicating that WT significantly outcompetes a partial lipoylation mutant in mice (Keeney *et al.,* 2007). After 24hrs, WT outcompeted the AlA2$^-$ mutant 9,872 and 23,391 fold in the liver and spleen of C57BL/6 mice; in some animals, no AlA2$^-$ mutant bacteria were recovered (Fig. 3.3). Therefore, *L. monocytogenes*

deficient in lipoyl ligase function is substantially less fit in a murine model of infection than bacteria with a partial lipoylation defect or WT bacteria.

Replication of *L. monocytogenes* in phagocytic cells requires LDMlipoate dependent metabolism

To determine where in the *L. monocytogenes* intracellular lifecycle lipoate dependent metabolism LDM was required, we measured the ability of the AlA2$^-$ mutant to escape and proliferate in phagocytes. In our initial studies, we measured hemolytic activity of the A1A2$^-$, and observed no defect in the bacterium's ability to lyse red blood cells, suggesting the bacterium would also be able to lyse the vacuole of phagocytic cells (Fig B.4). This observation was confirmed when we infected macrophages with WT or the AlA2$^-$ mutant, and quantified the colocalization of *L. monocytogenes* with polymerized host actin, as colocalization only occurs in the cytosol (Schnupf and Portnoy, 2007). The AlA2$^-$ mutant was able to escape from the vacuole into the cytosol, albeit with lower efficiency than WT, as it co-localized with host actin 2h post infection in bone-marrow derived macrophages (BMDM, Fig. 3.4A). However, the AlA2$^-$ mutant did not replicate in the nutrient limiting environment of the J774 or BMDM cytosol over an 8h infection (Fig. 3.4BC). When we examined the ability of the AlA2$^-$ mutant to survive and grow in the RAW264.7 peritoneal macrophage cell line, the AlA2$^-$ mutant was more susceptible to killing, demonstrating that lipoate dependent metabolism LDM may be important for survival as well as intracellular growth (Fig. 3.4D). We hypothesized lipoylation of *L. monocytogenes* could enhance the bacterium's ability to resist oxidative stress in the phagocyte, as WT

bacteria appear to have evolved resistance mechanisms against the phagocytic burst (Fig. A.3A). Additionally, lipoylated proteins are present on the surface of *L. monocytogenes*, and these lipoylated proteins appear to be present in both oxidized and reduced forms, suggesting they are capable of quenching oxidative stress (Fig. A.1). We also determined the A1A2$^-$ mutant, as well as lipoate starved WT bacteria, were susceptible to *in vitro* oxidative stress (Fig. A.2). However, a lipoate ligase mutant, *⊿lplA1*, which is deficient in lipoylation during growth *in vivo*, did not appear to enhance *L. monocytogenes* fitness against *in vivo* oxidative and nitrosative stress, so the role of lipoate in defending *L. monocytogenes* against host oxidative stress remains undetermined (Fig. A.3BC). However, all of this these data does indicate lipoate dependent metabolism LDM is vital for replication of *L. monocytogenes* inside macrophages.

Growth and spread of LDM lipoate dependent metabolism mutants in L2 cells

As different cell types may provide distinct nutrient environments for *L. monocytogenes*, we also measured bacterial growth in non-phagocytic cells. Murine L2 fibroblasts were infected with WT or the AlA2$^-$ mutant, and incubated for several days to allow necrotic foci to form. To measure bacterial replication and cell-cell spread, wWe visualized and measured plaques by Neutral Red staining, and measured plaque diameter as an indicator of bacterial replication and cell-cell spread. Notably, the AlA2$^-$ mutant was able to form plaques, although the plaques were substantially reduced in size compared with WT and only formed around the edge of the well (Fig. 3.5A). We also examined the

intracellular growth of WT, the AlA2$^-$ mutant, and a vacuole restricted LLO$^-$ deficient mutant in L2 cells by enumerating colony forming units over both a short and long period of infection (Fig. 3.5BC). After an initial lag, the AlA2$^-$ mutant could replicate in L2 cells, although its doubling time never approached WT (Fig. 3.5BC). The ability of the *L. monocytogenes* lipoylation mutant to grow in L2 cells suggests the possibility that the intracellular environment of some cell types may provide nutrients that bypass the requirement for lipoate dependent metabolismLDM.

Excess environmental amino and fatty acids bypass the requirement for LDMlipoate dependent metabolism

Critical nutrients for bacterial replication may be sequestered or absent in the host cytosol (Brown *et al.,* 2008). To determine if growth of *L. monocytogenes* requires lipoate dependent metabolism LDM to generate critical growth intermediates when the bacterium is in a nutrient limiting environment, we grew the A1A2$^-$ mutant and WT in improved minimal medium (IMM). This defined medium was developed for growth of WT *L. monocytogenes* (Fig. 3.6A) (Phan-Thanh and Gormon, 1997). While WT *L. monocytogenes* efficiently replicated in IMM, the AlA2$^-$ mutant did not grow, indicating that lipoate dependent metabolism LDM was necessary for bacterial growth under conditions of nutrient limitation. As the AlA2$^-$ mutant could grow in BHI, but not IMM, we hypothesized that one important function of lipoate dependent metabolism LDM is biosynthesis of key metabolites, which would be more critical in nutrient poor environments. We therefore reasoned that we could identify which biosynthetic pathways might be

supported by lipoate dependent metabolism LDM by taking a nutrient supplementation approach. WT or AlA2$^-$ mutant bacteria were inoculated into IMM supplemented with additional amino acids and fatty acids, and bacterial growth was measured over time. When amino acids were added to IMM in the form of soy protein hydrolysate, the AlA2$^-$ mutant exhibited a dramatic increase in growth (Fig. 3.6B). Soy protein hydrolysate somewhat enhanced the growth of WT bacteria as well. When an alternate source of amino acids, vitamin free casein hydrolysate, was used to supplement IMM, it also supported a similar increase in growth of the AlA2$^-$ mutant as observed with the soy protein hydrolysate (data not shown). When amino acids were added individually, growth did not occur, implying that lipoate dependent metabolism LDM controls multiple branches of amino acid biosynthesis. As the BCFA profile was altered in the AlA2$^-$ mutant after growth in BHI (Fig. 3.2D), we also added the BCFA precursors 2-methylbutyrate (2-MB), isovalerate (IV), and isobutyrate (IB)e. Addition of only BCFA precursors to IMM did not enable growth of the AlA2$^-$ mutant in the absence of soy protein hydrolysate. When a precursor for even carbon length iso-BCFAs, IVisovalerate, was added with soy protein hydrolysate, replication of the AlA2$^-$ mutant was not affected (Fig. 3.6B). However, addition of precursors for odd carbon chain length BCFAs, 2-MB2-methylbutyrate and IBisobutyrate, did partially enhance replication of the AlA2$^-$ mutant while not affecting growth of WT (Fig. 3.6B). Of the two odd carbon chain length BCFA precursors, 2-MB, a precursor for anteiso-BCFAs, better supported replication of the AlA2$^-$ mutant than IBisobutyrate, an iso-BCFA precursor (Fig. 3.6B). These data suggest that

in non-nutrient limiting conditions, such as BHI, *L. monocytogenes* uses lipoate dependent metabolism LDM for production of branched chain fatty acids. However, when the growth environment becomes limiting in amino and branched chain fatty acid precursors, such as found in IMM, lipoate dependent metabolism LDM becomes essential for growth.

As WT is able to generate substantially more BCFAs than the A1A2$^-$ mutant after growth in BHI (Fig. 3.2D) and BCAAs can be precursors for BCFAs, we sought to determine if addition of amino acid sources and short BCFA precursors would enhance the ability of the A1A2$^-$ mutant to generate BCFAs. Without supplementation, over 80% of the total fatty acids in WT were anteiso-BCFAs, whereas addition of soy protein hydrolysate and 2-MB methylbutyrate only increased WT anteiso-BCFA content to 90% of the total fatty acid content (Fig 3.6C, Table 3.3). In contrast, addition of soy protein hydrolysate to BHI substantially altered the BCFA profile of the A1A2$^-$ mutant nearly to levels found in WT, increasing the odd carbon length anteiso-BCFAs from 5% to 75% of the total fatty acid content (Fig. 3.6C,Table 3.3). Supplementation with 2-MB methylbutyrate in addition to amino acids increased the odd carbon length anteiso-BCFAs from 75% to 95% of total fatty acid content in the AlA2$^-$ mutant, while it remained constant at 90% in WT (Table 3.3). The total amino acid content of the AlA2$^-$ mutant after supplementation with soy protein and 2-MB methylbutyrate remained largely unaltered from the amino acid content of WT, with the exception of glutamine and glutamate (Glx), which was increased from

13 to 24% of the total recovered amino acids in the A1A2$^-$ mutant (Table 3.2). Together, soy protein hydrolysate and 2-MB restored the growth of the AlA2$^-$ mutant to near WT levels (Fig. 3.6B), while soy protein hydrolysate supplementation alone restored the branched chain fatty acid profile to near WT levels (Fig. 3.6C), but only partially restored growth. These data indicate that the requirement for lipoate dependent metabolism LDM mediated biosynthesis during *L. monocytogenes* replication in nutrient restricted medium can be bypassed by supplementation with amino acids, which not only provide building blocks for protein synthesis but also enhance anteiso-BCFA synthesis.

Intracellular growth and spread of *L. monocytogenes* is restored in A1A2$^-$ mutant with branched chain fatty acid precursor supplementation

L. monocytogenes is known to scavenge amino acids from the host environment, but scavenging of BCFA precursors from the cytosol has not been investigatedv\ (Marquis *et al.,* 1993). Biosynthesis of BCFA in *L. monocytogenes* is known to be partially dependent upon BCKD, which requires lipoylation for activity (Fig. 3.2A). If the A1A2$^-$ mutant cannot grow in the cytosol of host cells because it does not scavenge BCFAs from the host and it has a reduced ability to generate BCFAs, we hypothesized we would be able to bypass the requirement for lipoate dependent metabolism LDM in the A1A2$^-$ mutant by exogenously supplementing with BCFA precursors. We supplemented L2 cells with and without soy protein hydrolysate and 2-MB methylbutyrate and assessed the ability of the AlA2$^-$ mutant to grow and spread (Fig. 3.7). Addition of soy protein hydrolysate had an inhibitory effect on the growth of L2 cells (data not shown) and also had a slightly

inhibitory effect on the intracellular growth and spread of WT *L. monocytogenes*. We observed no enhancement of AlA2$^-$ mutant plaque formation in the presence of soy protein hydrolysate, compared to untreated L2 cells. We also tested whether 5mM 2-MBmethylbutyrate, which had no apparent effect on L2 cells up to a concentration of 500 mM, affected growth of the AlA2$^-$ mutant. In contrast to the behavior of the AlA2$^-$ mutant in IMM where amino acid supplementation was more stimulatory than 2-MBmethylbutyrate, addition of 5mM 2-MB methylbutyrate alone significantly enhanced the growth and spread of the lipoylation deficient mutant in L2 cells (Fig. 3.7). We infer from these data that in the host cell cytosol, *L. monocytogenes* can scavenge sufficient amino acids for growth, but requires lipoate dependent metabolism LDM for biosynthesis of anteiso-BCFAs, a major component of the listerial membrane.

DISCUSSION

In this study, we investigated the mechanism by which lipoate ligases in *L. monocytogenes* contribute to bacterial metabolism during intracellular growth and virulence. Disrupting both lipoate ligase genes resulted in a complete loss of detectable lipoylated proteins in bacterial cell extract. In the absence of lipoylation, the AlA2$^-$ mutant was able to grow in rich broth medium, but exhibited changes in acetoin and BCFA metabolism. Replication of the AlA2$^-$ mutant in nutrient limiting conditions and in the host cytosol was substantially impaired. Supplementation with non-essential amino acids and anteiso-BCFA precursors to the AlA2$^-$ mutant restored growth in minimal medium, and returned the profile of anteiso-BCFAs to a profile similar to WT. Notably, in the non-phagocytic L2 cell line, the metabolic block in growth was partially complemented by the addition of anteiso-BCFA precursors, but not by amino acids. These data imply that the cytosolic environment dictates bacterial biosynthesis of BCFAs, and suggest that lipoate dependent metabolism in *L. monocytogenes* enables this process to facilitate replication and virulence.

In *L. monocytogenes*, when genes encoding BCKD are disrupted, anteiso-BCFAs are synthesized at significantly reduced levels (Giotis *et al.,* 2007; Zhu *et al.,* 2005a). The inability to produce anteiso-BCFAs through BCKD can be bypassed by supplementation with 2-MB2-methylbutyrate, a precursor for anteiso-BCFAs. BCKD mutants exhibit defects in the cold stress response,

possibly because anteiso-BCFAs contribute substantially to the single membrane bilayer found in Gram-positive bacteria (Willecke and Pardee, 1971). Here, we find that in the absence of lipoylated metabolic enzymes, including BCKD, the A1A2$^-$ mutant produces very low levels of odd anteiso-BCFAs. We found that amino acid supplementation of the AlA2$^-$ mutant in minimal medium could substantially restore the anteiso-BCFA profile, even in the absence of functional BCKD. Thus, *L. monocytogenes* branched chain fatty acids must also be generated by a BCKD-independent mechanism. Although *L. monocytogenes* can scavenge amino acids from the cytosolic environment, replication of the A1A2$^-$ mutant was still significantly impaired (Marquis *et al.,* 1993). Since only 2-MB2-methylbutyrate, an anteiso-BCFA precursor, could supplement growth of the A1A2$^-$ mutant during intracellular growth, the scavenged cytosolic amino acids presumably were sufficient to replete bacterial amino acid pools, but not in sufficient excess to drive BCFA biosynthesis. Together, these data suggest the intracellular environment does not provide either enough amino acids or BCFA precursors to bypass the requirement for lipoate dependent metabolism LDM mediated fatty acid biosynthesis. The cytosol of different host cell types could offer distinct nutrient profiles, some of which could minimize the requirement for lipoate dependent metabolism LDM mediated biosynthesis in *L. monocytogenes* infection. It has already been suggested that the host may provide diverse nutrients to bypass metabolic pathway requirements in different cell types, as *L. monocytogenes* only requires a gene encoding glycerol kinase 1 in some cell types, but not others (Joseph *et al.,* 2008). It will be of interest to determine if *L.*

monocytogenes and other bacteria that require lipoate dependent metabolism LDM for growth exhibit tropism towards tissues rich in nutrients, as such tissues could lessen the energetic burden of biosynthesis.

The different mechanisms by which *L. monocytogenes* generates anteiso-BCFAs from BCAAs have not been well characterized, although studies in other bacterial species suggest that the requisite methyl-branch essential for BCFA biosynthesis is primarily derived from degradation of BCAAs into branched carboxylic acids (Kaneda, 1991). Alternatively, BCFA can directly be generated from branched short-chain carboxylic acids such as 2-MB2-methylbutyrate, or from β-oxidation of existing endogenous or exogenous branched chain fatty acids (Kaneda, 1991; Kunau *et al.,* 1995). These branched carboxylic acids can then either be activated to their CoA esters with BCKD or an acyl-CoA synthetase, or they can be activated by a decarboxylase. In bacteria such as *E. coli* and *Bacillus subtilis,* the branched primers then condense with malonyl-ACP derived from malonyl-CoA and eventually form elongated BCFAs (Kaneda, 1991). In *B. subtilis* lysate, when the large BCKD complex is removed by ultracentrifugation, the remaining lysate can still generate branched chain fatty acids using a branched-chain α-keto acid decarboxylase (KDCA)(Kaneda, 1988). KDCA decarboxylates α-keto acid derivatives of BCAAs, and in *B. subtilis*, this primer source then is thought to condense with a malonyl-ACP derivative to ultimately generate BCFAs (Kaneda, 1991). By homology to a characterized KDCA of *Lactococcus lactis, L. monocytogenes* EGD-e encodes a candidate KDCA (*lmo1984, ilvB*) that shares

21% identity and 66% similarity. Future experiments will determine if *L. monocytogenes* can generate branched chain fatty acids through KDCA as an alternative mechanism to BCKD.

The influence of nutrients on virulence is widespread among pathogens (Brown *et al.,* 2008). In *Vibrio cholerae* El Tor, the production of 2,3-butanediol enables survival under acidic growth conditions and enhances murine intestinal colonization (Yoon and Mekalanos, 2006). In *Salmonella enterica* serovar Typhimurium formate induces invasiveness, and in *Streptococcus pneumoniae* sucrose transport and metabolism contributes to colonization and disease (Huang *et al.,* 2008; Iyer and Camilli, 2007). Finally, in *Plasmodium falciparum,* lipoate scavenging contributes to mitochondrial lipoylation and enables growth in erythrocytes (Allary *et al.,* 2007; Günther *et al.,* 2005). Here, the ability of the nutrient 2-MB2-methylbutyrate, an anteiso-BCFA precursor, to partially bypass the need for lipoate dependent metabolism during intracellular growth demonstrates that BCFAs play a key role in *L. monocytogenes* survival and intracellular growth. High percentages of anteiso-BCFA are thought to increase *L. monocytogenes* resistance to the lantibiotic nisin, a peptide antibiotic naturally produced by non-pathogenic bacteria (Martínez and Rodríguez, 2005). Since BCFAs also contribute to the ability of *L. monocytogenes* to survive cold and alkaline stress, we speculate that they additionally allow the bacteria to survive the unfriendly environment of the host cytosol (Liu *et al.,* 2002; Stritzker *et al.,* 2004). Other studies also emphasize a key role for fatty acids in virulence. In

Staphylococcus aureus, insertional inactivation of BCKD disrupts BCFA and the ability of the bacterium to resist oxidative stress and alkaline pH (Singh *et al.,* 2008). In *M. tuberculosis*, microarray studies have revealed an upregulation of genes encoding fatty acid catabolic pathways during vacuolar growth in the macrophage (Schnappinger *et al.,* 2003). Finally, deletion of acyl carrier protein in *Toxoplasma gondii* leads to loss of PDH lipoylation and abrogation of apicoplast-localized fatty acid synthesis, resulting in attenuation of virulence (Mazumdar *et al.,* 2006). Collectively, our work and the work of others, suggest lipoate dependent metabolism LDM may play a critical role in the virulence of pathogens as it enables *de novo* biosynthesis of critical metabolic nutrients that may not be provided in the nutrient restrictive environment of the host.

EXPERIMENTAL PROCEDURES

Bacterial culture

Strains used in this study are described in Table 3.1. Prior to infection of cells in tissue culture, *L. monocytogenes* was grown overnight in brain heart infusion (BHI, Difco) at 37°C. For extracellular growth curves, bacteria were grown either in BHI or improved minimal medium (IMM) in a Bioscreen Growth Curve Analyzer (Growth Curves, USA). IMM was prepared as previously described, except where indicated, filter sterilized supplements were added (Phan-Thanh and Gormon, 1997).

Protein Analysis.

Bacteria were prepared and analyzed 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) or stationary phase cultures (OD_{600} 1.2) were used after growth in BHI at 37°C. Bacteria were pelleted and then lysed with FastProtein Blue Lysing Matrix (MP Biomedicals) by processing in a FastPrep machine for 40 seconds at a setting of 6.0.

Lactate, Fatty Acid Analysis, and Amino Acid Analysis.

Lactate was quantified using the Lactate Assay Kit (BioVision, K607-100), which was quantified by a colorimetric change at 570 nm. The amount of lactate in the supernatant was quantified by comparison with a standard curve. For fatty acid analysis, bacterial pellets were analyzed by gas chromatographic analysis of cellular fatty acid methyl esters with the Sherlock® Microbial Identification System (Microbial ID). Bacterial pellets were lysed with MP Fast Protein Blue matrix in a Bio101 FastPrep machine (40 seconds, setting 6.0). One mg bacterial protein was submitted for amino acid analysis to the Molecular Structure Facility of UC Davis, where lysate was hydrolyzed with 6N HCl for 24 hrsh at 110ºC, dissolved in sodium citrate buffer containing 40 nmoles/ml norleucine and 50.0 µl injected into a L-8800 Hitachi analyzer. Subsequent peaks and amino acid concentrations were identified with Beckman System Gold software.

Acetoin quantification.

Acetoin concentration in selected culture samples was determined using a previously validated derivatization method (Shirk *et al.,* 2002). 200 µL of reagent-grade acetonitrile (ACN) and 50 µL of a 2,4-dinitrophenylhydrazine (DNPH) stock solution in acidified methanol (0.5 % w/v DNPH, 4% v/v H_2SO_4) were added to 200 µL of filtered culture supernatant in 2-mL plastic micro-centrifuge tubes. Samples were then vortexed briefly and allowed to react for 6 min at room temperature in the dark. An Agilent 1100 series high pressure liquid chromatograph (HPLC) equipped with a UV diode array detector (UV-DAD) set to

345 nm and a Phenomenex C_{18} (reverse phase) column (250 mm × 2.0 mm, 5μ particle size) was used to detect the acetoin-DNP derivatization product, using injection volumes of 50 μL. Mobile phase was a mixture of ACN and distilled deionized water (DDI) pumped at 0.6 mL/min according to the following gradient: 0-13 min, 0:100 to 75:25 ACN:DDI; 13-17 min, 75:25 to 65:35 ACN:DDI; 17-17.5, 65:35 to 0:100 ACN:DDI; 17.5-21 min, 0:100 ACN:DDI. Under these conditions, acetoin-DNP exhibited a characteristic retention time (t_R) of 10.8 min. Peak identity was confirmed via derivatization and HPLC separation of acetoin standards prepared in fresh BHI media. Similarly, derivatized acetoin standards in BHI were used to generate a calibration curve for acetoin concentration as a function of acetoin-DNP peak area at t_R = 10.8 min over the range 0 to 5 mM acetoin.

BMDM isolation.

Primary bone marrow-derived macrophages (BMDM) were isolated from 6-8 week old C57Bl/6 female mice (Jackson Laboratory) and cultured as described (Portnoy *et al.,* 1988).

Bacterial infections.

Mammalian cells were infected as previously described (O'Riordan *et al.,* 2003). Bacterial cultures were grown at 37°C in BHI to stationary phase and washed. Per 24 well dish, $1.2x10^6$ RAW and $4x10^6$ BMDM or J774 macrophages were

infected for 30 minutes at an MOI of 0.6 for WT and 12 for AIA2$^-$ mutant in J774 and BMDM macrophages and an MOI of 20 for WT and 133 for the AIA2$^-$ mutant in RAW264.7 cells. Per 24 well dish, 5x10^6 L2 cells were infected for 60 minutes at an MOI of 1 for WT and 20 for the AIA2$^-$ mutant in L2 cells. Following infection, host cells were washed twice with D-PBS with Ca^{2+} Mg^{2+} and incubated with medium containing 10mg ml^{-1} gentamycin for the duration of the experiment. These conditions resulted in ~10% host cell infection at 1 h. Infected host cells were lysed in 5ml sterile water and plated on Luria-Bertani (LB) agar, where colony-forming-units (CFU) were counted after 72hrsh incubation at 37°C to permit growth of the AIA2$^-$ mutant.

Intracellular escape assays.

4x10^6 BMDM per 6-well plate were grown on coverslips and infected as described above. At 2h post infection, coverslips were removed and fixed in 3.7% paraformaldehyde in D-PBS. After washing 3 times with 0.1% Tween-20 in Dulbecco's PBS and blocking for 10 minutes with TBS-T (25mM Tris HCL, 150 mM NaCl, 0.1% Tween-20, 1% BSA), anti-*L. monocytogenes* antibody (Difco) was added to the coverslips for 1 h, washed, and then secondary antibody added for 30 minutes with rhodamine phalloidin. Intracellular bacteria were scored for the presence or absence of actin clouds. A minimum of 50 macrophages and at least 150 total bacteria were randomly scored per experimental replicate.

L2 plaque assays.

Plaque assays were performed as previously described (O'Riordan *et al.,* 2003). In L2 plaque assays, branched chain fatty acid precursors and soy protein hydrolysate supplements were added to 3 mls top agar per well after an initial 1h infection and developed for 5 days prior to addition of Neutral Red stain to visualize necrotic foci. Plaque diameters were measured (n=20) and expressed as percentage of the mean diameter of WT plaques using Canvas software (Deneba).

Mouse infections.

Competitive index (CI) infections were performed as previously described (Auerbuch *et al.,* 2001). Exponential phase bacterial cultures were diluted in Ca^{2+}- and Mg^{2+}- free Dulbecco's PBS, and $5x10^5$ WT bacteria and $5x10^7$ AlA2$^-$ mutant bacteria in 200 ml were injected i.p. into 4-6 week old C57Bl/6 J mice (Jackson Laboratories). At 24h and 72h post infection, the animals were sacrificed, livers and spleens homogenized in 0.1% NP-40 containing 0.1 mg ml^{-1} erythromycin (Erm), serially diluted, and plated on drug-free LB agar or LB agar containing 1 mg 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) after normalizing for the input ratio.

Statistical Analysis.

Samples were analyzed with a Student's *t*-test assuming unequal variance (Microsoft Excel). $P<0.05$ values were indicated in the figures with the * symbol.

TABLES AND FIGURES

Table 3.1 Strains used in Chapter 3

Name	Strain	Genotype/description	Reference
WT	10403S	*L. monocytogenes* serotype 1/2 a, parental strain used as wild type	(Freitag *et al.,* 1993)
AlA2- mutant	MOR125	104035S *lplA1::Tn917ΔlplA2*	(Keeney *et al.,* 2007)
ΔlplA1	DP-L4263	104035S *ΔlplA1*	(O'Riordan *et al.,* 2003)
ΔlplA2	MOR129	104035S *ΔlplA2*	(Keeney *et al.,* 2007)
LLO-	DP-L2161	104035S *Δhly*	(Ikonomidis *et al.,* 1994)
WTR	DP-L3903	104035S*::Tn917*, unknown site of insertion, wild-type phenotype	(Auerbuch *et al.,* 2001)

Table 3.2 Total amino acid content of bacterial lysate

Amino Acid[b]	WT growth environment[a]			A1A2 mutant growth environment[a]		
	BHI	SP	SP + MB	BHI	SP	SP + MB
aspartic acid	9.05%	8.19%	9.10%	9.23%	9.08%	7.72%
threonine	4.99%	5.10%	3.94%	5.23%	5.73%	4.75%
serine	3.08%	3.21%	3.77%	3.59%	3.84%	3.23%
glx[c]	21.92%	22.62%	13.75%	14.60%	12.60%	23.94%
proline	3.79%	3.16%	3.39%	3.60%	3.47%	3.10%
glycine	8.40%	9.94%	10.82%	10.82%	10.47%	9.54%
alanine	7.27%	8.61%	8.42%	8.53%	10.77%	9.17%
valine	5.85%	6.31%	7.63%	6.87%	7.50%	6.45%
cysteine	0.08%	0.03%	0.30%	0.06%	0.17%	0.06%
methionine	0.64%	0.33%	0.59%	0.60%	0.33%	0.48%
isoleucine	4.42%	4.66%	5.61%	5.20%	5.53%	4.72%
leucine	6.16%	6.40%	7.40%	7.18%	7.47%	6.37%
tyrosine	2.11%	2.04%	2.31%	2.29%	2.43%	2.17%
phenylalanine	2.79%	2.82%	3.27%	3.07%	3.38%	2.99%
b-alanine	0.24%	0.14%	0.68%	0.54%	0.23%	0.19%
homocysteine	1.31%	0.37%	0.19%	0.24%	0.21%	0.52%
hydroxylysine	0.06%	0.04%	0.10%	0.00%	0.06%	0.03%
ornithine	0.37%	0.17%	0.10%	0.09%	0.09%	0.07%
tryptophan	0.13%	0.06%	0.07%	0.17%	0.00%	0.08%
lysine	7.31%	7.32%	7.84%	7.52%	7.42%	6.09%
l-methylhistidine	1.07%	0.43%	1.12%	1.11%	0.24%	0.68%
histidine	1.70%	1.51%	1.67%	1.58%	1.66%	1.40%
arginine	3.44%	3.37%	4.50%	4.26%	3.86%	3.16%
hydroxyproline	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%
asparagine	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%
proline	3.79%	3.16%	3.41%	3.61%	3.47%	3.10%

[a]The innoculum was grown in brain heart infusion (BHI) or improved minimal medium containing 6% (w/v) soy protein hydrolysate (SP) with or without 5mM 2-methyl butyrate (2-MB)
[b]Individual amino acid percentages were calculated after quantification of total amino acid content in 1 mg bacterial protein lysate by amino acid analysis
[c]Glx represents both glutamine and glutamate

Table 3.3 Total fatty acid content of bacterial pellet

Fatty Acid Type[b,c]	WT growth environment[a]			A1A2 mutant growth environment[a]		
	BHI	SP	SP + MB	BHI	SP	SP + MB
Even SCFA[d]	1.24	0.94	0.67	57.6	3.49	1.23
Odd SCFA[d]	0	1.08	0.62	7.06	16.66	16.66
Even Iso BCFA[e]	10.81	6.98	6.39	15.25	1.81	0.21
Odd Iso BCFA[e]	10.25	1.59	1.74	1.22	0	0
Odd Anteiso BCFA[f]	83.83	88.8	90.07	5.1	75.25	95.22

[a]The innoculum was grown in brain heart infusion (BHI) or improved minimal medium containing 6% (w/v) soy protein hydrolysate (SP) with or without 5mM 2-methyl butyrate (2-MB)

[b]Omega alicyclic minor fatty acid components are not included in this table.

[c]Values are representative of two independent experiments.

[d]SCFA represent cumulative percentage of straight chain fatty acids with either an odd or even carbon chain length.

[e]Iso-BCFA represents cumulative percentage of iso-branched chain fatty acids with either an odd or even carbon chain length.

[f]Anteiso-BCFA represents cumulative percentage of anteiso-branched chain fatty acids with an odd carbon chain length. There were no detected even anteiso-BCFAs.

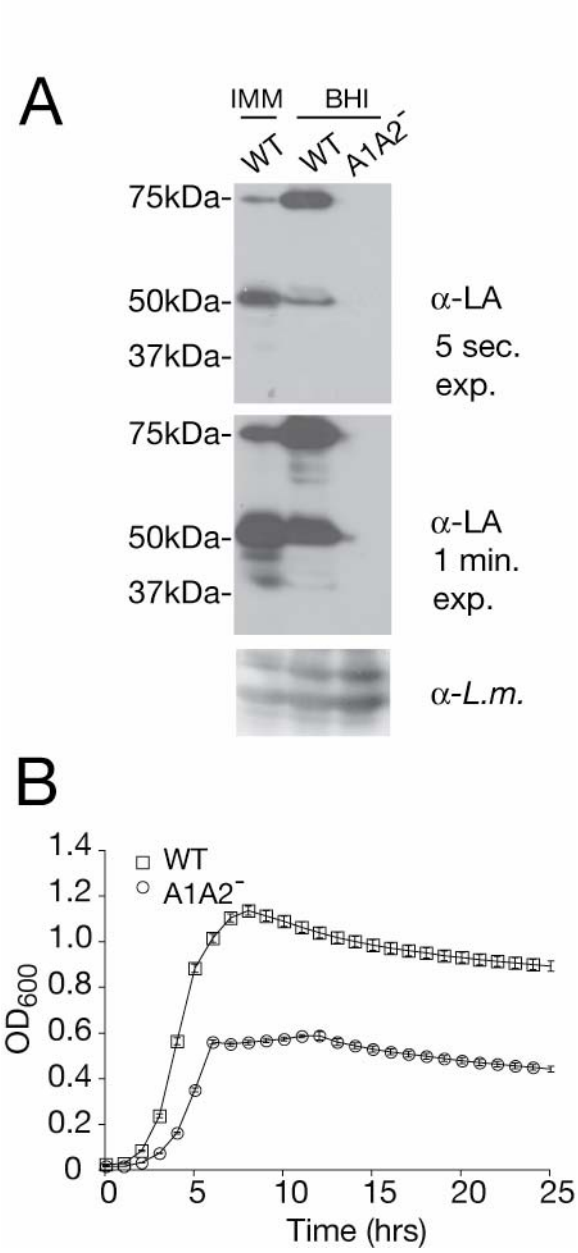


Figure 3.1 In the absence of lipoate ligases, *L. monocytogenes* can grow in nutrient rich conditions.

(A) WT and the lipoate metabolism mutant (AlA2$^-$ mutant) were grown in BHI or improved minimal medium (IMM) until stationary phase (OD_{600}=0.6 for A1A2$^-$ mutant in BHI, or 1.2 for WT in BHI and IMM). Lysates containing equivalent bacterial numbers were analyzed by SDS-PAGE and immunoblotted with an anti-LA antibody. The blot was exposed for 5 seconds (5 sec. exp.) or 1 minute (1 min. exp.). The anti-LA immunoblot is shown overexposed (1 min. exp.) to illustrate the lack of detectable lipoylation in the A1A2$^-$ mutant. The E2-subunit of bacterial PDH corresponds to the 75-kDa band. The blot was stripped and reblotted with polyclonal anti-*Listeria monocytogenes* antibody to confirm equivalent protein loading. (B) Bacterial growth was determined by measuring OD_{600} over time for WT and the AlA2$^-$ mutant inoculated in rich broth medium (BHI), and

grown at 37°C with aeration in a Bioscreen growth curve analyzer. Error bars represent standard deviation.

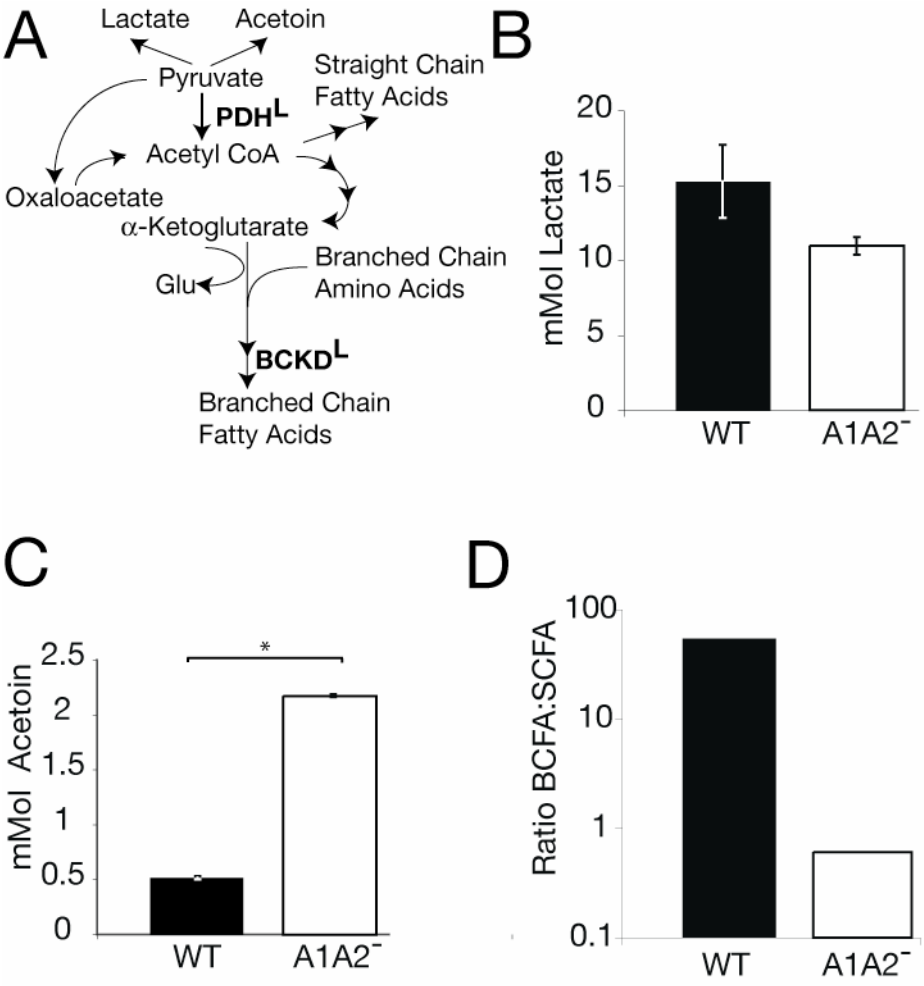

A
Lactate
Acetoin
Pyruvate
PDH^L
Straight Chain
Fatty Acids
Acetyl CoA
Oxaloacetate
α-Ketoglutarate
Glu
Branched Chain
Amino Acids
BCKD^L
Branched Chain
Fatty Acids
B
mMol Lactate
20
15
10
5
0
WT
A1A2⁻
C
mMol Acetoin
2.5
2
1.5
1
0.5
0
*
WT
A1A2⁻
D
Ratio BCFA:SCFA
100
10
1
0.1
WT
A1A2⁻

Figure 3.2 *L. monocytogenes* metabolism is altered in the absence of lipoylation.

(A) Model of lipoate dependent metabolism in *L. monocytogenes.* Both pyruvate dehydrogenase (PDH^L) and branched chain α-keto acid dehydrogenase ($BCKD^L$) require lipoate for function. In (B) and (C) WT and the AlA2- mutant were grown to stationary phase in BHI, pelleted, and the amount of secreted lactate (B) and acetoin (C) were quantified in the remaining supernatant (n=3). Error bars represent standard deviation. In (B) the amount of lactate in the supernatant was quantified by comparison with a standard curve. Lactate levels of WT and the $AlA2^-$ mutant were not significantly different. In C, filtered culture supernatant was derivatized and products detected by high pressure liquid chromatography. Acetoin peak identity and concentration was confirmed by HPLC separation of derivatized acetoin standards prepared in filtered BHI medium. Acetoin levels of WT and the AlA2- mutant were significantly different (Student's *t*-test $P<0.05$, as indicated by the symbol [*]). In (D) pellets of stationary phase WT and the $AlA2^-$ mutant grown in BHI (n=2) were analyzed by gas chromatographic analysis of cellular fatty acid methyl esters with the Sherlock® Microbial Identification System (Microbial ID), and the ratio of short chain fatty acids to branched chain fatty acids reported (SCFA:BCFA).

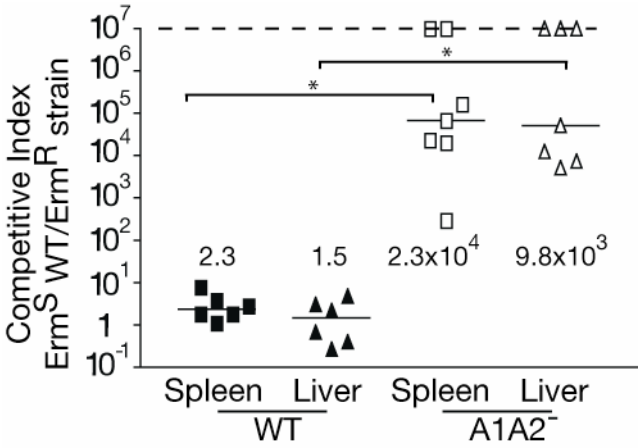


Figure 3.3 Lipoate dependent metabolism LDM is essential for *L. monocytogenes* pathogenesis.

Exponentially growing bacteria were mixed and injected i.p. into 4-6-week old female C57BL/6 mice. After 24hrsh, spleens and livers were harvested and plated onto LB agar with and without Erm. The competitive index was calculated by dividing the resulting drug resistant colony forming units (CFU) (WTR or $AlA2^-$ mutant) by the drug sensitive CFU (WT). The horizontal solid line represents the median value and the dotted upper line represents mice where no $AlA2^-$ mutant CFU was detected. The mice where CFU could not be detected were not used when CI were calculated. Statistically significant differences between two groups were determined by the Student's *t*—test at $P<0.05$ as indicated by the symbol [*].

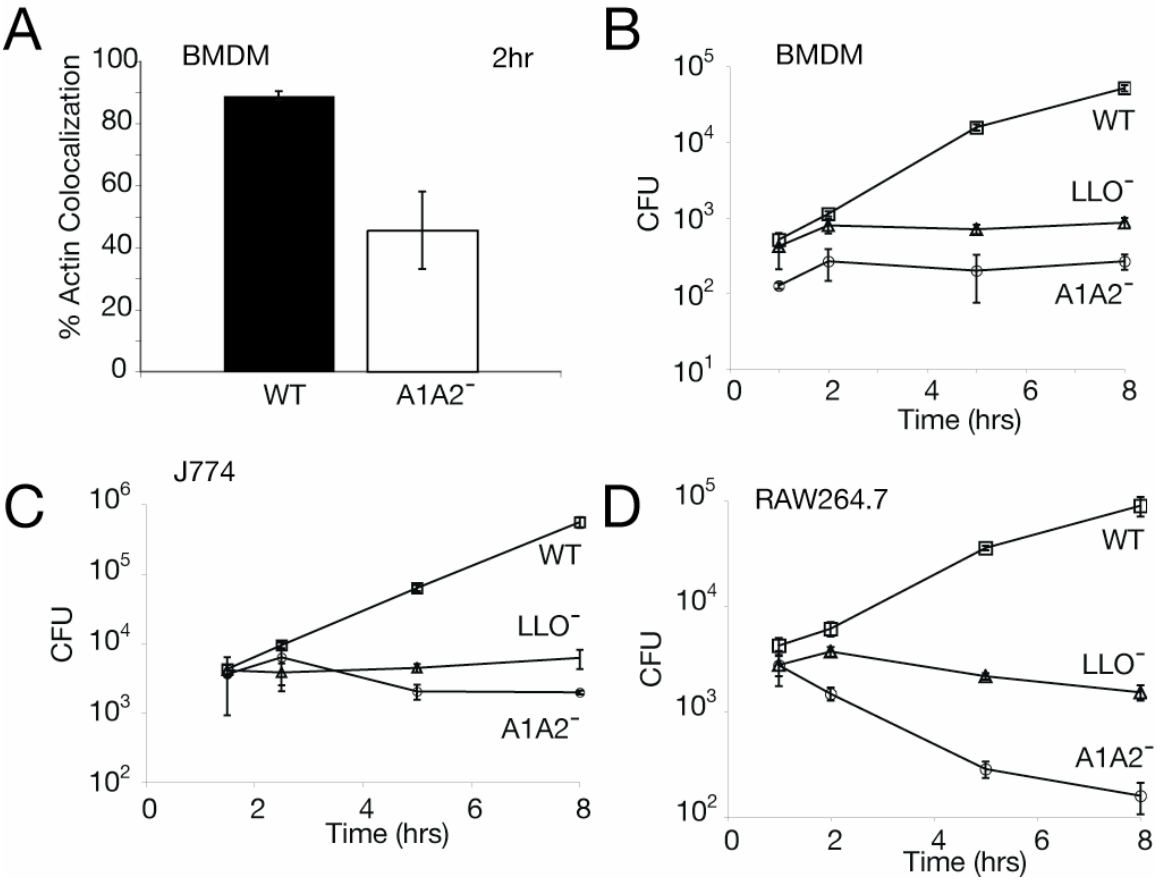


Figure 3.4 Lipoate dependent metabolism LDM is essential for growth during infection of phagocytes.

After overnight growth in the rich broth medium BHI, stationary phase cultures of WT, the AlA2- mutant, and an LLO- mutant of *L. monocytogenes* were washed and used to infect bone-marrow-derived macrophages (BMDM) (A, B), J774 macrophages (C) and RAW264.7 macrophages (D) for 30 minutes. Intracellular bacteria were visualized 2hrsh post-infection in BMDM by colocalization of *L. monocytogenes* with actin clouds by immunoflorescence (A). In (BCD), after washing away extracellular bacteria, the extracellular medium was treated with gentamycin. At the indicated times post infection cells were osmotically lysed and intracellular colony forming units plated on LB agar. Error bars represent standard deviation.

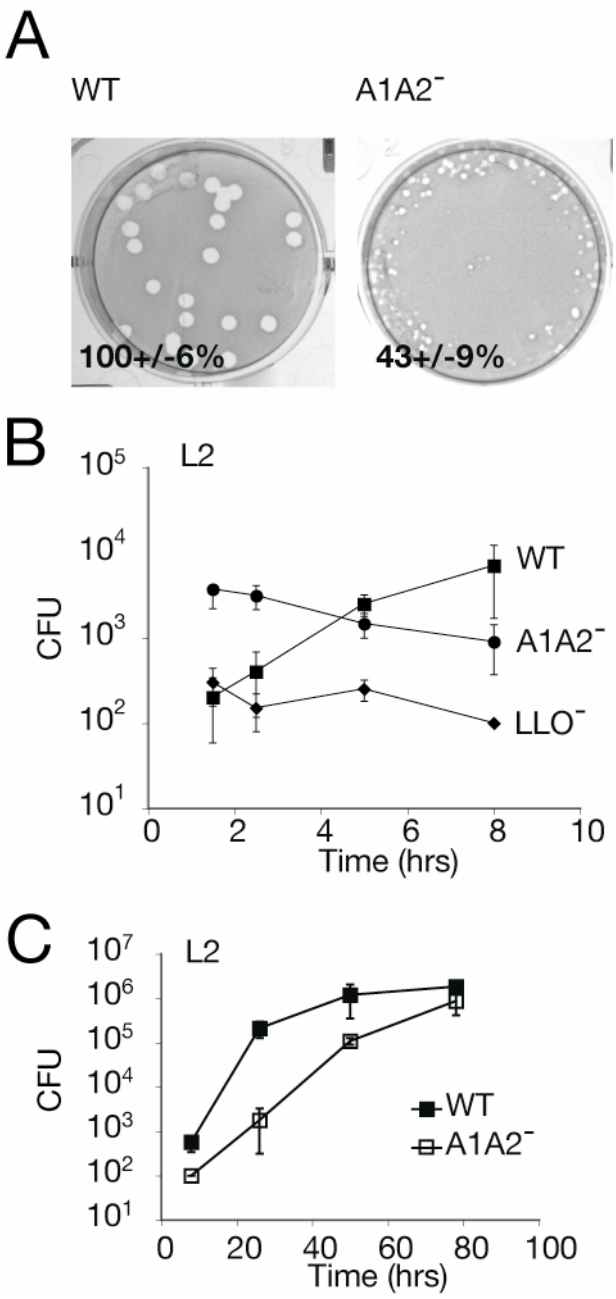
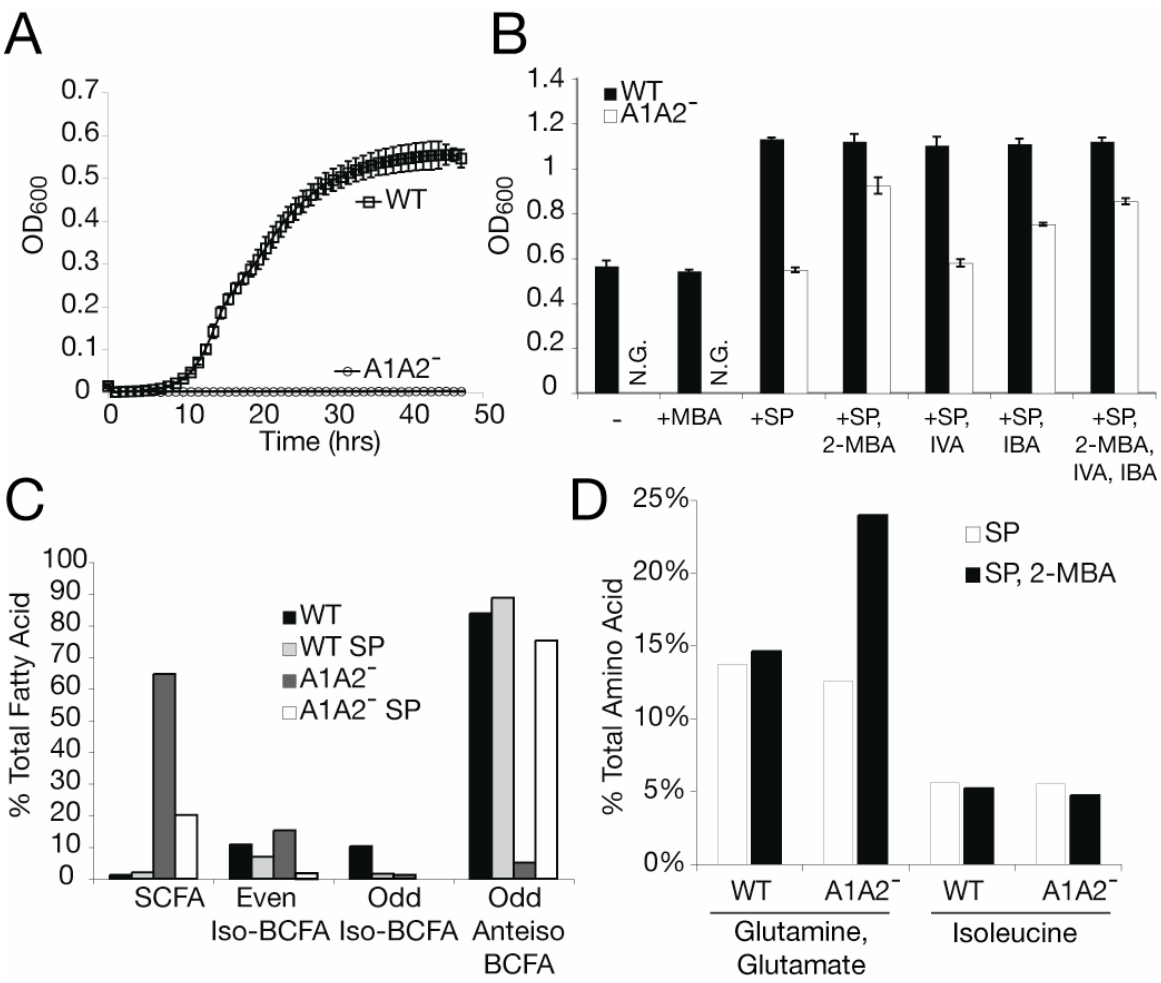


Figure 3.5 Lipoate dependent metabolism LDM is needed for optimal growth in L2 cells.

WT, the AlA2$^-$ mutant, and the LLO$^-$ mutant of *L. monocytogenes* were grown to stationary phase in BHI and used to infect L2 cells for 1h. Extracellular bacteria were washed and treated with gentamycin. In (A), a 3ml agar overlay was placed over infected cells and plaques allowed to develop for 5 days, where they were visualized by the addition of Neutral Red stain. Plaque diameters were measured (n=20) and expressed as percentage of the mean diameter of WT plaques. In (B) and (C), infected L2 cells were osmotically lysed and plated on LB agar to enumerate colony forming units. Error bars represent standard deviation.


A
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD600
WT
A1A2-
0
10
20
30
40
50
Time (hrs)
B
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
WT
A1A2-
N.G.
N.G.
-
+MBA
+SP
+SP, 2-MBA
+SP, IVA
+SP, IBA
+SP, 2-MBA, IVA, IBA
C
100
90
80
70
60
50
40
30
20
10
0
% Total Fatty Acid
WT
WT SP
A1A2-
A1A2- SP
SCFA
Even Iso-BCFA
Odd Iso-BCFA
Odd Anteiso BCFA
D
25%
20%
15%
10%
5%
0%
% Total Amino Acid
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A1A2-
Glutamine, Glutamate
Isoleucine

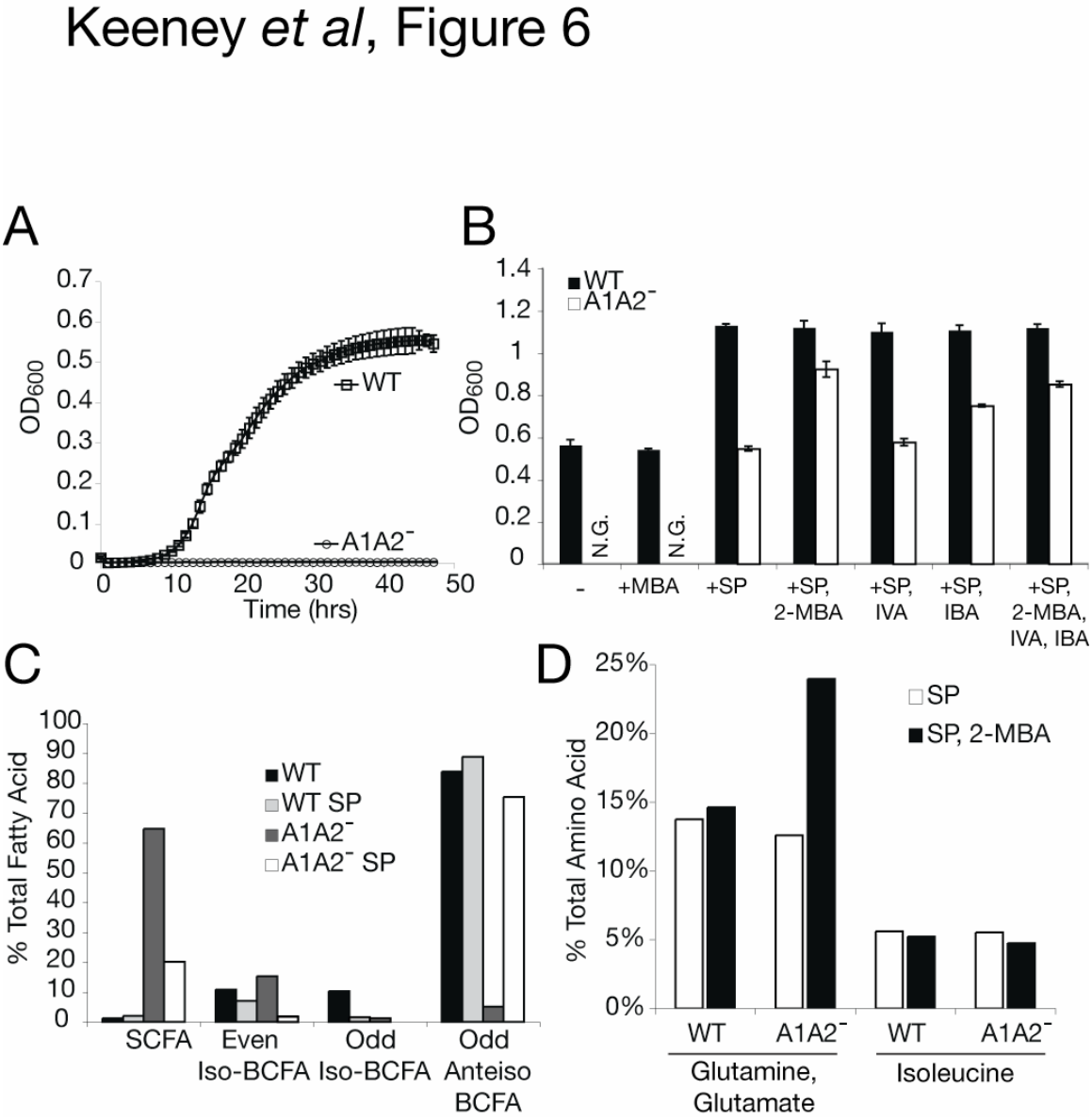


Figure 3.6 *L. monocytogenes* growth in the absence of excess environmental amino and fatty acids requires lipoate dependent metabolismLDM.

(A) Individual colonies picked from fresh BHI plates containing WT and the AIA2$^-$ mutant were inoculated into improved minimal medium and the OD_{600} was recorded over time at 37°C in a Bioscreen Growth Curve Analyzer. (B) Bacteria were grown as in (A) to stationary phase with or without additional supplementation with 6% (w/v) soy protein hydrolysate, 5mM 2-methylbutyrate (2-MB), 5mM isovalerate (IV) or 5mM isobutyrate (IB). Error bars in (A) and (B) represent standard deviation. (C) Fatty acid content of bacterial pellets after growth in BHI, or IMM supplemented with 6% soy protein hydrolysate (w/v) (SP). Percentages are reported as the percentage of total quantified fatty acids, and clustered based on the even or odd number of carbons as well as if the fatty acid branch was anteiso or iso in orientation. (D) Amino acid content of bacterial

lysate after growth in IMM supplemented with 6% (w/v) SP or 5mM 2-MB. Percentages are reported as the percentage of total quantified amino acids.

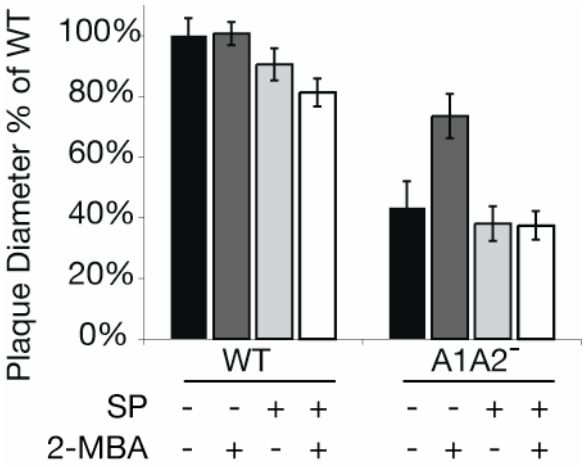


Figure 3.7 Branched chain fatty acid precursors bypass the requirement for lipoate dependent metabolism LDM in intracellular growth, spread, and infectivity.

WT and the AIA2$^-$ mutant of *L. monocytogenes* was grown to stationary phase and used to infect L2 cells for 1h. Extracellular bacteria grown in BHI were washed and treated with gentamycin. A 3ml overlay was placed over infected cells and plaques were allowed to developed for 4 days. Plaques were visualized by the addition of Neutral Red stain. Plaque diameters were measured (n=20) and expressed as percentage of the mean diameter of WT plaques. The overlay was supplemented with or without 0.75% soy protein hydrolysate (SP) and 5mM 2-methylbutyrate (2-MB). Error bars represent standard deviation. in structures are shown at the same magnification and orientation for each view.

Chapter 4

LPLA1 MEDIATED INTRACELLULAR GROWTH OF *L. monocytogenes* MAY REQUIRE SURFACE RESIDUE E94

SUMMARY

Listeria monocytogenes, an intracellular pathogen, scavenges lipoate from its growth environment using lipoate protein ligases to ligate it to metabolic enzyme complexes, such as pyruvate dehydrogenase. While *L. monocytogenes* encodes two lipoate ligases, LplA1 and LplA2, only LplA1 allows bacterial growth in limiting concentrations of host derived lipoyl-peptides. Our previous studies suggest that this function facilitates the growth of *L. monocytogenes* in the cytosolic environment of the host. In a structural model of the ligases, we observed two regions of clustered negatively charged residues on the surface of LplA1 that are less prominent on LplA2. To test the role of these charged surface regions on the ability of LplA1 to support growth of the bacterium using lipoyl-peptides, we employed a site directed mutagenesis approach. We created *ΔlplA1* mutant strains expressing LplA1 on a plasmid containing individual mutations in the surface patches. Here, we provide evidence thatprovide preliminary evidence that a single amino acid on LplA1 contributes to *L. monocytogenes* growth in limiting concentrations of host-derived lipoyl-peptides

and within the host cell. These preliminary data suggest that a single amino acid on LplA1 may confer a gain of function for *L. monocytogenes*.

INTRODUCTION

The ability to utilize nutrients derived from the host is critical for intracellular pathogens that replicate in the intracellular environment. Understanding the mechanisms evolved by these pathogens to take advantage of their biochemical environment will lead to a more complete understanding of the parameters that control a successful host-pathogen interaction. *Listeria monocytogenes* is an intracellular pathogen that replicates in the cytosolic compartment. One nutrient that *L. monocytogenes* must scavenge from its growth environment to support optimal growth is lipoate (Keeney *et al.,* 2007; O'Riordan *et al.,* 2003). This nutrient is a requisite cofactor for the acyltransferase (E2) subunit of metabolic enzyme complexes such as pyruvate dehydrogenase (PDH) and branched chain α-keto acid dehydrogenase (BCKD). Lipoate is attached to these complexes by a lipoate protein ligase, which facilitates the generation of an amide linkage between the carboxyl group of lipoate to the epsilon-amino group of a specific lysine on E2 (Perham, 2000). The ligated lipoamide arm protrudes from the E2 subunit and facilitates the generation and spatial shuttling of reaction intermediates between subunits of the metabolic enzyme complex, allowing the production of acyl-CoAs.

L. monocytogenes encodes two lipoate ligases, LplA1 and LplA2, which appear to differentially enable bacterial growth in the host cytosol. When LplA1 is disrupted, *L. monocytogenes* is impaired for intracellular replication and does not

lipoylate the E2 subunit of PDH in the cytosol of mammalian host cells. Additionally, *L. monocytogenes* is restricted for growth in limiting concentrations of host-derived lipoyl-peptides (O'Riordan *et al.,* 2003). The *lplA1* mutant can still grow and lipoylate PDH when the bacterium is grown in minimal medium with free lipoic acid as the sole source of lipoate. These data suggest that LplA1 can allow bacterial growth with limiting concentrations of host derived lipoyl-peptides, and this function facilitates the growth of *L. monocytogenes* in the intracellular cytosolic environment.

The mechanism for the gain of function that LplA1 appears to have is unknown, but we hypothesize that LplA1, when compared with LplA2, either has an increased ability to directly bind host derived lipoyl-peptides, or LplA1 can interact with a cofactor that increases its ability to support growth upon limiting concentrations of host-derived lipoyl-peptides. To begin to address these questions, we used our previously modeled structure of LplA1 and LplA2 to examine if there were any structural differences between the two ligases. The amino acid residues involved in the binding of the lipoyl group and the target domain are conserved in both *E. coli* LplA and the *L. monocytogenes* lipoate ligases, but there are two regions of clustered negatively charged residues on the surface of LplA1 that are not present in any of the other ligases (Fig. 2.7)(Fujiwara *et al.,* 2007; Keeney *et al.,* 2007). The negatively charged residues did not fall within regions of assigned function, and thus their role in LplA1 function was unknown. To test the role of these charged surface regions, we

used site directed mutagenesis to create a *⊿lplA1* mutant strain expressing a plasmid with LplA1 containing mutations in the surface patches. The surface amino acids chosen for mutation were not predicted to interfere with the lipoate binding pocket contained in the core of the model, and also were not predicted to interfere with the overall folding of LplA1. Here, we provide evidence thatidentify a single amino acid on LplA1 that is required critical for *L. monocytogenes* growth with limiting concentrations of host-derived lipoyl-peptides. Furthermore, this single amino acid is required for LplA1 mediated intracellular growth and spread. These data demonstrate a single amino acid on LplA1 may confer a gain of function for *L. monocytogenes*.

RESULTS

L. monocytogenes can utilize limiting concentrations of host-derived lipoyl-peptides to support growth in the absence of free lipoate (Keeney *et al.,* 2007). However, when the gene encoding LplA1 is removed, *L. monocytogenes* can no longer grow optimally, despite the expression of another functional lipoate ligase, LplA2. To determine if there are amino acid residues that contribute to the unique ability of the LplA1 lipoate ligase to enable utilization of lipoyl-peptide, we examined the amino acid sequences of LplA1, LplA2, and *E. coli* LplA (Fig. 4.1). LplA1 shared significant amino acid identity and similarity with LplA2 (*lmo0764*) and *E. coli* LplA (Fig. 4.1). Additionally, the amino acid residues predicted to interact with lipoate in the core of these ligases were conserved (Fig. 2A.1).

However, when comparing the electrostatic charge of LplA1 and LplA2, there were two patches of negative surface charge on LplA1 that were not present in LplA2 (Fig. 2.7). We hypothesized that these residues might be critical for LplA1-mediated *L. monocytogenes* growth on host-derived lipoyl-peptides.

To determine which residues were responsible for these two negative surface patches, patch 1 and patch 2, we analyzed our previously published model of LplA1 and identified five negatively charged amino acid residues within patch 1, including LplA1 amino acid residues E30, E90, D91, D92, and E94 (Fig. 4.1, Fig. 4.2) (Keeney *et al.,* 2007). In the second negative patch of LplA1, patch 2, seven negatively charged residues were identified, including D79, E80, D153, D187, D190, E192, and E196 (Fig. 4.1, Fig. 4.2). Both patches were located on the outer surface of LplA1, away from the hypothesized binding site of host-derived lipoyl-peptides within the core of the model (Fig. 4.2). Within patch 2, only four residues were different in amino acid sequence from LplA2, LplA1-D187, D190, E92 and E196. In patch 1, only two residues, LplA1-E90 and E94, were different.

Once identified, we individually replaced the six candidate LplA1 residues with the corresponding amino acid in LplA2 by site-directed mutagenesis. We then placed these LplA1 point mutants on an *L. monocytogenes* expression plasmid under the control of *lplA1's* native promoter. Expression of these distinct LplA1 mutant plasmids within an LplA1 null background will help clarify if any of these six residues individually confer the bacterium's unique ability to utilize limiting

concentrations of host-derived lipoyl-peptide. We predicted that LplA1 would likely tolerate mutating the residues of LplA1 to the corresponding residue on LplA2 without causing misfolding, as these residues are tolerated in the functional LplA2 lipoate ligase. Additionally, we wished to specifically test which residues facilitated bacterial utilization of lipoyl-peptide, a function that *L. monocytogenes* LplA2 containing strains do not have.

To determine if LplA1 mediated growth of *L. monocytogenes* is dependent upon the surface amino acid residues of LplA1, we grew bacteria pre-starved for lipoate in the presence of synthetic lipoyl-peptide (DK^LA) in improved minimal medium (IMM) with no other alternate sources of lipoate. At 0.5 µg/l, DK^LA supported growth of the strain containing wildtype LplA1 in the *ΔlplA1* mutant background, but the *ΔlplA1* mutant strain expressing empty vector could not grow. These data verified the specific contribution of LplA1 in utilizing lipoylated peptides for growth (Fig. 4.3). Of the six different LplA1 amino acid mutant alleles, only a residue switch within patch 1, LplA1-E94N, failed to complement the *ΔlplA1* mutant when grown in DK^LA (Fig. 4.3). Individual mutagenesis of each of the four candidate amino acid residues in surface patch 2 did not impair *L. monocytogenes* growth upon lipoyl-peptides, indicating this region of LplA1 may not be necessary for lipoyl-peptide dependent replication. Therefore, we concluded that glutamic acid at residue 94 of LplA1 in surface patch 1 might play a role in the ability of LplA1 to support *L. monocytogenes* growth in lipoyl-peptide.

As E94 of LplA1 appeared to enable *L. monocytogenes* growth with lipoyl-peptide, we wanted to determine if the LplA1 E94N allele could support growth and spread of the bacterium in a tissue culture model of *L. monocytogenes* infection. Murine L2 fibroblasts were infected with WT bacteria or the Δ*lplA1* mutant strain containing either empty vector or vector containing mutated LplA1, and then incubated for several days to allow necrotic foci to form. We visualized plaques by Neutral Red staining, and measured plaque diameter as an indicator of bacterial replication and cell-cell spread. As shown in Figure 4.4, the ability of the Δ*lplA1* mutant to form plaques was partially restored when complemented with a plasmid containing LplA1. Complete complementation did not occur, possibly because expression of *lplA1* on the high copy pAM401 plasmid may not be regulated or expressed similarly to endogenous *lplA1* in the WT bacterium. Of the six LplA1 mutant alleles, only LplA1-E94N failed to restore the plaque size of the Δ*lplA1* mutant to the level of the LplA1 plasmid. These data suggest that residue E94 is required for LplA1 to support intracellular growth and spread of *L. monocytogenes* when growing within the host cell. Further studies to confirm expression and function of the LplA1-E94N allele are ongoing, including mutagenesis of the endogenous *lplA1* allele, expression of the mutant allele in a double lipoate ligase disruption mutant, and growth of the mutant allele strains in a murine model of *L. monocytogenes* infection.

DISCUSSION

The *L. monocytogenes* lipoate ligase LplA1, but not LplA2, supports growth of the bacterium in limiting concentrations of host-derived lipoyl-peptides (Keeney *et al.,* 2007). However, the ability of a lipoate ligase to use substrates other than free lipoate has not been demonstrated in any other organism besides *L. monocytogenes* (Fujiwara *et al.,* 2007; Keeney *et al.,* 2007). Previously published crystal structures, as well as point mutations of lipoate ligases in other organisms, have identified a critical lipoate binding pocket within the core of these ligases (Fujiwara *et al.,* 2007). Here, we present evidence that the surface residue LplA1-E94 supports bacterial growth within the host cell and enables *L. monocytogenes* to utilize limiting concentrations of lipoyl-peptide in minimal medium. No other functional region has been identified in these ligases besides the lipoate binding pocket, indicating these findings may have significance towards understanding the function of lipoate ligases in other organisms. This is especially true for organisms that may encounter multiple lipoate sources.

Before we can conclusively state residue E94 of LplA1 is critical for the enzymes ability to support growth of *L. monocytogenes in vivo* and with lipoyl-peptides, it is imperative to perform further experiments to verify the LplA1-E94N protein properly folds and is expressed in *L. monocytogenes*. As previously mentioned, the potential for misfolding is unlikely, as we replaced a single amino acid residue on the surface of LplA1 with another amino acid residue that is present in the same region of LplA2, a functional lipoate ligase that can tolerate that particular residue on the surface of its structure. Additionally, the inability of the LplA1-

E94N allele to support *L. monocytogenes* growth in the host cytosol will also need to be verified in other tissue culture cells lines beside murine L2 fibroblasts, such as J774 macrophages, to determine if this mutant allele strain has a similar phenotype as the *ΔlplA1* mutant strain. Finally, mutation of the endogenous copy of *lplA1* will allow us to examine if LplA1-E94 is required to support the growth of *L. monocytogenes* in a murine model of listeriosis.

The structural mechanism for lipoate ligase mediated usage of host-derived lipoyl-peptide is not yet known, but E94 of LplA1 is within a cluster of negatively charged surface residues that may modulate the ability of LplA1 to support bacterial replication upon lipoyl-peptide. We initially hypothesized the interaction between the lipoate ligase and lipoyl-peptide would occur either directly, or with a cofactor that modulates LplA1 interaction with lipoyl-peptide. For the direct interaction model, we postulated the negative patches on the surface of *L. monocytogenes* LplA1 could increase the local concentration of lipoyl-peptide by charge affinity. For the cofactor model, we thought lipoyl-peptide could interact with a protein or cofactor that would interact with amino acid residues within the negative surface patches. However, we provide evidence that mutation of only one residue within surface patch 1 resulted in a loss in the ability of the LplA1 mutant allele to support replication of *L. monocytogenes* in lipoyl-peptide. Moreover, mutation of E90, which is also predicted to contribute to the electrostatic surface charge of the same negative patch E94 contributes to, did not result in a loss of the phenotype. This indicated to us it was unlikely that the

overall charge of these patches was important. Instead, our observation that a single amino acid is likely responsible for the ability of LplA1 to support lipoyl-peptide bacterial replication lends support to the cofactor model, where an interacting protein could bind LplA1 through an interaction with residue E94 of LplA1 and deliver host-derived forms of lipoate to the ligase. This cofactor could have lipoamidase activity that would liberate lipoate from lipoyl-peptides and increase the local concentration of free lipoate. Such lipoamidase activity would have to be strictly regulated, as it would oppose ligase function (Jiang and Cronan, 2005). Future studies to determine potential binding partners for LplA1 will ultimately clarify the role of this amino acid in the mechanism of this important lipoate ligase.

When aligned with the amino acid sequence of other bacterially encoded lipoate ligases, amino acid residue LplA-E94 is not conserved (Fig. C.1). It will be interesting to determine if the region corresponding with the location of E94 in other lipoate ligases confers preferential growth upon different lipoate substrates. Notably, *Staphylococcus aureus* and *Streptococcus pyogenes,* both pathogens that scavenge lipoate from their growth environment, have two different copies of *lplA*-like genes that could potentially recognize different forms of lipoate. In addition, despite their ability to synthesize lipoate, *Toxoplasma gondii* and *Plasmodium falciparum* still must scavenge lipoate from their growth environment to support intracellular replication (Allary *et al.,* 2007; Crawford *et al.,* 2006; Wrenger and Müller, 2004). These observations indicate lipoate scavenging and

the ability to utilize diverse lipoate sources may be an important adaptation for many diverse intracellular pathogens, even those that have the ability to synthesize lipoate.

EXPERIMENTAL PROCEDURES

Bacterial culture

Strains used in this study are described in Table 4.1. For intracellular growth, all *L. monocytogenes* strains were grown to late log phase (OD_{600} 0.8-1.2) in improved minimal medium (IMM) at 37°C unless otherwise specified. Improved minimal medium was prepared as previously described (Phan-Thanh and Gormon, 1997), using the concentrations listed in Table 1.1. Specifically, lipoic acid was used to supplement IMM at 5µg/L or with $DK^{L}A$ at 0.5µg/L. For growth curves in IMM, single colonies from freshly streaked BHI plates were inoculated into IMM without lipoic acid (IMM^{-L}) for 10-14 hours at 37°C with shaking to OD_{600} 0.2-0.4, back-diluted to an OD_{600} of 0.02 in IMM, and cultured at 37°C with shaking. Bacterial growth was determined by measuring changes in OD_{600} over time. All IMM growth curves were performed in a Bioscreen Growth Curve Analyzer (Growth Curves, USA). To ensure plasmids were retained in *L. monocytogenes*, the bacteria were grown with 10µg/mL chloramphenicol when grown in IMM, or 20µg/mL chloramphenicol when grown on BHI.

Site-directed mutagenesis of LplA1

QuikChange XL site-directed mutagenesis was performed as directed by the manufacturer (Stratagene). Briefly, PCR was performed with PfxPlatinum DNA polymerase using pCR2.1-LplA1 as template. Two synthetic oligonucleotide primers complementary to opposite strands of the vector containing the desired

mutation were used as primers. After extension, the product was treated with Dpn I endonuclease to select for mutation containing synthesized DNA, and then was transformed into *E. coli*. The final stage was transfer by restriction digestion and ligation of the mutated LplA1 to pAM401, a multi-copy *L. monocytogenes* expression plasmid.

L2 plaque assays.

Plaque assays were performed as previously described (O'Riordan *et al.,* 2003). In L2 plaque assays, an overlay of 3 mls top agar per well was added after an initial 1h infection and developed for 5 days prior to addition of Neutral Red stain to visualize necrotic foci. Plaque diameters were measured (n=20) and expressed as percentage of the mean diameter of WT plaques using Canvas software (Deneba).

TABLES AND FIGURES

Table 4.1 Strains used in Chapter 4		
Strains	**Genotype/description**	**Reference**
10403S	*L. monocytogenes* serotype 1/2a, parental strain used as wildtype	(Freitag *et al.,* 1993)
DP-L4263	10403S *ΔlplA1*	(O'Riordan *et al.,* 2003)
MOR03	10403S + pAM401	(O'Riordan *et al.,* 2003)
MOR88	10403S *ΔlplA1* + pAM401	(O'Riordan *et al.,* 2003)
MOR89	10403S *ΔlplA1* + pAM401-lplA1	(O'Riordan *et al.,* 2003)
MOR247	10403S *ΔlplA1* + pAM401-lplA1-E90K	This study
MOR248	10403S *ΔlplA1* + pAM401-lplA1-E94N	This study
MOR249	10403S *ΔlplA1* + pAM401-lplA1-D190A	This study
MOR250	10403S *ΔlplA1* + pAM401-lplA1-E196L	This study
MOR251	10403S *ΔlplA1* + pAM401-lplA1-E192D	This study
MOR252	10403S *ΔlplA1* + pAM401-lplA1-D187E	This study

Figure 4.1 Negative surface residues of LplA1

LplA1 and LplA2 align with 47% identify and 71% similarity using the Needleman-Wunsch global alignment (EMBOSS). *E. coli* LplA (Accession NP_418803) aligns with 28% identity to *L. monocytogenes* LplA1 and 23% identity to LplA2. Stars indicate identity between LplA1 and LplA2, while two dots indicate greater similarity than one dot. Surface residues predicted by structural modeling of LplA1 and LplA2 to be responsible for two electrostatic negative patches are highlighted in blue and green. Patch 1 consists of D79, E80, D153, D187, D190, E192, and E196. Patch 2 consists of G30, G90, D91, D92, G94.

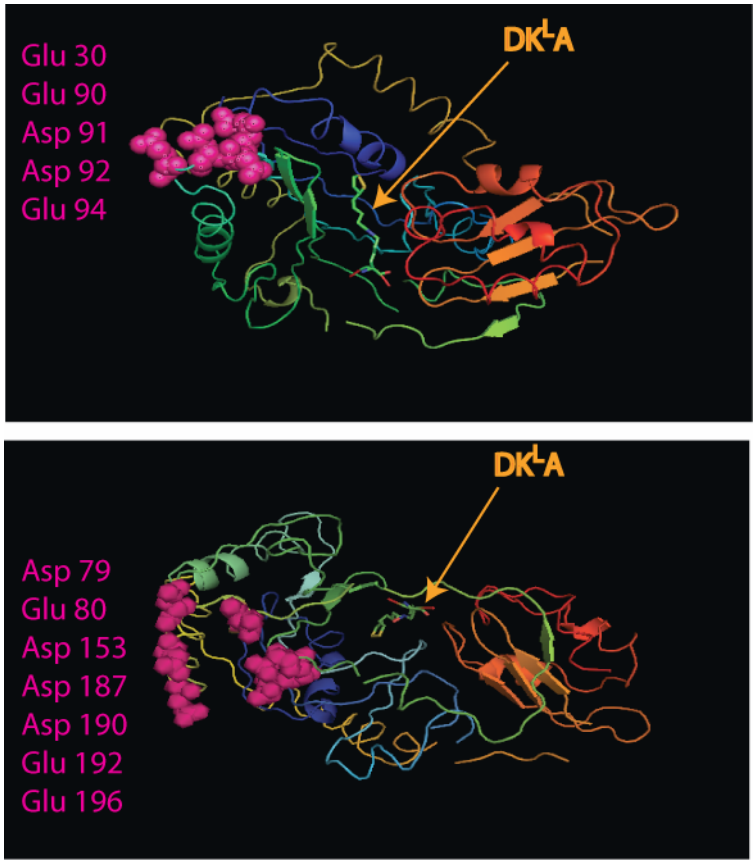


Figure 4.2 Surface amino acid residues within two putative electrostatically charged negative patches of LplA1

LplA1 surface residues attributed to electrostatic negative surface patch 1 (top panel) and negative patch 2 (bottom panel) illustrated in Figure 2.7. The protein structures are shown at the same magnification, but different orientations, for each view. The predicted binding site for lipoyl-peptide is filled with the lipoyl-peptide $DK^{L}A$, as indicated by the orange arrow. Each residue predicted to be involved in each patch is illustrated with a space filling amino acid representation in fuschia.

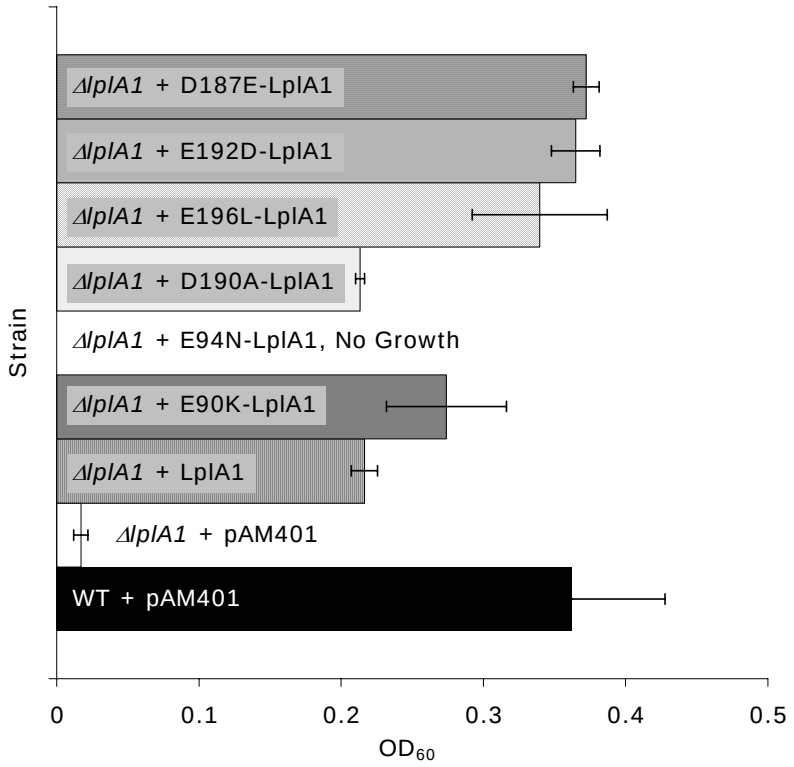


Figure 4.3 LplA1 residue E94 is essential for LplA1 mediated *L. monocytogenes* replication with lipoyl-peptide

WT *L. monocytogenes* containing empty vector and the *ΔlplA1* null strain containing an expression vector with LplA1 or point mutants of LplA1 were grown in improved minimal medium (IMM) containing synthetic lipoyl-peptide (DK^LA) at 0.5µg/L as the sole source of lipoate. Growth was measured by OD_{600} in a Bioscreen Growth Curve Analyzer until stationary phase was reached at 48hrsh. The mean value ± SD was calculated for each timepoint at 35hrsh, corresponding to early stationary phase (*n=3)*.

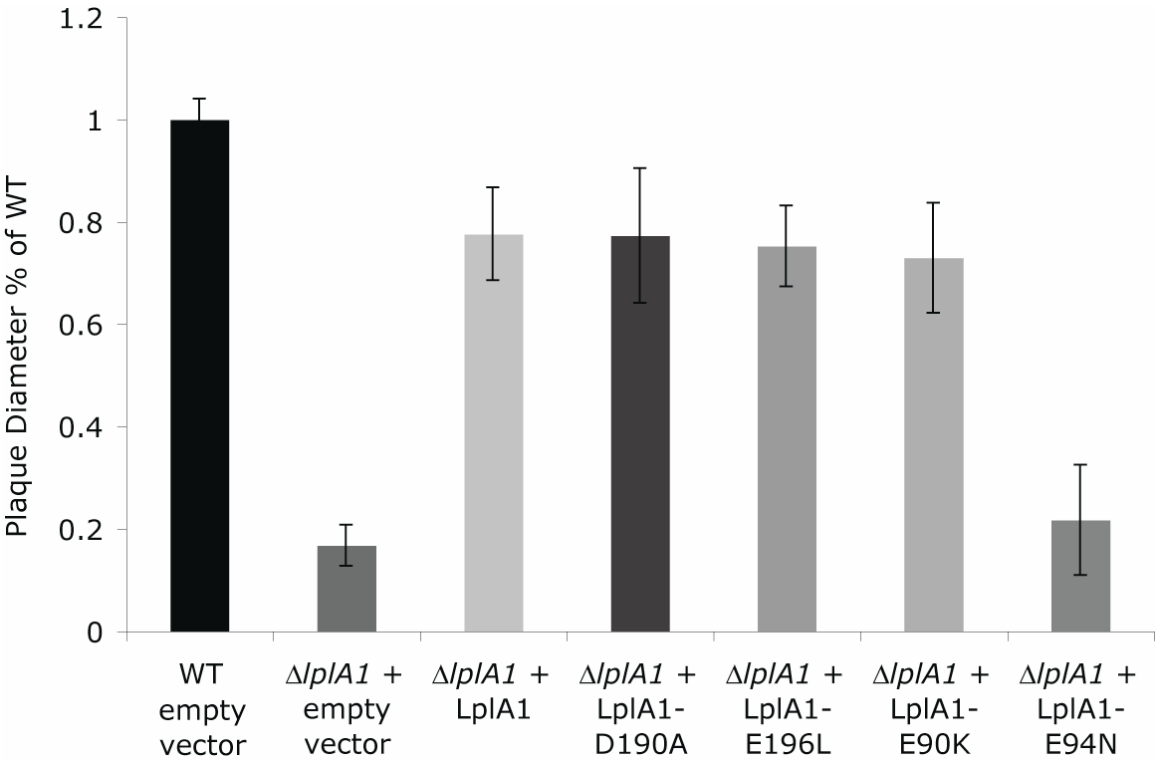


Figure 4.4 LplA1 residue E94 is required for LplA1 mediated plaque formation of *L. monocytogenes*

WT containing empty vector and the *ΔlplA*1 null mutant containing LplA1 vector or LplA1 point mutant vector were grown to stationary phase in BHI and used to infect L2 cells for 1h. Extracellular bacteria were washed and treated with gentamycin. A 3ml agar overlay was placed over infected cells and plaques allowed to develop for 5 days, when they were visualized by the addition of Neutral Red stain. Plaque diameters were measured (n=20) and expressed as percentage of the mean diameter of WT plaques. Error bars represent standard deviation.

Chapter 5

PERSPECTIVES

During infection, the substrates and metabolic pathways that pathogens use are largely unknown. However, these substrates and metabolic pathways allow the microbe to occupy diverse nutrient niches within the host. In 2003, O'Riordan and colleagues showed that a lipoate protein ligase, LplA1, in *L. monocytogenes* was important for intracellular growth of the bacterium, and suggested the lack of intracellular growth was due to the bacterium's inability to ligate lipoate to metabolic enzyme complexes while growing in the cytosolic compartment of its host. This first indication that lipoate dependent metabolism might be important in the pathogenicity of an intracellular pathogen has been confirmed with recent papers from our lab and others. Specifically, this thesis has shown LplA1 was important for intracellular replication of *L. monocytogenes,* likely because it enabled *L. monocytogenes* to utilize host derived forms of lipoate. Furthermore, this thesis has shown a loss in the ability to use lipoate resulted in the bacterium being unable to biosynthetically generate vital nutrients that enhance intracellular replication. In this concluding chapter, I will use the results of this thesis as a

background to interpret the influence and significance the host nutrient environment has upon intracellular pathogens.

It is apparent that metabolic adaptations to different nutrient environments are important for bacterial pathogenesis. For example, several biosynthetic enzymes are essential for systemic virulence of *S.* Typhimurium, including enzymes that generate products that the bacterium may not be able to obtain from its host (Becker *et al.,* 2006). The work in this thesis suggested the lipoylated metabolic enzymes PDH and BCKD are important for *L. monocytogenes* virulence. Previous observations of *L. monocytogenes* with mutations in the BCKD complex had shown that the activity of this metabolic enzyme was involved in the bacterium's ability to regulate its BCFA composition. Without active BCKD, *L. monocytogenes* was more susceptible to environmental stresses such as growth in acidic conditions, adherence to abiotic surfaces, and growth at cold temperatures. The enhancing effect we observed after supplementation with BCFA during intracellular growth demonstrated BCKD and the fatty acids it produces are central for *L. monocytogenes* growth in the host environment. The importance of BCKD in pathogenesis was also recently observed in *S. aureus* as well, as insertional inactivation of BCKD resulted in a loss of BCFA and also a loss of pathogen survival inside a murine host (Singh *et al.,* 2008). As BCKD is apparently important in the pathogenesis of *L. monocytogenes* and *S. aureus*, it is feasible BCKD could also play a role in the pathogenesis of other intracellular pathogens. This thesis highlights the possibility that similar enzymes considered

to be part of central metabolism could also have a dramatic effect upon the success of other bacterial pathogens.

While it has been assumed that central metabolic pathways are conserved between both pathogens and non-pathogens, my data and others suggest pathogens may acquire and metabolize nutrients with distinct enzymes and metabolic pathways. For example, recent work in *E. coli* demonstrated the pathogenic enterohemmorrhagic *E. coli* (EHEC) was dependent upon gluconeogenesis for persistence in the intestine, while commensal strains of *E. coli* did not have this requirement (Chang *et al.,* 2004; Miranda *et al.,* 2004). *L. monocytogenes* metabolism is also distinct from its host. The lack of a complete TCA cycle in *L. monocytogenes* indicates ATP normally synthesized by a complete TCA cycle in mammalian host cells is not produced in the pathogen by the same pathway. Therefore, the TCA cycle in *L. monocytogenes* likely only generates biosynthetic intermediates and metabolites. Additionally, while this thesis demonstrates LplA1 appears to be distinct from LplA2, it is unknown if LplA1 is also distinct from the lipoate protein ligases the host employs to lipoylate its own metabolic enzymes. If these lipoate protein ligases were different from one another, disrupting the function of LplA1 would be an attractive target for antimicrobial drug development, not just for *L. monocytogenes*, but also potentially for many other microbes. Our results suggest only a single amino acid residue on the surface of LplA1 enables this ligase to support *L. monocytogenes* growth upon limiting concentrations of lipoyl-peptide. When

examining the sequence of lipoate ligases in a variety of other pathogens, the region corresponding to residue E94 of LplA1 does exhibit significant diversity (Fig. C.1). Thus, it is possible other pathogens besides *L. monocytogenes* also have multiple lipoate ligases that enable bacterial growth upon diverse lipoate substrates if this region is indeed involved in substrate utilization by the bacterium. As most current antimicrobial therapies are directed at halting bacterial growth, breaking the assumption that central metabolism is conserved throughout all organisms will likely open the door for the development of new targets to generate therapies against pathogenic diseases.

Bacterial growth and persistence in different environments suggest microbes must be able to metabolize nutrients predominant during different stages of infection. For example, the sugars *E. coli* encounters during colonization of the intestine change based upon the composition of the intestinal microbiota; mutants that cannot catabolize glucosamine or sialic acid were not impaired for growth during initial stages of colonization, but did have a defect in late stage colonization (Chang *et al.,* 2004). Similarly, the work from this thesis suggested *L. monocytogenes* might have multiple mechanisms to exploit and utilize different sources and concentrations of lipoate. It is conceivable that different tissues within the host may contain higher or lower concentrations of host lipoyl-peptide, and as such, may influence *L. monocytogenes* tissue tropism. Recent studies examining pregnant Guinea pigs infected with *L. monocytogenes* suggested bacterial colonization of placental tissue results in massive reseeding of maternal

tissues, eventually resulting in spontaneous abortion (Bakardjiev *et al.,* 2006). Knowing the basis for tissue preference, and determining if lipoyl-peptide availability contributes to this tropism, will lead to a more complete paradigm of how this disease works. This knowledge potentially could lead to new discoveries on how to limit bacterial growth in key tissues of not just *L. monocytogenes*, but also other pathogens that must scavenge nutrients from the host.

The ability of exogenous metabolites to act as signaling molecules also influences the outcome of infection. In *L. monocytogenes* and other pathogens, the ability to respond to amino acid starvation with the stringent response is an essential process for virulence, emphasizing that the ability to respond to exogenous metabolites influences the success of a bacterial pathogen (Taylor *et al.,* 2002*)*. In this thesis, supplementation of *L. monocytogenes* lipoate ligase mutants with lipoate or BCFAs also altered the intracellular replication of the pathogen, illustrating those two nutrients can also influence the outcome of infection. Conversely, exogenous metabolites can also act as signaling molecules toward the host, eventually altering the outcome of infection as well. Enteropathogenic *E. coli* (EPEC) inhibits epithelial uptake of the short chain fatty acid butyrate, which ultimately can effect EPEC virulence (Borthakur *et al.,* 2006; Cook and Sellin, 1998; Inan *et al.,* 2000). These data, and the data from this thesis, indicate that fatty acids could potentially also act as signaling molecules to ultimately effect pathogen invasion, infectivity, and pathogenicity.

As mentioned previously, recognition and utilization of host-derived nutrients by pathogens can influence their expression of virulence determinants through the stringent response and potentially other undefined mechanisms. When *L. monocytogenes* was grown in a carbon source found in soil, virulence gene expression was repressed (Brehm *et al.,* 1999). These studies and other more recent ones suggested some carbon sources and the metabolic pathways involved in their catabolism were involved in PrfA activity modulation. By demonstrating intracellular growth and virulence of *L. monocytogenes* required the modification and activation of metabolic enzyme complexes, this thesis work implied those enzymes and the metabolites they produce may also influence the activity of other virulence determinants essential for *L. monocytogenes* pathogenesis. Nevertheless, what those virulence determinants might be are still unknown. One virulence determinant that PrfA regulates is LLO, a hemolysin that enables bacterial escape from the vacuole. However, in the absence of lipoylation, the bacterium could still escape from the vacuole of macrophages. The work done in this thesis suggests while lipoate mediated metabolism may not alter LLO activity, it is still possible lipoate dependent metabolism influences the expression of other unidentified virulence determinants essential for intracellular growth and survival of the bacterium.

One defense strategy intracellular bacteria have likely developed to infect phagocytic mammalian cells are mechanisms to defend against host generated

stress. Lipoic acid scavenged from the host may enhance the bacterium's resistance to oxidative stress. Lipoic acid contains a disulfide bond that can act as an electron acceptor in both its reduced or oxidized forms (Biewenga *et al.,* 1997). Others and we have observed that the surface of *L. monocytogenes* is associated with bacterial lipoyl-E2 in both an oxidized and reduced form (Fig. B.2) (Lenz *et al.,* 2003). *Mycobacterium tuberculosis*, another pathogen that encounters nitrosative and oxidative stress during infection, has strong antioxidant activity that is dependent upon lipoyl-E2 (SucB) (Bryk *et al.,* 2002). It is possible that lipoyl-E2 on the surface of *L. monocytogenes* may act as an electron sink to protect bacteria from oxidative stress. However, as depletion of lipoylation has such a strong and immediate effect upon intracellular *L. monocytogenes* growth, it may be difficult to demonstrate *in vivo* that bacterial lipoylation can also facilitate resistance to host oxidant stress. Utilization of a nutrient to enhance survival against host defense mechanisms, and not just replication, is not unprecedented. Biofilm communities also position themselves within nutrient gradients that physically facilitate survival of the bacterial community against environmental stress (Parsek and Tolker-Nielsen, 2008). Nutrients can act in other ways besides just supporting replication; the implications of this observation support the possibility that lipoate could help an intracellular pathogen defend itself against oxidative stress.

Restriction of nutrients could also be considered a defense strategy of the host, as many pathogenic microorganisms do not have the ability to generate all of the

nutrients they need to support bacterial replication. For example, *Chlamydia trachomatis* and *C. pneumoniae* are obligate intracellular bacteria that form inclusion bodies within epithelial and endothelial cells. A defense strategy that host cells appear to have developed to defend against growth of chlamidiae is depletion of tryptophan from infected cells (Rottenberg *et al.,* 2002). This depletion strategy is effective because these pathogens are auxotrophic for tryptophan. Similarly, *L. monocytogenes* is a lipoate auxotroph, indicating it must scavenge this vital nutrient from the host environment to enable lipoate dependent metabolism. However, this thesis work demonstrated that free lipoate is limiting in the host environment, and the ability of *L. monocytogenes* to utilize host-derived lipoyl-peptides with LplA1 may enhance the bacterium's intracellular replication. Therefore, the sequestration of lipoate by the host in the form of lipoyl-peptides could be interpreted also as a defense strategy exhibited by the host. *L. monocytogenes* has overcome this host restriction of a vital nutrient by encoding LplA1.

The requirement to scavenge host-derived lipoate to support optimal replication may be widespread among intracellular pathogens, including those that can synthesize their own lipoate (Allary *et al.,* 2007; Crawford *et al.,* 2006; Reed *et al.,* 1951; Wrenger and Müller, 2004). The nutrient environment that surrounds the pathogen may act as a distinct signal to trigger biosynthesis, scavenging, and even expression of virulence factors. In the future, a detailed understanding of each distinct nutrient compartment that a microbe is contained within will be

important in determining what metabolic pathways are critical for growth of pathogenic bacteria. The findings in this thesis highlight the dynamic interaction between a microbe and its host, and illustrate that to successfully establish and sustain infection in the host environment, pathogens must adjust their metabolism. In the future, a more comprehensive understanding of *in vivo* microbial ecology will lead to promising new anti-microbial therapeutics designed to limit nutrients intracellular pathogens require.

Appendix A

LIPOATE MAY ENHANCE *L. monocytogenes* ABILITY TO RESIST OXIDATIVE STRESS

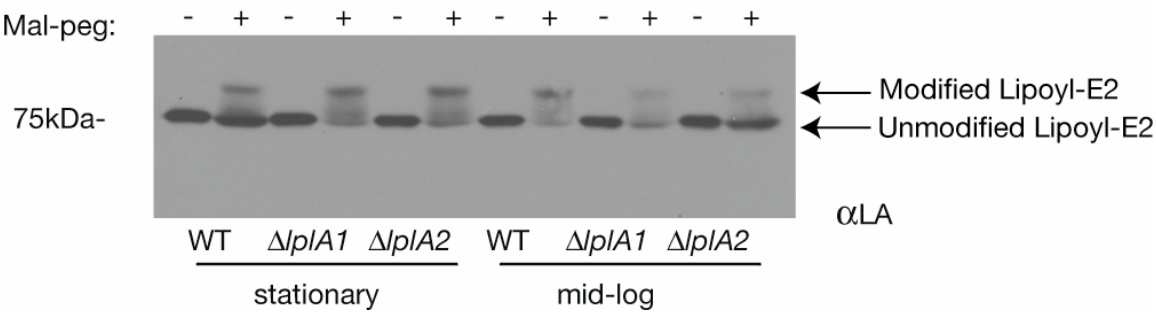
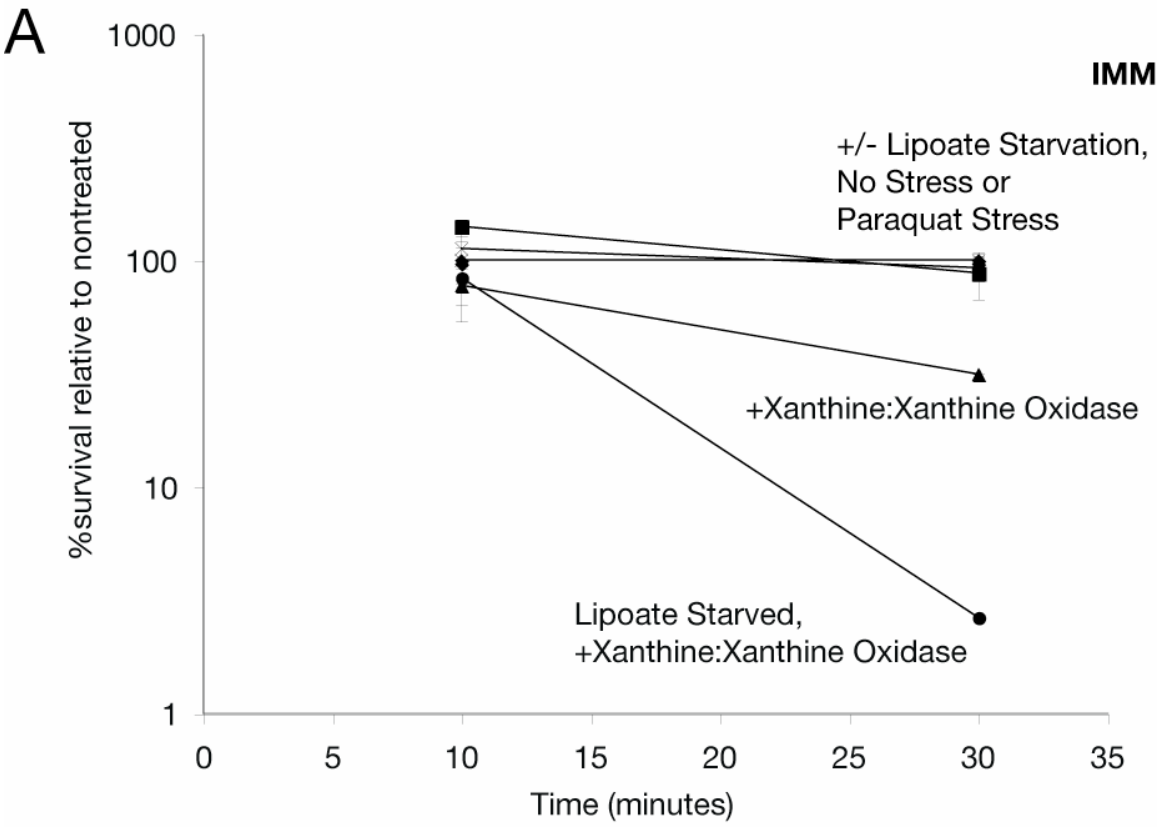


Figure A.1 Lipoate on the surface of *L. monocytogenes* is both oxidized and reduced

MAL-PEG (Mr = 5,000) was used to tag free thiols on the surface of *L. monocytogenes.* After growth at 37°C in BHI with aeration, stationary phase bacteria were harvested at OD_{600}=1.2, while mid-log phase cultures were harvested at OD_{600}=0.5. Equivalent bacterial pellets were pelleted, and then incubated in 20 mM Tris·HCl and 1 mM EDTA with 1 mM MAL-PEG at 25°C. After 1 h, the reactions were terminated by addition of equal volume of 2x SDS sample buffer and immediately subjected to SDS/PAGE. Non-modified and modified lipoylated proteins were analyzed by immunoblot with an anti-lipoic acid antibody. The 75kDa protein is the E2 subunit of *L. monocytogenes*, which only no thiol groups from a cysteine in its amino acid sequence, but does contain thiol groups from ligated lipoate. The upper band corresponds to the predicted shift in size if a free thiol on surface E2 reacts with two Mal-peg reagents. **This western-blot indicates lipoyl-E2 may have the capacity of being both oxidized and reduced on the surface of the bacterium.**


A
IMM
1000
100
10
1
%survival relative to nontreated
+/- Lipoate Starvation,
No Stress or
Paraquat Stress
+Xanthine:Xanthine Oxidase
Lipoate Starved,
+Xanthine:Xanthine Oxidase
0
5
10
15
20
25
30
35
Time (minutes)

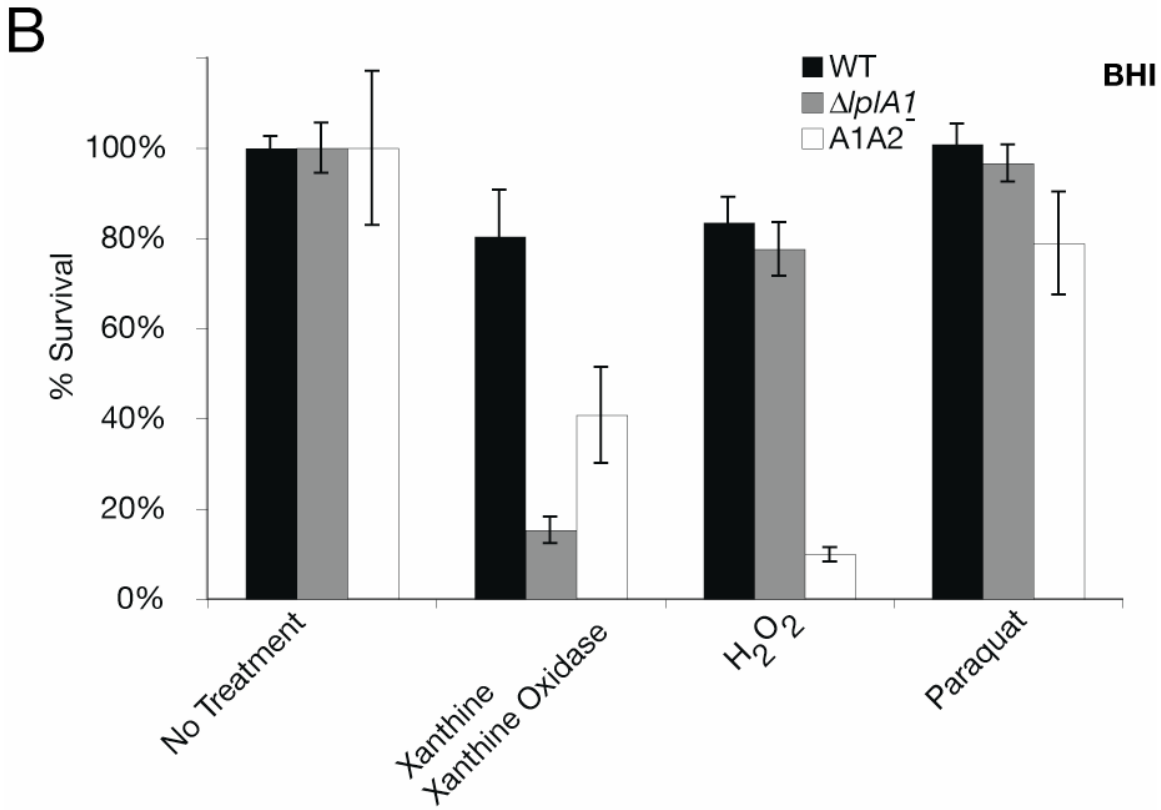

B
BHI
WT
ΔlplA1
A1A2
100%
80%
60%
40%
20%
0%
% Survival
No Treatment
Xanthine
Xanthine Oxidase
H2O2
Paraquat

Figure A.2 *In vitro* susceptibility to H_2O_2 and Xanthine:Xanthine Oxidase.

In (A), WT bacteria were grown in improved minimal medium with or without lipoate. In (B), WT, the *ΔlplA1*, and the A1A2$^-$ mutant strain of *L. monocytogenes* were grown in BHI. In (A), washed midlog phase bacteria grown at 37°C shaking with aeration were incubated with 17.2mM Xanthine and 1200mU Xanthine Oxidase, or 40mM Paraquat in PBS for 30 minutes at 37°C before 20mM HEPES was added. Surviving bacteria were then plated immediately and CFU enumerated after 2 days growth on LB agar plates. Percent survival was calculated by dividing average CFU of treatment by the average CFU of untreated sample. In (B), washed midlog phase bacteria grown at 37°C shaking with aeration were incubated with 4.3mM Xanthine and 300mU Xanthine Oxidase, 10mM H_2O_2 (Riedel-deHaen), or 10mM Paraquat in PBS for 30 minutes at 37°C before 20mM HEPES was added. Percent surviving bacteria were enumerated and calculated as in (A). Standard deviations for each average value were combined by rms error propagation.

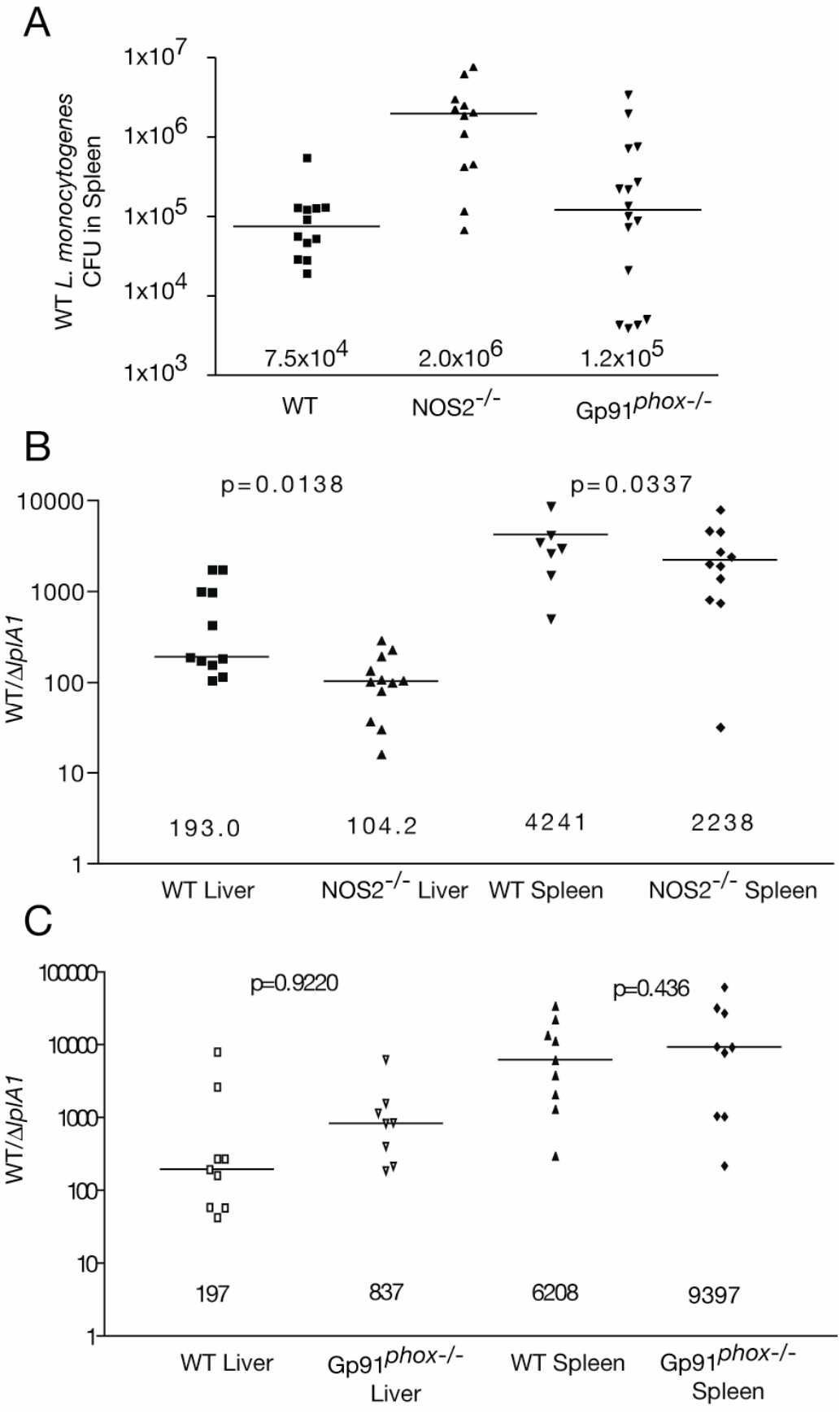

A
WT L. monocytogenes CFU in Spleen
1x10^7
1x10^6
1x10^5
1x10^4
1x10^3
7.5x10^4
2.0x10^6
1.2x10^5
WT
NOS2^-/-
Gp91^phox-/-
B
p=0.0138
p=0.0337
WT/ΔplA1
10000
1000
100
10
1
193.0
104.2
4241
2238
WT Liver
NOS2^-/- Liver
WT Spleen
NOS2^-/- Spleen
C
p=0.9220
p=0.436
WT/ΔplA1
100000
10000
1000
100
10
1
197
837
6208
9397
WT Liver
Gp91^phox-/- Liver
WT Spleen
Gp91^phox-/- Spleen

Figure A.3 LplA1 does not appear to enhance *L. monocytogenes* fitness against *in vivo* oxidative and nitrosative stress

In (A) 6-8 week male C57Bl/6 mice were injected IP with $2x10^5$ CFU wildtype *L. monocytogenes.* 72hrsh post infection, spleens were harvested and enumerated for CFU. The median value for the resulting CFU are presented as a horizontal line and also represented numerically below the data points. $NOS2^{-/-}$ mice have a defect in the ability to produce large amounts of nitric oxide over prolonged periods of time (Jackson stock number 002596). $Gp91^{phox-/-}$ mice have a defect in their phagocyte respiratory burst oxidase (Jackson stock number 002365). These data confirm previously published data suggesting *L. monocytogenes* is susceptible to nitric oxide stress, and may have evolved resistance mechanisms towards the phagocytic respiratory burst, as removal of these defense mechanisms in the mouse enabled greater *in vivo* growth of the bacteria in the $NOS2^{-/-}$ background, but there was no difference in *in vivo* growth in the $Gp91^{phox-/-}$ background {Shiloh, 1999, p11320}. In (B) and (C) 6-8 week male C57Bl/6 mice were injected IP with $2x10^5$ CFU wildtype and $2x10^6$ *ΔlplA1 L. monocytogenes.* 72hrsh post infection, spleens and livers were harvested and enumerated for CFU. The median value for the resulting CFU are presented as a horizontal line and also represented numerically below the data points. **If *lplA1* conferred an advantage to *L. monocytogenes in vivo* fitness against the phagocyte respiratory burst, we would expect the competitive index of infection (WT/*ΔlplA1*) to be decreased in the $Gp91^{phox-/-}$ mouse. Similarly, if *lplA1* conferred an advantage to *L. monocytogenes* fitness against nitric oxide stress, the CI should be reduced in the $NOS2^{-/-}$ mouse. However, there was no statistically significant difference in fitness between WT and the mutant mice** ($p<0.05$, unpaired Students *t*-test with unequal variance).

Appendix B

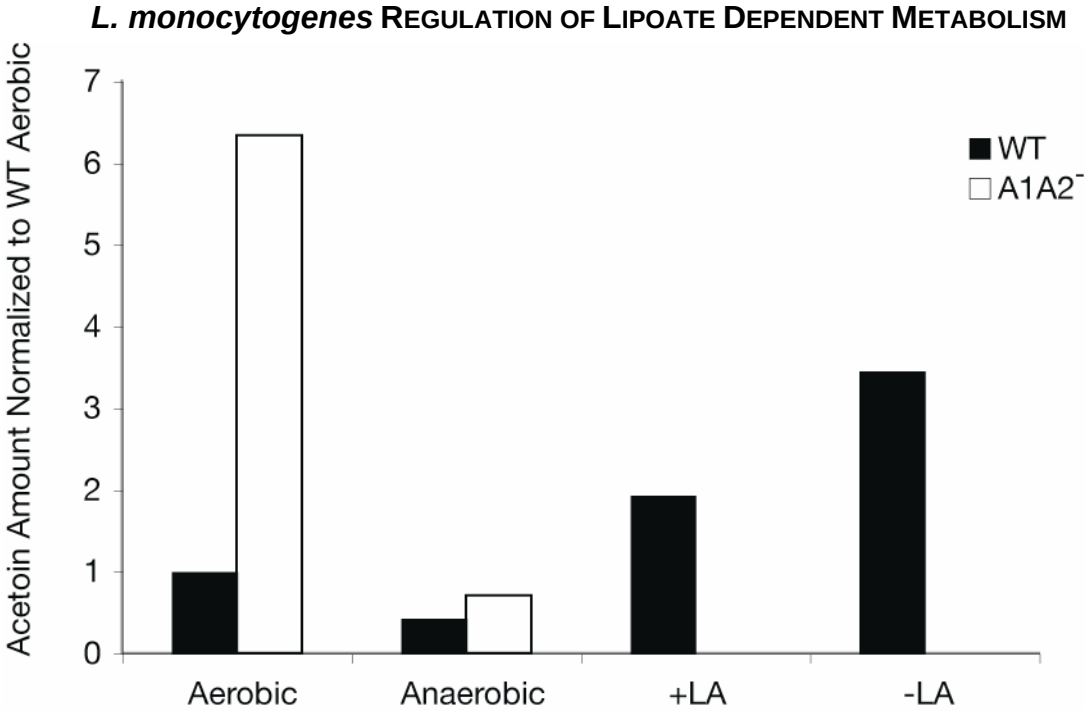


Figure B.1 Acetoin secretion is inhibited during anaerobic growth and well as by lipoylation and growth in lipoic acid

WT and the A1A2$^-$ mutant were grown to stationary phase in BHI aerobically or anaerobically at 37°C. Additionally, WT was also aerobically grown in minimal medium with lipoic acid (+LA) or without lipoic acid (-LA). Filtered culture supernatant was derivatized and products detected by high pressure liquid chromatography. Acetoin peak identify and concentration was confirmed by HPLC separation of derivatized acetoin standards prepared in filtered BHI medium. Dark bars represent WT levels of acetoin, while open boxes represent acetoin levels in the A1A2$^-$ lipoylation mutant. **Acetoin secretion is highest in the lipoylation A1A2$^-$ mutant, and also increased when lipoate is withheld from the growth medium, indicating lipoate dependent metabolism may influence acetoin metabolism.**

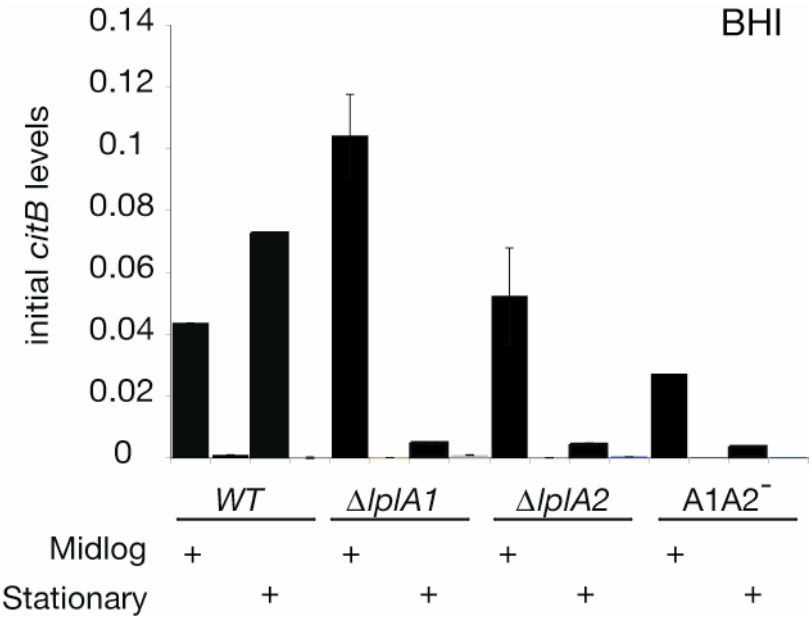


Figure B.2 Aconitase RNA transcript levels are regulated by lipoate protein ligase genes as well as by growth phase.

Quantitative RT-PCR was performed in triplicate on isolated bacterial RNA to determine template quantities of *rpoA* and *citB.* Template quantities were normalized against *rpoA* levels (data not shown). *CitB* encodes aconitase, an enzyme that functions in the oxidative branch of the TCA cycle. Levels of *citB* RNA were increased during midlog growth of the bacterium in all mutant strains. Additionally, deletion of a single lipoate protein ligase gene, *lplA1* or *lplA2*, resulted in decreased levels of *citB* during stationary phase. Finally, a double disruption mutant in both lipoate ligases (A1A2$^-$) results in overall decreased *citB* RNA levels. **These data reveal an underappreciated complexity in regulation of enzymes involved in the TCA oxidative branch of *L. monocytogenes* by lipoate protein ligase genes.**

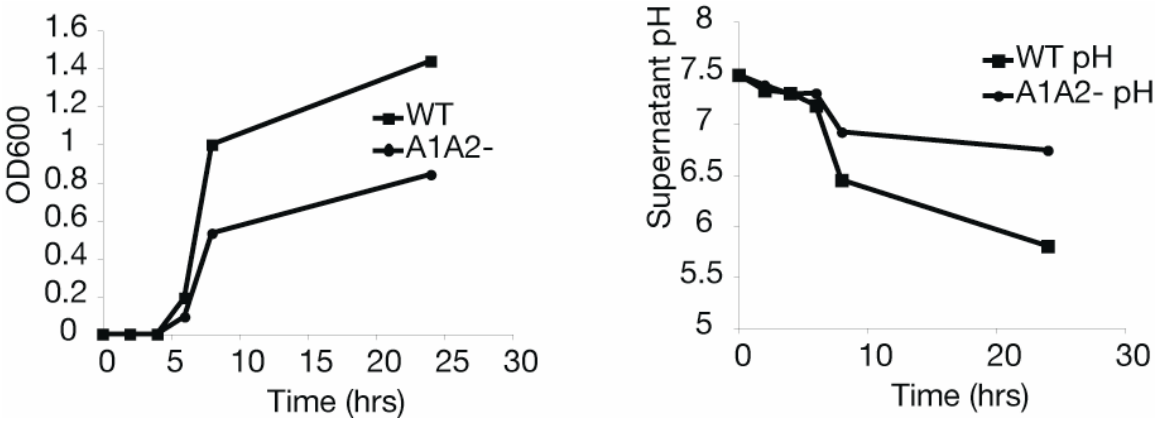


Figure B.3 The A1A2$^-$ mutant is defective in acidification of its growth medium

In the left panel, WT and the A1A2$^-$ mutant were grown with aeration in BHI at 37°C and OD_{600} measured over time. The pH of the corresponding time points in the left panel was measured and graphed over time in the right panel. **Together, these data suggest the A1A2$^-$ mutant is defective in acidification of its growth medium when compared with WT. However, it is possible this defect in acidification is because of the decreased density of the bacterial culture.**

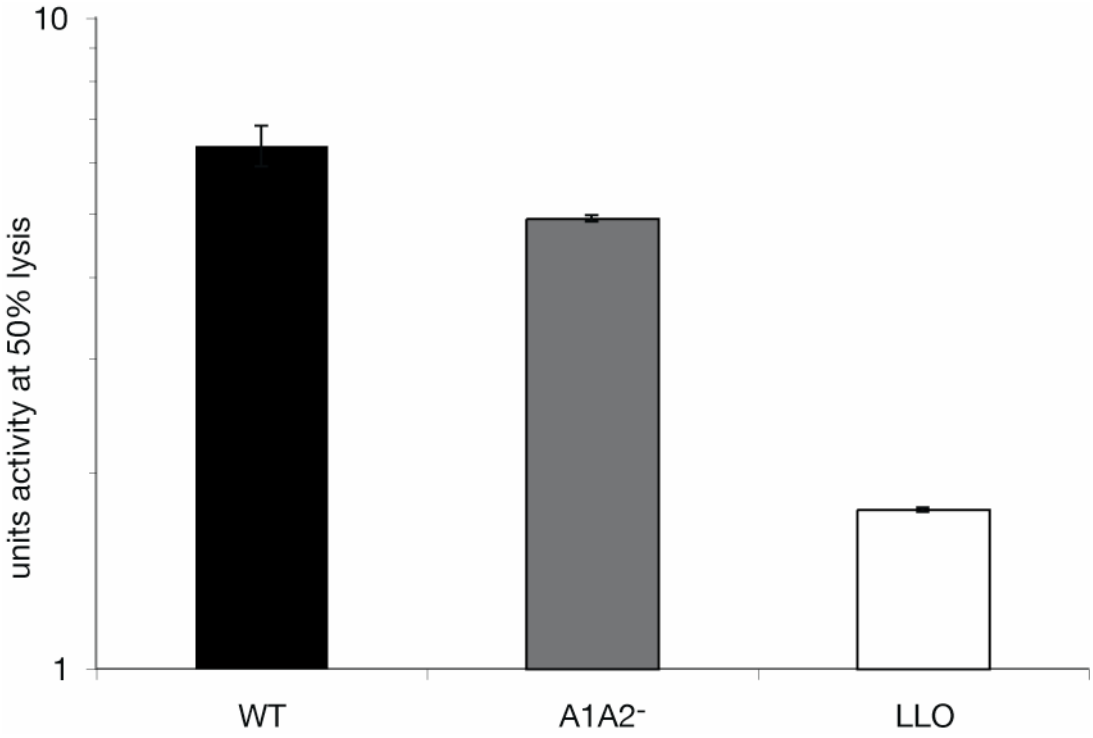


Figure B.4 Loss of lipoylation does not appreciably impair hemolytic activity

WT and the A1A2$^-$ lipoylation mutant were grown for 20hrsh in BHI at 37°C with aeration. Equivalent bacterial pellets were then resuspended in depleted and cleared BHI medium used to grow the *hly* strain of *L. monocytogenes* deficient in LLO production. After 24hrsh incubation at 37°C with shaking, bacteria were pelleted and the supernatant was treated with 0.1mM DTT for 1hr at 37°C. Twofold serial dilutions of samples were made in hemolysis assay buffer, pH 5.5 (125mM NaCl, 35mM Na_2HPO_4, 0.1mg/ml BSA) with sheep red blood cells and incubated at 37°C for 30 minutes. Unlysed red blood cells were then pelleted and the OD_{541} of the supernatant measured. 50% lysis was calculated as 50% the value of the complete lysis control (10% Triton X-100). Units activity at 50% lysis was calculated with the following formula: Units activity 50% lysis = UL + (AL – AH/AL – AR) (UR-UL), where UL = units left of 50% lysis, AR = absorbance right of 50% lysis, UR = units right of 50% lysis, AH = absorbance of 50% lysis, and AL = absorbance left of 50% lysis. **These data indicate the A1A2$^-$ lipoylation mutant is capable of lysing red blood cells nearly to WT *L. monocytogenes* levels.**

Appendix C

LIPOATE LIGASES IN OTHER MICROBES

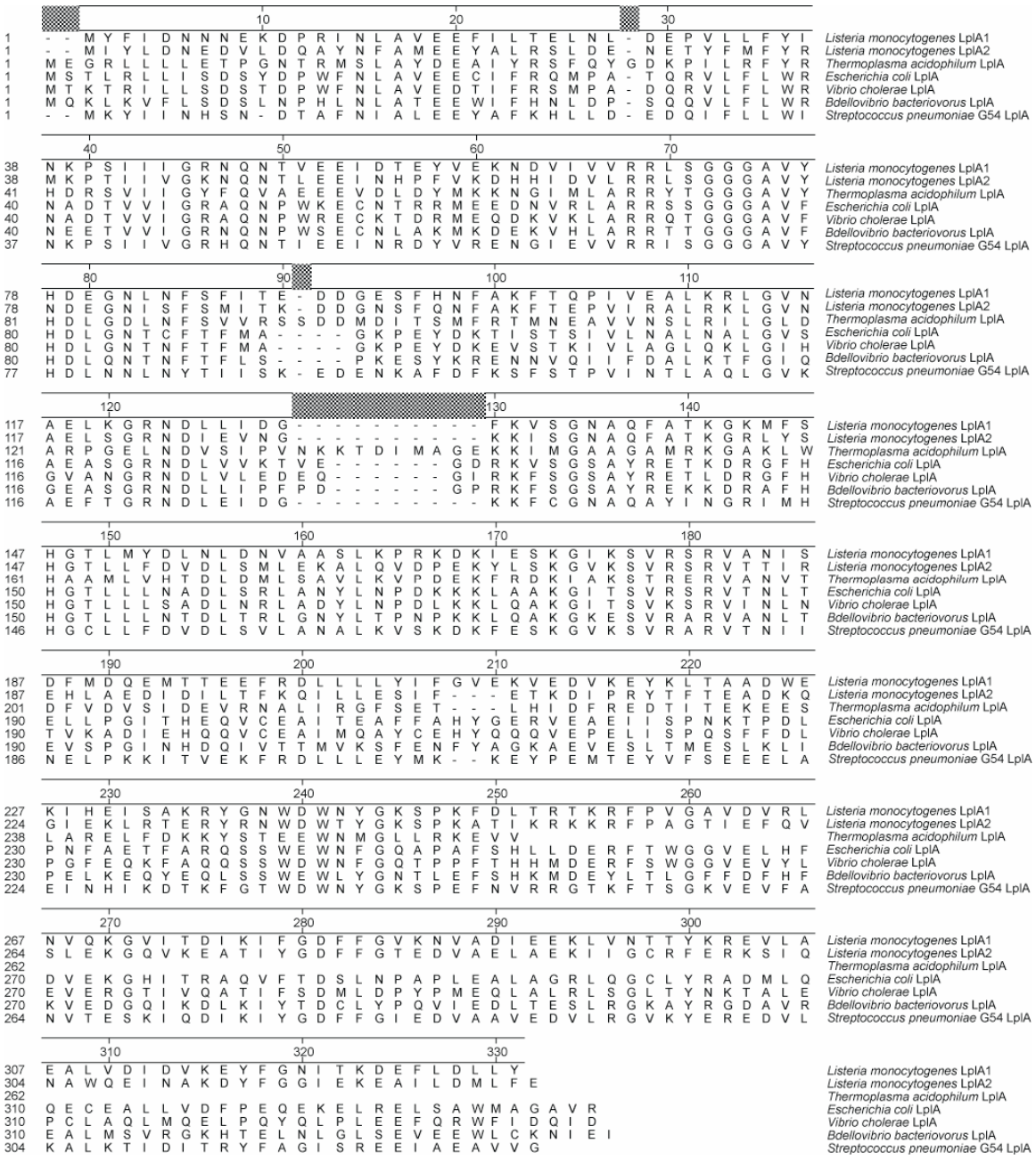


Figure C.1 Lipoate protein ligase diversity

Lipoate protein ligase protein sequences were aligned by the Clustal W method. *Thermoplasma acidophilum* (accession CAC11654), *Bdellovibrio bacteriovorus* HD100 (accession ACC CAE80307), *Vibrio cholera* (accession YP_002175805), *E. coli* DH10B (accession ACB05314), *Streptococcus pneumoniae* G54 (accession YP_002037771) aligned with *L. monocytogenes* LplA1 and LplA2.

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