A Complex Lipoic Acid Utilization Pathway in *Listeria monocytogenes*

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Although a complete pathway of lipoic acid metabolism has been established in *Escherichia coli*, lipoic acid metabolism in other bacteria is more complex and incompletely understood. *Listeria monocytogenes* has been shown to utilize two lipoate-protein ligases for lipoic acid scavenging, whereas only one of the ligases can function in utilization of host-derived lipoic acid-modified peptides. We report that lipoic acid scavenging requires not only ligation of lipoic acid but also a lipoyl relay pathway in which an amidotransferase transfers lipoyl groups to the enzyme complexes that require the cofactor for activity. In addition, we provide evidence for a new lipoamidase activity that could allow utilization of lipoyl peptides by lipoate-protein ligase. These data support a model of an expanded, three-enzyme pathway for lipoic acid scavenging that seems widespread in the Firmicutes phylum of bacteria.

*Listeria monocytogenes* is a Gram-positive bacterium of the phylum Firmicutes that is a serious human pathogen. Like a number of other bacteria, it is a natural lipoic acid auxotroph and relies upon exogenous sources of lipoic acid for growth. Utilization of lipoic acid requires that the cofactor become covalently attached to the ε-amino group of a conserved lysine residue of the lipoyl domain(s) of the enzyme complexes that require the cofactor for activity. The only known route for attachment of exogenous lipoic acid is by lipoic acid ligase action (1). *L. monocytogenes* employs two lipoate protein ligases for this purpose, LplA1 and LplA2 (2). Although either ligase suffices for lipoylation in cells grown in a rich medium, only lplA1 is essential for intracellular growth and virulence (2, 3). The largest lipoyl peptide substrate utilized by *L. monocytogenes* is the DKA tripeptide, where lipoate is attached to the ε-amino group (DKLA) by an amide linkage. LplA1 is required for efficient use of DKA4A as a lipoate source (2). Prior work (2) indicated that expression of LplA1 but not LplA2 in *Escherichia coli* functionally replaced the host LplA ligase, the most thoroughly characterized lipoate ligase. The enzymatic properties that differentiate LplA1 from LplA2 were unknown.

It was proposed that LplA1 and LplA2 may have different protein interaction partners or that LplA1 may be able to transfer the lipoyl group from a synthetic lipoylated peptide, DKA4A, to lipoate requiring enzymes by functioning as a lipoyl-amidotransferase (2). We conducted the present study to improve our understanding of lipoil scavenge by *L. monocytogenes* and to determine the mechanism of DKLA utilization. It was possible that the DKA4A lipoil moiety could be utilized either by direct transfer of the lipoil moiety or by hydrolysis with subsequent ligation of the lipoic acid released.

In *Bacillus subtilis* a novel amidotransferase, called LipL, was recently shown to be required for lipoic acid biosynthesis (4, 5). Moreover, LipL homologues are present in all Firmicutes that use lipoic acid including those, such as *L. monocytogenes*, that lack the ability to synthesize the cofactor (and thus are natural lipoate auxotrophs). Moreover, although growth of *B. subtilis* lipl null mutants is supported by lipoic acid, the rate of growth is slow, and the E2 subunits of the pyruvate and branched chain dehydrogenase proteins (pyruvate dehydrogenase and BkdB, respectively) are only partially modified (5). Because this slow growth requires the LplJ lipoate ligase, it seems that the presence of LipL increases the efficiency of scavenging by the ligase, although it is unclear if LipL transfers lipoic acid or octanoic acid (or both) to the dehydrogenase proteins of this bacterium. It also remains to be seen if LipL confers any competitive advantage beyond its requirement for biosynthesis of lipoic acid. The inability of *L. monocytogenes* to synthesize lipoic acid provides a more straightforward system than *B. subtilis* to study lipoate scavenging in a Firmicutes bacterium. Moreover, it seemed possible that the amidotransferase activity of the putative *L. monocytogenes* LipL might allow direct utilization of DKLA to modify the lipoic acid-requiring dehydrogenases essential for growth and pathogenesis of this bacterium.

It also remained possible that DKLA utilization as a lipoate source proceeds by hydrolysis of the amide linkage to give free lipoic acid. Such a lipoamidase activity is present in *Enterococcus faecalis* (6), another Firmicutes that lacks the ability to make lipoic acid. The *E. faecalis* lipoamidase activity was discovered as an activity that allowed a lipoate ligase to use various lipoamide compounds as substrates. Moreover, the lipoamidase was also able to cleave lipoic acid from intact lipoil domains. *E. coli*,

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†‡§¶ The abbreviations used are: BkdB, branched chain dehydrogenase E2 subunit; GCV, glycine cleavage system; Ni-NTA, nickel-nitrilotriacetic acid; TCEP, (tris(2-carboxyethyl)phosphine); TMS, trimethylsilane; TEV, tobacco etch virus.
A Complex Lipoate Utilization Pathway

a bacterium that lacks lipoamidase activity, was the host used in a cosmid library screening approach to isolate an *E. faecalis* gene (called *lpa*) that encoded a lipoamidase (7). Close homologues of *E. faecalis* Lpa are not widely distributed in bacteria, and it remains unclear if other isozymes or more divergent homologues having the same function exist. Unlike *E. faecalis*, *L. monocytogenes* cannot utilize intact lipoyl domains (2). The ability to use lipoylated peptides is dependent on LplA1 (2), but the mechanism of utilization was unknown (2). Although no obvious orthologue of the *E. faecalis* lipoamidase is encoded in the *L. monocytogenes* genome sequences, the possibility of utilization of DK²-A by a lipoamidase of unrelated sequence remains.

In this study we report that *L. monocytogenes* LplA1 is a lipoyl ligase that is largely specific for modification of the H protein of the glycine cleavage system and that *L. monocytogenes* LipL is a reversible lipoylamidotransferase that participates in lipoic acid scavenging. We also provide evidence for a novel lipoamidase activity, which together with LplA1 provides a pathway for utilization of the lipoyl moieties of lipoyl peptides.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—Bacterial strains, plasmids, and oligonucleotides used are given in Table 1. Standard molecular biology methods were used unless otherwise indicated (8). Cloned genes were verified by sequencing performed by ACGT, Inc. *L. monocytogenes* EDGe gcvH (lmo2425), LipL (lmo2566), and the lipoyl domain of bkdB (lmo1374 and E2_{bdB}) were placed under the control of a T7 promoter for heterologous expression in *E. coli*. Gene sequences were amplified using Phusion polymerase (New England Biolabs) according to the manufacturer’s recommendations. The amplified products were inserted into the expression vector such that the protein products would be produced with a N-terminal hexahistidine tag. The tag could be removed by cleavage with tobacco etch virus (TEV) protease. *L. monocytogenes* EDGe genomic DNA from the American Type Culture Collection was used as a template. Primers Q155 and Q156 were used to amplify gcvH, Q159 and Q160 for the bkdB lipoyl domain (E2_{bdB}), and Q161 and Q162 for LipL. The PCR products were inserted into plasmid pMCSG21 by ligase independent cloning as described (9) except for a modified T4 polymerase treatment. The treatment was performed with 1.5 μg of DNA and 5 units of T4 polymerase in the supplied buffer (New England Biolabs) plus the appropriate deoxynucleotide triphosphates (2.5 mM) for 2 h at 37 °C. The products were purified using a Qiagen plasmid mini kit (Qiagen).

**TABLE 1**

| Strain | Genotype | Source |
|--------|----------|--------|
| Tuner DE3 | ompT hsdS_{F} (r_{F} m_{F} {\_}_{F}) | EMD |
| BL21 | hsdS_{F} | New England Biolabs |
| BH285 | rpl-1 ΔlpaA::FRT ΔlpaE::FRT | Ref. 10 |
| QC113 | ΔlpaA::FRT | This study |
| QC146 | ΔlpaA::FRT | Ref. 12 |
| QC239 | ΔlpaA::FRT | This study |
| QC240 | ΔlpaA::FRT | This study |
| QC241 | ΔlpaA::FRT | This study |
| QC242 | ΔlpaA::FRT | This study |
| QC244 | ΔlpaA::FRT | This study |

Strains, plasmids, and oligonucleotides used in this study

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were cultured in M9 minimal medium with 0.4% glycerol, 5 mM acetate, 5 mM succinate, 10 μM FeCl₃ (from a stock solution in HCl), and 100 μg/ml sodium ampicillin overnight at 37 °C. The cells were collected by centrifugation and washed three times with medium lacking acetate and succinate. The final culture density was measured and adjusted to an A₆₀₀ of 0.05 in the same medium lacking acetate and succinate with additional supplements as indicated. Growth assays were carried out in a Bioscreen C (Growth Curves USA) at 37 °C with very strong shaking, and growth was measured by A₆₀₀ readings taken every 15 min.

To further purify lipoamide for use as a growth factor, it was dissolved in ethyl acetate and extracted three times with aqueous sodium bicarbonate, and the ethyl acetate solution was then taken to dryness. The product was dissolved in a minimal volume of ethanol at 42 °C and filtered through a sintered glass funnel. The filtrate was then allowed to slowly cool first to room temperature and then to −20 °C. The fluffy needle-like yellow crystals were collected by filtration and washed with cold ethanol. A second crystallization was then carried out.

Purification of Proteins—Buffered solutions were prepared at room temperature. Apolipoyl domains GcvH and E2_E2BkdB were prepared from strains QC235 and QC237, respectively, whereas apoLipL was prepared from strain QC243. The strains were cultured in 1 liter of LB supplemented with 5 mM acetate, 5 mM succinate, 0.2% glucose, 25 μg/ml chloramphenicol, 50 μg/ml streptomycin, and 50 μg/ml spectinomycin overnight. These cultures were subcultured in the same medium except for the presence of 0.1% glucose and grown at 37 °C to an A₆₀₀ of 0.5 when phage T7 RNA polymerase expression was induced (11) by the addition of arabinose to 0.2%. After 4 h of incubation, the cells were harvested by centrifugation at 8500 × g for 7 min and frozen at −80 °C. Hexahistidine-tagged proteins containing a TEV cleavage site (GcvH₉₂₆_GcvH₉₂₆, LipL₉₄₉₄) were purified by a modification of a subtractive immobilized metal affinity chromatography steps. Lipoyl domains were further purified by anion exchange on a 1.8-ml POROS HQ 20 column using an AKTA Purifier 10 (GE Healthcare) at 5 ml/min in 25 mM sodium-MES (pH 6.1). The proteins were eluted with a 0–1 M gradient of LiCl in the same buffer. The MALDI-MS determined masses of the E₂_E2BkdB and the GcvH protein were 9,179.9 and 13,942.7, values that were greater by 1.7 and 0.4 atomic mass units, respectively, from the theoretical masses. The bands of the proteins that copurified with apoLipL were cut from an SDS-PAGE gel, the proteins were digested with trypsin, and the resulting peptides were subjected to liquid chromatography-mass spectrometry analysis for identification as previously described for lipoyl domains (5).

Lpa1 and Lpl2 were purified from strains QC239 and QC240, respectively. The strains were grown at 37 °C in LB with 50 μg/ml streptomycin and 50 μg/ml spectinomycin to an A₆₀₀ of 0.5 at which point they were shifted to 30 °C for 1 h. Protein expression was induced with 10 μM isopropyl β-D-1-thiogalactopyranoside for 16 h, and the cells were harvested and lysed as described above except in Halo buffer: 50 mM sodium HEPES (pH 7.5), 150 mM NaCl, 0.01% Tergitol Nonidet P-40, and 5 mM TCEP. Purification steps using Halo and Ni-NTA resins were carried out as recommended by the manufacturer. The pure untagged protein was dialyzed against storage buffer and flash-frozen for storage at −80 °C. Lpl1A was quantified using the calculated extinction coefficient of 34,380 M⁻¹ cm⁻¹ and had a mass of 38,382 as determined by MALDI-MS.

The multimeric state of LipL was determined using size exclusion chromatography with a Superdex 200 10/300 GL column (GE Healthcare) with 50 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl at a flow rate of 0.5 ml/min. A standard curve was used to estimate size using chy-
A Complex Lipoate Utilization Pathway

motrypsin A, albumen, aldolase, and catalase as standards. The reported value is the average of three injections.

Lipoic Acid Ligase Assay—The reactions were performed in assay buffer (50 mM sodium MOPS (pH 7.2), 100 mM NaCl, 5 mM TCEP) with 1 mM ATP, 1 mM MgCl₂, 20 μM GcvH, 1 μM LipL, and 1 mM acyl substrate. The acyl substrates were sodium lipoate, lipoamide, or DK²A where indicated. The reaction was separated by native PAGE on a 20% acrylamide gel and visualized with Coomassie Blue R-250. Modification with lipoic acid was assayed by Western blotting using anti-lipoic acid antibody (Calbiochem) and anti-mouse IgG conjugated to horseradish peroxidase (Calbiochem). The modification state of the lipoyl domain was also analyzed by electrospray ionization mass spectrometry as previously described (12).

Assay of Amidotransfer—To directly measure lipoal amidotransfer by LipL, lipoal-GcvH was synthesized as a substrate. The synthesis and purification was the same as for [1-¹⁴C]octanoyl-E²BkdB except GcvH and E. coli LplA were used in place of E²BkdB and LplA1. Transfer of lipoate from 100 μM lipoal-GcvH to 20 μM hexahistidine-tagged E²BkdB by 1 μM LipL was measured in assay buffer and incubated at 37 °C for 1 h with a total reaction volume of 100 μL. In place of lipoal-GcvH, 1 mM lipoamide or 1 mM DK²A was used where indicated. Reactions were analyzed by SDS-PAGE and Western blotting as described for lipoal ligation assays.

Octanoylation of lipoal domains by LplA1 and subsequent amidotransfer by LipL was assayed using radiolabeled octanoate. The reaction was performed in assay buffer (50 mM sodium-MOPS (pH 7.2) and 5 mM TCEP with the addition of 1 mM ATP, 1 mM MgCl₂, 250 μM sodium [1-¹⁴C]octanoate, 20 μM E²BkdB, 20 μM GcvH, 1 mM LplA1, and 1 μM LipL) and incubated at 37 °C for 1 h. Reaction components were omitted where indicated. Proteins were separated by SDS-PAGE on 4–20% gradient acrylamide gels, stained with Coomassie Blue R-250, soaked in Amplify (GE Healthcare), dried on filter paper, and exposed to film at −80 °C for 8 to 20 h. The reaction products were analyzed by the method of Laskey and Mills (13).

To directly measure octanoyl amidotransfer by LipL in the reverse of previous assays, [1-¹⁴C]octanoyl-E²BkdB was synthesized as a substrate using E. coli LplA. E. coli LplA was purified by successive immobilized metal affinity chromatography and ion exchange chromatographic steps as described above for lipoal domains (without cleavage and subtractive immobilized metal affinity chromatography). The reaction contained assay buffer with 1 mM sodium [1-¹⁴C]octanoate, 2 mM ATP, 2 mM MgCl₂, 0.5 mM E²BkdB, and 10 μM LplA and was incubated at 37 °C for 2 h. [1-¹⁴C]octanoyl-E²BkdB was purified by ion exchange chromatography as described above for lipoal domains. Transfer of octanoate from 20 μM [¹⁴C]octanoyl-E²BkdB to 20 μM GcvH by 1 μM LipL was measured in assay buffer with incubation at 37 °C for 1 h in a total reaction volume of 25 μL. The reaction was analyzed as described above.

Assay of Lipoamidase Activity in Crude Extracts—Half-liter cultures of L. monocytogenes were grown to mid-log phase in brain heart infusion medium (Difco), pelleted, resuspended in 20 ml of 50 mM sodium MOPS (pH 7.2), 200 mM NaCl, 10% (v/v) glycerol, and 5 mM 2-mercaptoethanol and lysed by passage through a French pressure cell twice at 20,000 p.s.i.. Ammonium sulfate was added to 85% of saturation to precipitate proteins, and samples were gently agitated for an additional 30 min on ice. The precipitated protein was pelleted at 14,000 × g for 10 min, flash-frozen in a dry ice ethanol bath, and stored at −80 °C indefinitely. For use, the extract pellet was thawed on ice and resuspended in assay buffer containing only 1 mM TCEP and 0.1 mM EDTA. The slurry was dialyzed twice for 2 h, and insoluble material was removed by centrifugation at 14,000 × g for 10 min. Protein contents were assayed by Coomassie Blue binding with the Bio-Rad protein assay kit using bovine gamma globulin as the standard. The lipoamidase assay reaction contained assay buffer with 0.5 mM MnCl₂, 2.5 mM DK²A, and 100 μg of extract protein. The assay was heated to 37 °C for 5 min before the addition of extract. MnCl₂ was replaced with other metal salts where indicated, and to avoid oxidation of redox-sensitive metals, the reactions were performed in anaerobic conditions (Coy Laboratory Products Inc.) under an atmosphere of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. Samples of 50 μl were removed at various time intervals, and the reaction was quenched by the addition to 150 μl of ice-cold acetone. Nonadecanoic acid (5 μg) was added to each of the quenched samples as an internal standard. The samples were acidified with 10 μmol of HCl. The samples were derivatized for gas chromatography-mass spectrometry (GC-MS) by first drying under a stream of nitrogen, then adding 100 μl of the silylation reagent N-methyl-N-(trimethylsilyl)trifluoroaceticamide. The derivatization reaction was carried out for 1 h at 65 °C. The derivatized samples were analyzed by GC-MS at the Carver Metabolomics Center of the University of Illinois. Samples (1 μl) were injected in split mode (5:1) into a GC-MS system that consisted of an Agilent (Palo Alto, CA) 7890A gas chromatograph, an Agilent 5975s mass selective detector, and an Agilent 7683B autosampler. Injections were performed on a 60-m HP-5 column with a 0.25-mm inside diameter and a 0.25-mm film thickness (Agilent) with an injection port temperature of 250 °C, the interface set to 250 °C, and the ion source adjusted to 230 °C. The helium carrier gas was set at a constant flow rate of 1.5 ml/min. The temperature program consisted of isothermal heating at 180 °C for 5 min followed by an oven temperature increase of 5 °C/min to 310 °C for 2 min. The mass spectrometer was operated in positive electron impact mode at a 69.9-eV ionization energy in the m/z 50–800 scan range. The spectra of all chromatogram peaks were evaluated using HP ChemStation (Agilent) and AMDIS (National Institute of Standards and Technology, Gaithersburg, MD). The spectra of all chromatogram peaks were compared with electron impact mass spectrum libraries NIST08 (National Institute of Standards and Technology) and WILEY08 (Palisade Corp.). This procedure was modified to detect the alanine and aspartate residues of the DK²A peptide using a temperature program consisting of isothermal heating at 70 °C for 5 min followed by an oven temperature increase of 5 °C/min to 310 °C for 2 min.

RESULTS

The lplA1 and lplA2 Genes Differentially Complement an E. coli lplA Strain—To investigate the proposed mechanism of lipoal peptide utilization, we conducted a series of genetic complementation experiments. LplA1 has been shown to be critical
for lipoyl peptide scavenging during *L. monocytogenes* infection, whereas LplA2 was dispensable (2). Also, lplA1 expression was found to allow growth of a ΔlplA ΔlplA strain of *E. coli* (2).

To further examine the functions of the lplA1 and lplA2 gene products, complementation of the growth of a ΔlplA ΔlplA strain of *E. coli* was assayed using differing derivatives of lipoic acid at different concentrations. The *E. coli* strain carried a ΔlplA mutation because in this bacterium lipoic acid biosynthesis must be disrupted for growth to be affected by the ΔlplA mutation (14, 15). Expression of the *L. monocytogenes* lplA1 and lplA2 restored growth of the *E. coli* strain when the minimal medium was supplemented with 25 μM sodium lipoate, which provided the first evidence that LplA2 functions as a lipoyl ligase. However, when the lipoate concentration was lowered to the more physiologically relevant concentration of 25 nm, only lplA1 allowed growth (Fig. 1A). LplA1 also allowed growth when the medium was supplemented with either lipoamide or DK3-A at 25 μM, although neither compound was active when added at 25 nm (Fig. 1, B and C). The finding that the amide-linked lipoate compounds were only active at high concentrations raised the possibility that the growth observed was due to the presence of contaminating lipoic acid in these preparations (either carried over from synthesis and/or the result of hydrolysis).

To test this possibility, we purified the commercial lipoamide by extraction followed by recrystallization and found that the purified lipoamide preparation was an appreciably poorer supplement. The commercial lipoamide preparation was due to trace contamination of the growth of a lipoate auxotroph (*E. coli* strain of *L. monocytogenes* lplA1 and DK3-A) in *C. jejuni*. Lipoamide was purified by three extractions followed by crystallization and recrystallization. The purified lipoamide was assayed using differing derivatives of lipoic acid at 25 μM, and scavenging pathways of *B. subtilis* and *E. coli* lipA lplA (2). Also, LplA2 was dispensable (2). However, given the diverse enzymatic activities of this family of proteins, it could not be assumed that the *L. monocytogenes* LplA has the same enzymatic activity as *B. subtilis* LplA. Moreover, given the parallel chemistry, it seemed possible that the putative *L. monocytogenes* LplA might be able to catalyze transfer of lipoyl moieties from DK3-A to apolipoyl domains. To test this possibility in vitro, recombinant *L. monocytogenes* LplA was expressed in *E. coli*. Previous work with *B. subtilis* LplA showed that the protein expressed in *E. coli* was modified with lipoate and octanoate. Therefore, we attempted to purify the unmodified form (apoLplA) from an *E. coli* lipoate auxotroph and unexpectedly found that apoLplA copurified with several larger proteins (Fig. 2A). Attempts to separate LplA from the larger proteins by ion exchange chromatography resulted in insoluble LplA aggregates. Excision of the bands of contaminating proteins from the gel, trypsin digestion, and LC-MS/MS analysis of the resulting peptides revealed the bands were the E1, E2, and E3 subunits of the *E. coli* pyruvate dehydrogenase complex. This suggests that like *E. coli* LipB (10), *L. monocytogenes* LplA binds to the pyruvate dehydrogenase complex. Upon LplA expression in a prototrophic *E. coli* strain, the protein was isolated with increased yield and purity (Fig. 2B). Size exclusion chromatography and MALDI-MS analysis of LplA indicates this preparation is monomeric (Fig. 2C) and is

A Complex Lipoate Utilization Pathway

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**FIGURE 1. Complementation of an *E. coli* lipA1 lipA2 strain by either lipA1 or lipA2 of *L. monocytogenes*.** The strains were grown on M9 minimal medium with 0.4% glycerol as a carbon source and supplemented with either a low (25 nm) or high (25 μM) concentration of a lipoate compound. The conditions are indicated by color for panels A, B, and C. The supplement was lipoic acid in A, lipoamide in B, and DK3-A in C. Lipoamide was purified by three extractions followed by crystallization and recrystallization. D, growth (absorbance at 600 nm) of the lipA1-complemented strain with 25 μM lipoamide (LD) before (green) and after (blue) purification is shown.

**FIGURE 2. Purification and properties of *L. monocytogenes* LipL, E2b, GcvH, and LplA1.** A, LipL purified from an *E. coli* lipoic acid auxotroph (−) or from a wild type strain grown with 5 mg/ml lipoic acid added to the medium (+) is shown. The pyruvate dehydrogenase subunit identity was determined by trypsin digestion, and LC-MS/MS of the peptides produced was as indicated followed by the percent sequence coverage of the peptides detected. B, SDS-PAGE analysis of purified proteins from A is shown. The mass spectra confirm that excepting LipL, the proteins were purified as apoproteins. C, size exclusion chromatogram of LipL is shown. LipL eluted as a single peak between chymotrypsinogen A and albumin with an estimated molecular mass of 32 kDa and thus appears monomeric. D, MALDI mass spectrum of LipL from the lipoate supplemented cells is shown. The protein appears to be in three major modification states having masses of 30,960 (+2) kDa, 31,147 (+189) kDa, and 31,344 (+386) kDa. The first peak corresponds to the unmodified form of 30,958 kDa, whereas the second, most abundant peak corresponds with the single-lipoylated form of 31,146 kDa. The third peak may be a double-lipoylated protein of 31,335 kDa.
A Complex Lipoate Utilization Pathway

primarily in the single lipoylated holo form, although the spectra suggest that some apoLipL and some double-lipoylated LipL may also be present (Fig. 2D). In our analysis of *B. subtilis* LipL evidence was found for modification of a secondary site that was not required for catalysis, which may be the larger species detected in the mass spectra of Fig. 2D.

The holo-LipL preparation was assayed for amidotransferase activity using lipoyl-GcvH as the lipoyl donor for modification of pyruvate dehydrogenase E2 subunit and was found to have good activity (Fig. 3A). However, DKL-A and lipoamide were inactive as substrates (Fig. 3A), indicating that interaction with the substrate lipoyl domain was required for catalysis. As expected, free lipoic acid was also not a LipL substrate.

**LplA1, LipL, and GcvH Are Required for a Lipoylation Relay—**

*In vitro* assays performed with the pure proteins showed that LplA1 modified GcvH but was unable to significantly modify E2<sub>BkdH</sub> (Fig. 3B). These results are similar to those observed in *in vitro* assays with *B. subtilis* LplJ (4) but are in contrast to *E. coli* LplA, an enzyme that modifies many different lipoyl domains (13). Modification of E2<sub>BkdH</sub> was only detected when both LipL and GcvH were present in the assay. Moreover, octanoyl-amidotransfer was reversible. LipL transferred the octanoyl moiety of pure [1-<sup>14</sup>C]octanoyl E2<sub>BkdH</sub> to GcvH, and the transfer required LipL (Fig. 3C). These data support that a lipoylation relay occurs during lipoic acid scavenging in *L. monocytogenes* and that two enzymatic activities are required for scavenging due to the specificity of the ligase.

**LplA1 Is Unable to Utilize Amide-bound Lipoic Moieties as Substrates—**

The inability of DKL-A and lipoamide at physiological conditions to provide lipoyl moieties to *E. coli* strains expressing LplA1 or LplA2 could be explained by an inability of the compounds to enter the test bacterium. To more directly test the functions of LplA1 or LplA2, we expressed the proteins in *E. coli* and attempted their purification. The solubility of LplA1 was improved by expression at low temperatures and use of an N-terminal Halo tag (Promega) (Fig. 2B). We also attempted to purify LplA2 but found it was insoluble when expressed in *E. coli* under a variety of conditions.

Lipoate-protein ligases require a lipoate moiety having a free carboxyl group to form the mixed anhydride intermediate, lipo-yl-adenylylate. One solution to this requirement would be to bypass lipoyl-adenylate formation by LplA1-catalyzed transfer of the lipo-yl moiety to a target protein as previously suggested (2). In this scenario LplA1 would have both ligase and amidotransferase activities. The possibility of transfer of lipoate from DKL-A or lipoamide to a GcvH target domain in *vitro* was directly tested with purified LplA1. When free lipoate was the substrate, lipo-ylation of GcvH was detected using both an anti-LA antibody and by mass spectrometry, whereas no lipoic acid attachment was seen when either DKL-A or lipoamide were tested as substrates (Fig. 4). This together with the evidence of the presence of free lipoate in the lipoamide and DKL-A preparations (see above) strongly suggested that *L. monocytogenes* contains a lipoamidase activity.

**Crude Extracts Assay of *L. monocytogenes* Contain Lipoamidase Activity—**

Given that neither *L. monocytogenes* LplA1 nor LplL were able to use DKL-A as a substrate, the most straightforward explanation for the prior observations was that *L. monocytogenes* encodes a lipoamidase activity that generates free lipoic acid by cleavage of the amide bond linking lipoate to the host peptides and thereby provides a substrate for LplA1. The only well characterized lipoamidase, a member of the Ser-Lys family of amidohydrolases, is found in *E. faecalis*, another Firmicutes bacterium that is a human pathogen and auxotrophic for lipoate (7). However, no recognizable lipoamidase orthologue is encoded in the *L. monocytogenes* genome. To determine whether *L. monocytogenes* contains a divergent lipoamidase activity, we added DKL-A to protein extracts of wild type *L. monocytogenes* and assayed release of free lipoate by GC-MS (Fig. 5A). The addition of *L. monocytogenes* protein extracts to DKL-A followed by extraction and silylation of the products resulted in a peak having the retention time and mass spectra of the expected silylated lipoate, the formation of which was dependent on the presence of extract. Free amino acids

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**FIGURE 3. Specificities of LplA1 and LipL.** A, Coomassie Blue staining (top image) or anti-lipoic acid Western blotting (bottom image) analyses of LipL reaction products after separation by native PAGE are shown. LipL was assayed for modification of E2b in the presence of one of the indicated acyl donors as shown: lipoyl-GcvH (lip-H), lipoyl-tripeptide (DKL-A), lipoamide (LD), or lipoic acid (LA). B, shown is an assay of ligation and transfer by LplA1 and LipL. [1-<sup>14</sup>C]Octanoate was used to detect protein modification by SDS-PAGE followed by autoradiography. C, LplA1 specificity for GcvH *in vitro* and amidotransfer of the 1-<sup>14</sup>C-octanoyl moiety from E2b to GcvH in the presence of LipL (± LipL) are shown.
were also produced from DK^{1}A. The lysine residue was completely silylated and eluted near lipoic acid (Fig. 5A), whereas the aspartate and alanine residues eluted earlier and were seen only when a lower starting temperature was employed (Fig. 5B). The major peaks from lipoic acid are the molecular ion of m/z 278 and the fragment of m/z 123 (Fig. 5C). A peak was found at m/z 155 indicating that the previous assignment (16) of this peak as a methyl ester fragment is incorrect. We also found strong peaks at m/z 73 and 75 that are characteristic TMS fragments (17). Consistent with what is observed with free fatty acids (17), the TMS derivative of lipoic acid yielded a more intense molecular ion peak than the methyl ester (16), allowing us to select for the m/z 278 species with good sensitivity.

To test if the lipoamidase activity was metal-ion dependent, the effects of EDTA on lipoamidase activity were tested, and the chelator was found to block formation of free lipoic acid. When EDTA-treated L. monocytogenes extracts were supplemented with various metal ions and lipoamidase activity was assayed by GC-MS, manganese ion proved to be the most effective ion, whereas magnesium ion was completely inactive (Table 2) (Fig. 5D). Cobalt and reduced iron ions gave intermediate activities.

These data suggest that L. monocytogenes, when located within eukaryotic host cells, relies on metal-dependent lipoamidase activity to liberate lipoate from lipoyl peptide substrates. The metal dependence of DK^{1}A cleavage indicates that the lipoamidase activity of L. monocytogenes is distinct from E. faecalis lipoamidase, consistent with the lack of an lipoamidase orthologue. To confirm that lipoamidase activity is independent of LplA1, LplA2, and LipL, we assayed extracts of mutants deficient in these enzymes. Lipoamidase activity was present in a strain lacking LplA1-catalyzed lipoic acid attachment was specific for modification of GcvH. This specificity engenders a novel gene product (or products) having lipoamidase activity.

**DISCUSSION**

In this study we define a novel pathway for scavenging of lipoic acid by L. monocytogenes (Fig. 6). Previous work has shown that LplA1, but not LplA2, is required for intracellular utilization of lipoic acid (2). Although direct in vitro comparison of the two enzymes was precluded by the insolubility of LplA2, LplA1 was able to complement E. coli with much lower levels of lipoic acid supplementation than was LplA2. Thus, LplA1 may have a higher affinity for lipoate than LplA2, although there are other possible reasons for the apparent lower scavenging ability of LplA2. However, the apparent high affinity of LplA1 for lipoate would seem important for growth within host cells where the levels of free lipoic acid are limited. We found that LplA1-catalyzed lipoic acid attachment was specific for modification of GcvH. This specificity engenders a requirement for amidotransfer of lipoyl moieties from GcvH to the 2-oxoacid dehydrogenases required for growth (18). Lipoyl

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**TABLE 2**

### Lipoamidase activity of L. monocytogenes extracts

The assays were performed as described under “Experimental Procedures,” and data are per mg of extract protein.

| Extract | Metal          | pmol/min |
|---------|----------------|----------|
| WT      | MnCl₂          | 146 ± 25 |
| lplA1   | MnCl₂          | 104 ± 51 |
| lplA2   | MnCl₂          | 172 ± 41 |
| lipL    | MgCl₂          | <1       |
| lplA1   | EDTA           | <1       |
| None    | MnCl₂          | <1       |

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**FIGURE 5. Lipoamidase activity and products of the reaction.** A, shown is a total ion chromatogram from GC-MS analysis of lipoamidase reaction products after silylation formed in extracts containing MnCl₂. The intensity was normalized and displayed as percent of the internal standard. Each chromatogram is offset by 10% from the next. The peaks are labeled as follows: 1, MOPS-TMS; 2, lysyl with three TMS adducts; 3, lipoyl-TMS; 4, hexadecanoyl-TMS; 5, octadecanoyl-TMS; 6, nonadecanoyl-TMS (internal standard); 8, GC-MS at reduced starting temperature of a lipoamidase reaction catalyzed by the extract of a lplA null mutant strain is shown. Peaks corresponding to silyl derivatives of alanine (1) and aspartate (2 and 3) are indicated. C, electron impact mass spectrum from GC-MS of lipoyl-TMS synthesized from pure lipoic acid is shown. The intensity was normalized and displayed as the percent of maximum. D, extracted 278 m/z ion chromatogram of silylated lipoamidase products formed by an extract of a lplA1 lplA2 strain of L. monocytogenes supplemented with the indicated metal ion is shown. Chromatograms (offset by 5%) are normalized based on internals standard peak area and displayed as relative percent of maximum.
transfer is catalyzed by the LipL amidotransferase and defines a new role for LipL amidotransferases in scavenging of lipoic acid. From previous studies in *B. subtilis*, it was clear that LipL was required for lipoic acid biosynthesis and was capable of catalyzing amidotransfer of octanoyl moieties. However, the advantages, if any, of the amidotransfer relay systems of *L. monocytogenes* and *B. subtilis* over the more direct pathways used by *E. coli* are unclear. We initially supposed that *L. monocytogenes* LipL might be involved in transfer of lipoyl groups from small lipoylated peptides to lipoyl domains. However, no such activity could be demonstrated. We report that LipL action is reversible, which opens up the possibility that transfer of lipoyl groups between 2-oxoacid dehydrogenases may be beneficial in adaptation to growth on different carbon sources. In this study we report that LipL is capable of lipoyl-amidotransfer between lipoyl domains, which are the only substrates present in *L. monocytogenes*.

Recently, a transposon insertion into the gene encoding the *L. monocytogenes* LipL ortholog was isolated. The strain was attenuated in its ability to grow in bile (19). Growth was not restored upon the addition of lipoic acid to the medium, which is surprising given the two lipoate protein ligases of *L. monocytogenes*. These results provide physiological evidence that LipL is required for lipoyl scavenging in *Listeria* and are consistent with our findings that lipoyl ligation is specific for GcvH, a specificity that engenders a requirement for LipL to activate the essential dehydrogenases (Fig. 4B). The reduced tolerance of the lipL mutant strain to acidic bile salts (19) is likely due to a decreased branched fatty acid content of the membrane phospholipids, which results when *L. monocytogenes* is deficient in lipoic acid (18).

Although much attention has been given to lipoic acid ligation, lipoamidase action is poorly defined. Lipoic acid is assembled covalently bound to cognate enzymes on the enzymes themselves (20), and yet lipoate-protein ligases are widely distributed. This suggests that lipoamidases, which cleave the amide bond that links lipoic acid to its cognate enzymes, must also be widely distributed. Early studies of *E. faecalis* found that lipoamidase activity was present in two separate fractions, suggesting there may be two enzymes (6). This was also seen in a later purification of lipoamidase (21). Jiang and Cronan (7) found only a single lipoamidase-encoding gene, but it is possible that other lipoamidase genes were not detected because their protein products have different specificities.

*L. monocytogenes* extracts contain significant lipoamidase activity. This activity is independent of *lplA1*, *lplA2*, and *lipL*,...
A Complex Lipoate Utilization Pathway

demonstrating that at least one additional gene is required for lipoic acid scavenging. The strong manganese ion dependence of this lipoamidase activity indicates hydrolysis is performed by a mechanism that differs from that of the metal-independent \textit{E. faecalis} enzyme lipoamidase (7). Note that recent evidence demonstrates under oxidative stress conditions manganese can functionally replace reduced iron in iron-dependent enzymes (22). Thus, bacteria can mitigate the oxidative stress defense that mammalian hosts use against infection (23). The use of manganese by the \textit{L. monocytogenes} lipoamidase may be a means to combat oxidative stress and also an adaptation to the iron limitation of the intracellular environment (23). It is clear from this study that more work is required to fully appreciate the diversity and abundance of lipoamidases, although the activity has been known for more than 50 years. However because the known enzymes are from pathogenic bacteria, biosafety considerations make enzyme discovery by fractionation of cell extracts highly problematical.

An interesting possibility is that a LipL relative could function as a lipoamidase by transferring the acyl group to water instead of to another lipoyl domain. We considered that \textit{L. monocytogenes} LipL may be capable of employing water as an acyl acceptor and, therefore, acting as a lipoamidase but did not see appreciable loss of acyl groups from lipoyl domains in the presence of LipL. However, it remains possible that this activity exists in one of the many uncharacterized clades of this protein family (see below). The likelihood of this possibility could be tested by engineering a lipoamidase from an amidotransferase, which would be essentially the reverse of peptide transfer by proteases (24).

Finally, from characterization of the LipL amidotransferases of \textit{L. monocytogenes} and \textit{B. subtilis}, we can now better predict the function of related proteins. Amidotransferases are members of the cofactor transferase family (Pfam entry PF03099) and are distinct from lipoic acid ligases (Fig. 7). Also, the LipL clade presented has a significant bootstrap value that defines the clade and its relationship to other clades (Fig. 7B). LipL orthologues can be found in all Firmicutes that use lipoic acid. Given that the gene distribution is somewhat independent of taxonomy, lipL was presumably distributed by ancient horizontal gene transfer events. The overall phylogeny of LipL and relatives combined with new functional information yields some interesting observations. In the LipL family, the more deeply branching clades also include LipL lipoamidotransferases, the LipM octanoyltransferase (12), and bipartite lipoate protein ligases (25). These enzymes do not have an attached accessory domain, although the bipartite lipoate ligases use a separate protein as the accessory domain. The related LipB octanoyltransferases (Fig. 7A) also do not have an accessory domain. This suggests the ancestral protein lacked an attached accessory domain, which would presumably be required for efficient ligase activity. The function of the ancestral protein is not clear, although a transferase active in modification of lipoyl or biotinoyl domains would be expected. This function is common to all character-

ized members of this family. The reversibility of this reaction is used by LipL to transfer modifications.

Another characterized transferase is present in the LplA family, the mammalian lipoamidotransferase (Fig. 7B). Lipoamidotransferase transfers lipoic acid from lipoyl-adenylate to lipoyl domains but unlike the bacterial ligases is unable to form the adenylate intermediate (26). However, we lack evidence demonstrating the physiological role of lipoamidotransferase. A lipoamidotransferase orthologue from yeast, LIP3, has been shown to be required for lipoic acid biosynthesis (27). Loss of LIP3 results in a yeast strain that accumulates lipoylated H protein but shows no lipoylation of 2-oxoacid dehydrogenases. This is the same result observed in \textit{B. subtilis} upon inactivation of \textit{lipL} (5). Because LIP3p and lipoamidotransferase are present in the same small clade (Fig. 7B), it appears that these proteins may represent another class of lipoamyl-amidotransferases.

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