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Understanding the Dynamics of Protein Lipoylation in Staphylococcus Aureus

Sarah C. Flury
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LOYOLA UNIVERSITY CHICAGO

UNDERSTANDING THE DYNAMICS OF PROTEIN LIPOYLATION IN
STAPHYLOCOCCUS AUREUS

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY
SARAH FLURY
CHICAGO, ILLINOIS
AUGUST 2018
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<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>PDH</td>
<td>Pyruvate dehydrogenase</td>
</tr>
<tr>
<td>OGDH</td>
<td>2-oxoglutarate dehydrogenase</td>
</tr>
<tr>
<td>GcvH</td>
<td>H subunit of the Glycine cleavage system</td>
</tr>
<tr>
<td>GcvH-L</td>
<td>H subunit of the glycine cleavage-like system</td>
</tr>
<tr>
<td>BCODH</td>
<td>Branched chain 2-oxoacid dehydrogenase</td>
</tr>
<tr>
<td>BCFA</td>
<td>Branched chain fatty acid</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>LA</td>
<td>Lipoic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptic soy agar</td>
</tr>
<tr>
<td>Gcs</td>
<td>Glycine cleavage system</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline and tween</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethane sulfonyl fluoride</td>
</tr>
<tr>
<td>AMP</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>CM</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>AnTet</td>
<td>Anhydrous tetracycline</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl-phosphate</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitro blue tetrazolium</td>
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ABSTRACT

*Staphylococcus aureus* is a commensal bacterium that also acts as an opportunistic pathogen. The pathogenicity of *S. aureus* has often been attributed to the wide range of virulence factors that the bacterium produces. While virulence factors do contribute a great deal, there is a growing field of research that aims to investigate the role of metabolism in bacterial virulence.

My project focuses on the necessity of a metabolic cofactor, lipoic acid. To ensure sufficient amounts of lipoic acid are available for enzyme activity, *S. aureus* has evolved two pathways to obtain the important nutrient. The lipoic acid salvage pathway is composed of two lipoic acid ligases, LplA1 and LplA2, that sequester environmental lipoic acid. The *de novo* biosynthesis pathway allows for the transfer of octanoic acid from an acyl carrier protein to GcvH. The octanoic acid is converted to lipoic acid, and LipL facilitates the transfer to the bacterial metabolic protein.

While a significant amount is known about these pathways from recent studies in our lab, some questions remain. So far, the Alonzo lab studied the function of LplA1 and LplA2 in the presence of free lipoic acid. While this is valuable in understanding ligase activity, it does not necessarily model the host environment in which there is limited free lipoic acid. A more prevalent substrate is lipoylpeptides. This led me to ask if LplA1 and LplA2 ligases can use lipoamide as a source of lipoic acid for protein lipoylation. Through biochemical lipoylation assays, we discovered that
these enzymes are unable to directly use lipoylated peptides; instead, *S. aureus* harbors lipoamidase functions that allow for generation of the free acid from its peptide-bound form.

My thesis also interrogates the activity of LipL. Previously attempts to purify LipL and study it in a lipoylation assay led to no observable function of the protein, and complementation of a ΔlipL strain with *lipL* did not restore of growth defects. I evaluated the *lipL* gene sequence and determined that the LipL coding sequence in NCBI yields a truncated non-functional gene. Use of an extended LipL in biochemical assays showed that LipL can utilize free lipoic acid alone or in conjunction with LplA1 to lipoylate a limited range of substrates.
CHAPTER ONE
LITERATURE REVIEW

Introduction

*Staphylococcus aureus.*

*S. aureus* is a Gram-positive commensal bacterium that is commonly found in the nasal cavity of approximately 30% of the population as well as on the skin of an additional 20% of the population\(^1\text{-}\text{3}\). The probability that an individual is a carrier of *S. aureus* increases if they have recently been hospitalized or if the individual works in a hospital\(^2\text{-}\text{4}\). This is due to the frequency with which the bacterium is found in a hospital setting. However, the bacterium is also found throughout the community, leading to both hospital- and community-acquired infections. *S. aureus* can spread easily in both environments from direct person-to-person contact, contact with a contaminated surface, or, less frequently, from aerosolized bacteria after coughing or sneezing\(^5\).

Those with chronic infections, such as those affecting the skin or lung, diabetes, cancer or an otherwise weakened immune system, are at higher risk for *S. aureus* infection\(^2\text{-}\text{5}\). However, *S. aureus* often causes infection in otherwise healthy individuals when the skin is breached due to a cut or burn\(^2\). The bacteria can also gain access into the body through surgical procedures, either by infection of the surgical site or by contamination of a medical implant. Once inside the human body, *S. aureus* can cause a variety of different diseases, including skin and soft tissue infections, endocarditis, osteomyelitis, pneumonia and sepsis\(^5\). Depending on the type of infection,
treatment can vary from drainage of an abscess, surgical removal of infected tissue or surgical implant, and/or the use of antibiotics².

While antibiotics are often useful in treating bacterial infections, *S. aureus* is known to acquire resistance to beta-lactam antibiotics such as penicillin, ampicillin, and methicillin, and has shown a rise in resistance to glycopeptides such as vancomycin, which is commonly used to treat MRSA ²,³,⁶. The ability of *S. aureus* to become resistant to a variety of antibiotics provides insight into how adept this bacterium is at evasion of killing by pharmacological intervention. *S. aureus* also combats the host immune system through an arsenal of different virulence factors⁷–⁹. At least 40 different genes have been identified and are predicted to play a role in virulence, with potentially more yet to be determined ¹,¹⁰.

Inside the host, the bacterium not only needs to evade the host immune system and combat any medical treatment, but also must be able to access limiting nutrients to survive¹¹,¹². The work described in this thesis fits into a growing field of research that is interested in understanding how components of bacterial metabolism are related to virulence¹². My project specifically examines the ability of *S. aureus* to obtain a critical metabolic cofactor, lipoic acid.

**Bacterial Requirement of Lipoic acid**

As with any living organism, it is necessary for bacteria to metabolize nutrients and produce energy in the form of ATP ¹³. To allow for this process, bacteria must acquire nutrients that supply carbon, nitrogen and essential cofactors that enable enzyme function. One of the critical cofactors is lipoic acid, which is required for the function of metabolic enzyme complexes such as pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (OGDH), branched chain 2-oxo-acid dehydrogenase (BCODH), and the glycine cleavage system (Gcs)¹⁴. Two of these enzymes function as a component of glycolysis and the TCA cycle. At the end of
the glycolysis pathway, PDH acts to produce acetyl-CoA from pyruvate, which serves as the starting substrate of the TCA cycle or is utilized in the process of fermentation\textsuperscript{15}. OGDH is an enzyme complex of the TCA cycle, which produces succinyl-CoA from $\alpha$-ketoglutarate\textsuperscript{16,17}. Of the remaining lipoyl enzyme complexes, BCODH is responsible for the breakdown of branched amino acids\textsuperscript{18} and facilitates the biosynthesis of branched chain fatty acids, whereas Gcs is responsible for the degradation of glycine\textsuperscript{17,19}. Each one of these enzyme complexes is composed of several subunits. In the case of PDH, OGDH and BCODH, there are three subunits, E1, E2, and E3\textsuperscript{20,21}. In the case of the GcvH (and in \textit{S. aureus} GcvH-L) of the glycine cleavage system, the subunits are H, P, L and T \textsuperscript{14}. The lipoylated subunits in these proteins are the E2 and H subunit respectively. These subunits contain what is known as a lipoyl domain, which characteristically contains charged amino acids, proline, alanine and, importantly, a conserved lysine\textsuperscript{22}. This lysine is the location of the lipoic acid attachment by an amide bond. Each of these enzymes contains at least one lipoyl domain; however, PDH has been shown to have several in some species\textsuperscript{23}, suggesting variability in the quantity of lipoic acid needed for enzyme activity of certain complexes. Once bound to the lipoyl domain, lipoic acid aids in enzyme function in two ways: the lipoic acid is able to bind intermediates produced by the enzymes, and it also is able to aid in the movement of substrates between subunits of the enzyme \textsuperscript{14} (Figure 1).
Figure 1. Lipoylation of E2 and H Subunits of Metabolic Protein Complexes. A) Lipoylated BCODH. The E2 subunit is lipoylated and also has the catalytic activity of transferring the acyl group from the reduced lipoic acid to coenzyme A. B) lipoylated acetoin dehydrogenase complex. This protein is similar to that of PDH, however it uses acetoin as a substrate. The E2 subunit is lipoylated and has the catalytic activity of transferring an acetyl group from the lipoic acid to coenzyme A. This enzyme is not present in *S. aureus*. C) Lipoylated glycine cleavage complex. The H subunit of GcvH is lipoylated and does not contain catalytic activity itself. (Reprinted with permission from Spalding and Prigge<sup>14</sup>)

### Lipoic Acid Salvage Pathway

The requirement for lipoic acid is a trait of phylogenetically diverse species including bacteria like *S. aureus, Escherichia coli, Bacillus subtilis*, and *Listeria monocytogenes*, as well as eukaryotic species from yeast to mammals<sup>14,24–26</sup>. One of the ways that some of these organisms are able to lipoylate their enzymes is through a salvage pathway. Generally, the salvage pathway allows for the organism to sequester lipoic acid, which can be found in two different forms in the environment<sup>14</sup>. The first form is the free acid in which there is a free hydroxyl that allows for
direct attachment to the conserved lysine by an amide bond (Figure 2). The more prevalent form of lipoic acid is the lipoylated peptide or protein\textsuperscript{27,28}. In this case, lipoic acid is already attached to a lysine and therefore needs to be hydrolyzed before use.

The enzymes responsible for the salvage process are lipoic acid ligases. However, the exact salvage mechanisms vary from species to species. The most direct salvage pathway is that of \textit{E. coli} in which a single enzyme, LplA, is able to lipoylate any lipoyl domain\textsuperscript{14,30,31,19,20}. While the ligation reaction is carried out by a single enzyme, it is composed of two steps. The first step requires ATP to form a lipoyl-AMP intermediate, which is then attacked by the lipoyl domain lysine and the formation of an amide bond between the conserved lysine and the lipoic acid\textsuperscript{20} (Figure 3).

In \textit{L. monocytogenes} additional enzymes are required in the salvage pathway. There are two ligases, LplA1 and LplA2, which are utilized at different points in the bacterial life cycle\textsuperscript{31,34,35}. LplA1 is required while the bacterium is living intracellularly and is able to use lipoylated
peptides as a substrate, while LplA2 is only able to use free lipoic acid as a substrate and is not required for intracellular growth. It has been shown that the reason LplA1 is able to use lipoylated proteins as a substrate, is through coordinated activities with a bacterial-encoded lipoamidase. In *L. monocytogenes*, once the lipoic acid is accessible, the ligases function similarly to that of LplA in *E. coli* in that they require ATP to allow for the formation of a high energy lipoyl-AMP intermediate, which then allows for the lipoylation of a lipoyl domain. However, in this pathway, LplA1 is used to lipoylate an H subunit of the glycine cleavage system protein instead of an E2 subunit directly. After the lipoylation of GcvH, LipL is used to transfer the lipoic acid to the E2 subunit (Figure 3). These final steps are similar to that of the salvage pathway in *B. subtilis*. LplJ is able to utilize free lipoic acid to lipoylate GcvH, the lipoic acid is then transferred to E2 subunits.

Finally, a third category of salvage exists in mammals as well as *T. acidophilum*, in which a separate enzyme is first required for the formation of the lipoyl-AMP intermediate, followed by a second enzyme that transfers lipoic acid from lipoyl-AMP to the E2 subunit (Figure 3). In *T. acidophilum* the enzymes responsible for this stepwise process are LplA and LplB.
Figure 3. Lipoic Acid Salvage Pathways. The lipoic acid salvage pathways are involved in acquiring lipoic acid from the environment for lipoylation of E2 and H subunits. In *E. coli*, depicted in the middle pathway, LplA directly lipoylates all E2 subunits. In *L. monocytogenes* there is an additional step in which LplA1 is able to lipoylate the H subunit of the glycine cleave system. LipL is then able to transfer the lipoic acid o the E2 subunit. Finally, in the mammalian-like pathway, the E2 subunit is directly lipoylated, but two enzymes are required. (Adapted from Storm and Muller31)

Salvage Pathway in *S. aureus*

*S. aureus* is unique in the fact that it contains two lipoic acid ligases, LplA1 and LplA2, that can independently lipoylate E2 and H subunits with environmental lipoic acid25. This is unusual because, as we have seen with other organisms’ salvage pathways, there does not seem to be a requirement for multiple enzymes unless they aid in the other’s function24. In the case of *S. aureus*, both LplA1 and LplA2 have distinct specificities for lipoylation of either E2 or H subunits25 (Figure 4). LplA1 lipoylates the two glycine cleavage system proteins, GcvH and GcvH-L, in addition to limited lipoylation of the E2 subunit of OGDH 38. LplA2 lipoylates all E2 subunits, including PDH, OGDH, and BCODH, as well as the H subunit of GcvH-L. The enzymes are also differentially expressed in vitro and in vivo25. LplA2 is not expressed in vitro, however both LplA1 and LplA2 seem to be expressed in vivo 25. The divergent targets and different regulatory patterns of these ligases suggest that *S. aureus* has used complex lipoic acid
acquisition strategies to promote its survival in a range of different environments\textsuperscript{39}. However, we do not know if the ligases are able to use substrates other than free lipoic acid. As mentioned, free lipoic acid is not present in high quantities within the host environment\textsuperscript{28}. Therefore, it would be beneficial for the bacterium to have a mechanism to lipoylate proteins using more prevalent sources of lipoic acid.

**Figure 4. Lipoic Acid Salvage and *de novo* Biosynthesis Pathways in *S. aureus*.** The salvage pathway of *S. aureus* is composed of two enzymes, LplA1 and LplA2. LplA1 is able to lipoylate the H subunits of GcvH and GcvH-L as well as the E2 subunit of OGDH using free lipoic acid. LplA2 is able to lipoylate the E2 subunits of PDH, OGDH and BCODH, as well as the H subunit of GcvH-L. LplA1 and LplA2 can also attach octanoic acid to the H subunits of GcvH and GcvH-L respectively. The *de novo* biosynthesis pathway starts with an octanoic acid on an acyl carrier protein (ACP), which is then transferred to GcvH by LipM. LipA is converts the octanoic acid on the GcvH to lipoic acid, which is then transferred to an E2 subunit of PDH, OGDH, or BCODH by LipL.\textsuperscript{25}

**Lipoic Acid Biosynthesis Pathways**

An alternate to the salvage pathway is the *de novo* biosynthesis pathway in which lipoic acid is produced by the organism itself\textsuperscript{31}. This is useful in environments that have a low concentration of exogenous lipoic acid. This process uses a byproduct of fatty acid biosynthesis, octanoic acid bound to an acyl carrier protein (ACP), which is converted to lipoic acid by the
addition of two sulfur groups, and then transferred to either an H or E2 subunit\textsuperscript{40–42}. As with the salvage pathway, there is variability in the exact steps of the de novo biosynthesis pathway between species. In \textit{E. coli}, this process is fairly simple and occurs in two steps. First, LipB transfers octanoic acid from the acyl carrier protein and attaches it to the lysine of any lipoyl domain \textsuperscript{20,33}. From there, LipA adds to sulfur atoms to generate a dithiolane ring, converting octanoic acid to lipoic acid (Figure 5). In \textit{B. subtilis}, there is an additional step that first requires the transfer of the octanoic acid from an acyl carrier protein to GcvH by LipM \textsuperscript{30,36}. Then, the octanoic acid is transferred to the lipoyl domain of any E2 subunit by LipL. LipL transfers the octanoic acid by creating an intermediate in which the octanoic acid is attached to a sulfur of a cysteine residue on LipL. From there, the octanoic acid is attached to the lipoyl domain through an amide bond. Once attached to the lipoyl domain, lipoic acid is generated by LipA-mediated sulfur insertion, resulting in the lipoylation of the E2 subunits (Figure 5).
**Figure 5. Lipoic Acid Biosynthesis Pathways.** Lipoic acid biosynthesis occurs when octanoic acid is transferred from an acyl carrier protein (ACP) to an E2 or H subunit and subsequently converted to lipoic acid by LipA. In *E. coli* LipB is responsible for octanoic acid transfer and LipA converts octanoic acid to lipoic acid. In *B. subtilis*, LipM transfers the octanoic acid from ACP to GcvH where it is then transferred again to the E2 subunit by LipL and finally converted to lipoic acid by LipA. (Adapted from Storm and Muller31)

**Role of LipL in Lipoic Acid de novo Biosynthesis and Pathogenesis of *S. aureus***

The biosynthesis pathway in *S. aureus* is similar to that of *B. subtilis* in that GcvH is used as an intermediate substrate. Octanoic acid is transferred from the acyl carrier protein to GcvH by LipM 25 (Figure 4). However, a distinct feature of this pathway in *S. aureus*, compared to that of *B. subtilis*, is that the octanoic acid is first converted to lipoic acid on GcvH by LipA. This is followed by LipL-mediated transfer of lipoic acid from GcvH to an E2 subunit similar to that of LipL in *B. subtilis* and *L. monocytogenes*, although this has not been directly tested. The work in this thesis focuses in part on the function of LipL.

Previous members of the lab conducted a preliminary study to determine the role of LipL in pathogenesis. A Δ*lipL* mutant strain was used in a systemic infection model in mice (Figure
When bacterial load in the heart and kidney were evaluated, the ΔlipL mutant was found to be dramatically attenuated compared to that of wild type *S. aureus*. This phenotype seems to be distinct from simply the disruption of the *de novo* biosynthesis pathway as a ΔlipA mutant shows attenuation only in the heart and not the kidneys during systemic infection. These data indicate that lipL is required for bacterial persistence in a systemic infection model and highlights the importance of studying LipL activity in the context of bacterial pathogenesis.

**Figure 6. A ΔlipL Mutant is Attenuated in a Systemic Infection Model.** Swiss webster mice were infected by retro orbital injection with either a wildtype or ΔlipL strain. After 96 hours, organs were harvested and resulting bacterial burden was evaluated by calculating CFU/mL. A ΔlipL mutant is attenuated in the kidney and heart.

**Pta/ AckA Pathway**

An unusual genetic feature of the *S. aureus* de novo biosynthesis enzymes is that the gene immediately upstream of lipL, is the phosphotransacetylase gene, *pta*. In many other species of bacteria, the *pta* gene is either linked to the *ackA* gene of the Pta/AckA pathway in an operon, or the two genes are separated, but monocistronic. The Pta/AckA pathway is involved in ATP production in bacteria during carbon overflow metabolism and fermentation. Normally, when bacteria are in the presence of their preferred carbon source, they are able to import that carbon source and perform glycolysis to make pyruvate. Pyruvate is then brought into the TCA cycle where bacteria use it to produce energy in the form of ATP and NADH. However, when a
bacterium is in an environment where there is a surplus of a carbon source, the bacterium goes through the acetate switch. In this case, the TCA cycle is halted, at least in part, by the global regulator CcpA, which inhibits citrate synthase. The acetate switch then involves the feeding of acetyl CoA, the product of glycolysis, into the Pta/AckA pathway instead of the TCA cycle. The Pta/AckA pathway is comprised of Pta, which is responsible for converting acetyl CoA into acetyl phosphate thereby recycling the CoA that is required for glycolysis and allowing the bacterium to continue to metabolize the excess carbon. The pathway is completed by AckA, which is responsible for using acetyl phosphate to generate ATP, producing acetate in the process. This pathway is reversible, allowing the bacterium to reassimilate acetate and use it as a carbon source for the TCA cycle. While less ATP is produced in the Pta/AckA pathway, the speed at which ATP is produced is much faster than in the TCA cycle. This is beneficial to the bacterium because it can generate free CoA and, therefore, continue to use glycolysis and take up carbon sources from the environment at an increased rate. While bacteria may have other metabolic pathways that allow for the production of acetate, the Pta/AckA pathway is the dominant one.
Figure 7. Pta/AckA Pathway. The starting substrate of the Pta/ AckA pathway is acetyl-CoA, a product of glycolysis. Pta is able to convert acetyl-CoA to acetyl-phosphate, which is then used by AckA to produce acetate and, more importantly, ATP. This process is reversible, so the acetate produced can later be converted back into acetyl-CoA for further use by the cell. (Adapted and reprinted with permission from Wolfe et al 50).

**Role of Pta in *S. aureus* Bacterial Growth and ATP Production.**

Sadykov et al. previously examined the role of *pta* in the growth of *S. aureus* 51. In this study, the authors examined the effects of *pta* on bacterial growth and survival as well as bacterial energy production. Through the use of a Δ*pta* and Δ*ackA* mutants, Sadykov et al. determined that interfering with the Pta/AckA pathway leads to decreased levels of growth in the presence of glucose 51. They also showed that when the pathway was inhibited, there was an increase in cell death when compared to wildtype cells (Figure 8). Due to this growth defect as well as the increased cell death in the Δ*pta* mutant, they hypothesized that an *S. aureus* mutant with a defective Pta/AckA pathway would produce ATP at a slower rate and in reduced levels. However, when the levels of ATP in wildtype, Δ*pta*, and Δ*ackA* strains were evaluated, wildtype cells actually had a reduced level of ATP compared to those of the Pta/AckA pathway mutants.
Furthermore, it was observed that there were higher levels of NAD and NADH present in the cell when this pathway was inhibited\textsuperscript{52}. This implies that the growth defect of the Δpta mutant is not due to the inability of the strain to sufficiently produce ATP but is instead due to other downstream effects of the inhibition of the pathway.

**Figure 8. Deletion of pta or ackA Results in a Bacterial Growth Rate Defect.** Bacterial strains, wildtype, Δpta, and ΔackA were grown in TSB, a rich medium, and the optical density and pH of the culture were recorded every hour. Open shapes indicate the OD of each strain while the closed shapes indicate the pH of the culture. (Reprinted with permission from Sadykov et al.\textsuperscript{51}).

**Potential Connection Between Pta and LipL in S. aureus.**

Azul Zorzoli noted an interesting potential relationship between Pta and the lipoic transferase, LipL, when conducting complementation studies of a ΔlipL mutant. Azul noted that the expression of lipL alone was not sufficient to restore the growth defect of a ΔlipL mutant in RPMI\textsuperscript{25} (Figure 9). This medium does not contain any free lipoic acid and therefore the ΔlipL mutant strain would not be expected to grow due to its inability to transfer lipoic acid from GcvH to essential E2 proteins. It was surprising to find that complementation of the ΔlipL mutant growth defect is only achieved when both pta and lipL are present. These data suggest a possible link between pta and lipL.
The possibility that LipL requires additional enzymes to function is supported by studies of the lipoic acid salvage enzyme, LplA, in *T. acidophilum* 37. The researchers found that the lipoyl transferase, LplA, of *T. acidophilum* was missing a critical C terminal domain that rendered it unable to perform lipoyl transfer. However, with the addition of a secondary protein known as LplB, exposure of appropriate substrate binding domains was achieved, and transfer was completed 37. This two-enzyme requirement for lipoic acid ligation is also present in mammals as described above 31. This highlights the potential importance of secondary proteins in promoting functionality of a lipoyl transferase. Therefore, it is possible that the requirement of pta-lipL for complementation of a ΔlipL mutant growth defects is akin to *T. acidophilum* LplA, where LipL of *S. aureus* requires the presence of a secondary enzyme, possibly Pta, to confer functional activity.

An alternative possibility is that the gene for lipL may be incorrectly annotated leading to generation of incorrect complementation vectors. According to its NCBI annotation, there is a
gap of approximately 70 nucleotides between \textit{pta} and \textit{lipL} (Figure 10). However, in the upstream noncoding region between \textit{pta} and \textit{lipL}, there is another potential start codon that is only 2 base pairs downstream from the TAA stop codon of the \textit{pta} gene, yet in-frame with the remainder of the predicted \textit{lipL} coding sequence.

\textbf{Figure 10. Potential Start Sites of \textit{lipL}}. A) The end of the \textit{pta} sequence in red, and the beginning of \textit{lipL} in green according to the annotation in NCBI. B) The end of the \textit{pta} sequence in red and the beginning of \textit{lipL} in blue, starting at the upstream start codon.

A BLAST analysis of either the annotated LipL amino acid sequence or the LipL amino acid sequence including translation from this novel putative upstream start codon (extended LipL) was conducted and compared to the LipL amino acid sequence of \textit{B. subtilis}. From the alignment it was noticed that there is additional amino acid sequence identity between full length \textit{B. subtilis} LipL and the extended LipL of \textit{S. aureus} (Figure 11). When blasting the annotated LipL amino acid sequence, I observed that the regions of high amino acid identity start far into the \textit{B. subtilis} LipL sequence at amino acid 30. This gap in sequence identity is reduced when comparing to the extended LipL amino acid sequence, where the homology starts at amino acid 12 and 13. Thus, truncation of the \textit{lipL} gene in our complementation constructs, due to an incorrectly annotated open reading frame, might explain why complementation of a \textit{ΔlipL} mutant
was unsuccessful and could indicate that *pta* is not required for function of LipL, but that instead the *lipL* coding sequence upstream of the current *lipL* start site is required.

Figure 11: Sequence Homology Between LipL in *S. aureus* and *B. subtilis*. A) BLAST results comparing the sequence of LipL in *B. subtilis* to that of LipL in *S. aureus* as it is annotated in NCBI. B) BLAST results comparing the sequence of LipL in *B. subtilis* to that of LipL in *S. aureus* using the upstream start codon (extended LipL). Green font shows the sequence homology using the sequence as annotated in NCBI while blue font shows the sequence homology when the upstream start site is used.

Concluding Remarks

*S. aureus* is a commensal bacterium that is present on approximately 30% of the population. While generally harmless to those that carry the bacterium in their noses or on their skin, *S. aureus* can also act as an opportunistic pathogen when it is able to pass through the barrier of the skin and enter into the host. The bacteria can cause a variety of diseases, including some that...
mild, such as skin infection, and others that are more severe and life-threatening like sepsis. While *S. aureus* pathogenesis has been studied in the context of its ability to produce a variety of virulence factors and its ability to develop resistance to antibiotics, understanding the relevance of bacterial metabolism to virulence has been relatively neglected. In this project, the requirement of the metabolic cofactor, lipoic acid, as a critical nutrient during infection is studied.

It has previously been established that *S. aureus* uses two pathways to meet its requirement for lipoic acid. The first is the salvage pathway in which two enzymes, LplA1 and LplA2, are able to use free lipoic acid from the environment to lipoylate the bacterial metabolic enzymes. The second pathway is the *de novo* biosynthesis pathway, in which lipoic acid is derived from octanoic acid on an acyl carrier protein and then transferred to bacterial metabolic enzymes by LipM, LipA, and LipL. While these pathways have been studied in the lab, a major question remains: How is *S. aureus* able to use substrates other than free lipoic acid to lipoylate its metabolic enzyme subunits? Free lipoic acid is very limiting in the host; therefore, bacteria must either use alternative lipoyl sources, such as lipoylated peptides, or generate lipoic acid from octanoic acid. We attempted to address this overarching question in two different ways.

We first asked if LplA1 and LplA2 have lipoamidase or amidotransferase functions that would allow for the direct use of lipoylated peptides from the environment, or if *S. aureus* encodes a secondary protein with lipoamidase function. This was tested with the use of purified LplA1, LplA2, and E2 subunit proteins, in the presence of lipoylated lysine in biochemical assays and with the use of mass spectrometry analysis.

We then sought to analyze the function of LipL and its possible connection to neighboring Pta. This was accomplished by testing the function of extended lipL in genetic complementation
experiments and biochemical lipoylation assays. We also analyzed the connection between Pta and LipL through assessment of complex formation between the two proteins. Overall, the assessment of lipoic acid requirement in *S. aureus* is beneficial due to the essential requirement of the cofactor. Understanding in totality the ability of the bacterium to acquire or produce this cofactor could enable us to inhibit these pathways and therefore lead to treatment of this potentially life-threatening organism 53.
CHAPTER TWO
MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Strains used in all experiments are listed in Table 1. *E. coli* strains were grown in Lysogeny Broth (LB), whereas *S. aureus* strains were grown in Tryptic Soy Broth (TSB) or Roswell Park Memorial Institute medium (RPMI) with 1% casamino acids. TSB or RPMI were also supplemented with branched chain fatty acids (BCFA) -10.8 mM isobutyric acid, 9.2 mM 2-methylbutyric acid, and 9 mM isovaleric acid with 10 mM sodium acetate - to alleviate the requirement of lipoic acid for mutant stains when necessary\(^5^4\). Bacteria in liquid culture were grown at 37°C, shaking at 220 rpm at an angle or in a 96-well plate at 37°C. Growth under other conditions is specified in some of the following methods. Where necessary antibiotics were supplemented at the following concentrations: 100 µg/ml of ampicillin (AMP), 10 µg/ml of chloramphenicol (CM), 1 µg/ml of anhydrous tetracycline (AnTet).
Table 1. List of Strains Used in This Study.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Description</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td><em>S. aureus</em> USA300 Strain LAC. Plasmid cured.</td>
<td>USA300 LAC</td>
</tr>
<tr>
<td>DH5α</td>
<td><em>E. coli</em> strain used to propagate plasmid</td>
<td>DH5α</td>
</tr>
<tr>
<td>DC10B</td>
<td><em>E. coli</em> strain used to propagate plasmid</td>
<td>DC10B</td>
</tr>
<tr>
<td>RN4220</td>
<td>Restriction deficient <em>S. aureus</em>. Used for plasmid propagation</td>
<td>RN4220</td>
</tr>
<tr>
<td>RN9011</td>
<td>Contains the SaPI1 Integrase in the pRN7023 plasmid</td>
<td>RN9011</td>
</tr>
<tr>
<td>Δpta</td>
<td>LAC with an in-frame deletion of <em>pta</em></td>
<td>FA-S1675</td>
</tr>
<tr>
<td>Δpta+ pta</td>
<td>LAC with an in-frame deletion of <em>pta</em>, complemented with pJC1111-<em>pta</em></td>
<td>FA-S1668</td>
</tr>
<tr>
<td>Δpta+ lipL</td>
<td>LAC with an in-frame deletion of <em>pta</em>, complemented with pJC1111-<em>exlipL</em></td>
<td>FA-S1693</td>
</tr>
<tr>
<td>ΔlipL</td>
<td>LAC with an in-frame deletion of <em>lipL</em></td>
<td>FA-S1169</td>
</tr>
<tr>
<td>ΔlipL+pta</td>
<td>LAC with an in-frame deletion of <em>lipL</em>, complemented with pJC1111-<em>lipL</em></td>
<td>FA-S1190</td>
</tr>
<tr>
<td>ΔlipL+pta</td>
<td>LAC with an in-frame deletion of <em>lipL</em>, complemented with pJC1111-<em>lipL</em></td>
<td>FA-S1258</td>
</tr>
<tr>
<td>ΔlipL+exlipL</td>
<td>LAC with an in-frame deletion of <em>lipL</em>, complemented with pJC1111-<em>exlipL</em></td>
<td>FA-S1257</td>
</tr>
<tr>
<td>ΔlplA1</td>
<td>LAC with an in-frame deletion of <em>lplA1</em></td>
<td>FA-S841</td>
</tr>
<tr>
<td>ΔlplA2</td>
<td>LAC with an in-frame deletion of <em>lplA2</em></td>
<td>FA-S837</td>
</tr>
<tr>
<td>ΔlplA1 ΔlplA2</td>
<td>LAC with an in-frame deletion of <em>lplA1</em> and <em>lplA2</em></td>
<td>FA-S1754</td>
</tr>
<tr>
<td>ΔgcvH</td>
<td>LAC with an in-frame deletion of <em>gcvH</em></td>
<td>FA-S1038</td>
</tr>
<tr>
<td>ΔlplA1 ΔlplA2 ΔgcvH</td>
<td>Gene replacement mutant (*ΔgcvH:*kan) transduced into double <em>lplA1</em> and <em>lplA2</em> mutant</td>
<td>FA-S1739</td>
</tr>
<tr>
<td>ΔlipL ΔlplA1</td>
<td>LAC with an in-frame deletion <em>lplA1</em> and <em>lipL</em> replaced with the kan cassette</td>
<td>FA-S977</td>
</tr>
<tr>
<td>ΔlplA1 ΔlplA2 ΔlplA Δlpm</td>
<td><em>lipM</em> transposon mutant (<em>lipM:erm</em>) transduced into Δ<em>lplA1</em> Δ<em>lplA2</em> Δ<em>lplA</em> Δ<em>lplL</em> quadruple mutant</td>
<td>FA-S1476</td>
</tr>
<tr>
<td>6x-His-GcvH</td>
<td>pET15b encoding 6x-His-GcvH, transformed into Δ<em>lplL</em>:kan <em>LysY</em></td>
<td>FA-S1357</td>
</tr>
<tr>
<td>6x-His-GcvH-L</td>
<td>pET15b encoding 6x-His-GcvH-L, transformed into Δ<em>lplL</em>:kan <em>LysY</em></td>
<td>FA-S1383</td>
</tr>
<tr>
<td>6x-His-OGDH</td>
<td>pET15b encoding 6x-His-OGDH, transformed into Δ<em>lplL</em>:kan <em>LysY</em></td>
<td>FA-S1363</td>
</tr>
<tr>
<td>6x-His-PDH</td>
<td>pET15b encoding 6x-His-PDH, transformed into Δ<em>lplL</em>:kan <em>LysY</em></td>
<td>FA-S1359</td>
</tr>
<tr>
<td>6x-His-LplA1</td>
<td>pET15b encoding 6x-His-LplA1, transformed into <em>LysY</em></td>
<td>FA-S1284</td>
</tr>
<tr>
<td>6x-His-LplA2</td>
<td>pET15b encoding 6x-His-LplA2, transformed into <em>LysY</em></td>
<td>FA-S1278</td>
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<tr>
<td>6x-His-LipL</td>
<td>pET15b encoding 6x-His-LipL, transformed into <em>LysY</em></td>
<td>FA-S1277</td>
</tr>
<tr>
<td>6x-His-exLipL</td>
<td>pET15b encoding 6x-His-exLipL, transformed into <em>LysY</em></td>
<td>FA-S1547</td>
</tr>
<tr>
<td>6x-his-Pta</td>
<td>pET15b encoding 6x-His-Pta, transformed into <em>LysY</em></td>
<td>FA-S1467</td>
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</table>
### Table 2. List of Primers Used in This Study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>NEWlipLcompSOE2</td>
<td>ATTTACTCGCTAAATCCATGGGTTTCACTCTCCTTCTA</td>
</tr>
<tr>
<td>NEWlipLcompSOE3</td>
<td>TAGAAGGAGAGTGAAACCATGGATTTAGCGAGTAAA</td>
</tr>
<tr>
<td>NEWlipLcompSOE4</td>
<td>ATATGCTGAC (SalI) CTATTGCAATTTGATCTATCATT</td>
</tr>
<tr>
<td>ptaSOE1</td>
<td>ATATGGTACC (KpnI) AGAAACAAATATAATCAAGATG</td>
</tr>
<tr>
<td>ptaSOE2</td>
<td>CTGCCTAAATCCATCCTCCTGTAATA</td>
</tr>
<tr>
<td>ptaSOE3</td>
<td>TATTACAGGAGGATTGATTAGCGAG</td>
</tr>
<tr>
<td>ptaSOE4</td>
<td>ATATGAGCTC (SacI) CTTCACCTTTTTAAAGCATG</td>
</tr>
<tr>
<td>Nthis-LipLlongF</td>
<td>ATATCATATG (Nde1) GATTTAGCGAGTAATATTTA</td>
</tr>
<tr>
<td>Nthis-LipLlongR</td>
<td>ATATGGATCC (BamHI) CTATTGCAATTTGATCTATCATTT</td>
</tr>
<tr>
<td>ptaN/C-F</td>
<td>ATATCATATG (Nde1) GCTTTAAATGTATTAA</td>
</tr>
<tr>
<td>ptaN-R</td>
<td>ATATCTCGAG (XhoI) TTAGTAAAAGGCTTGC</td>
</tr>
</tbody>
</table>

**Molecular Genetic Techniques**

To isolate chromosomal DNA from *S. aureus*, overnight cultures were inoculated in 5ml of TSB. The following day, the cultures were spun down at 15,000 rpm for 3 minutes. Supernatant was discarded, and bacterial pellets were resuspended in 200 µl TSM (50 mM Tris, 0.5 M sucrose, 10 mM MgCl₂, pH 7.5). 2.5 µl of lysostaphin (2 mg/ml in 0.5M Tris, pH 8.0) was added and incubated for 15 minutes at 37°C. Bacteria were pelleted and the protocol for the Wizard Genomic DNA purification kit by Promega was used to complete the genomic DNA isolation.

Plasmids were isolated from *E. coli* with the use of QIAGEN mini and midi preps kits and their respective protocols. Additional preliminary steps were taken to isolate plasmids from *S. aureus*. Overnight cultures were inoculated in 5 ml of TSB (mini prep) or 120 ml TSB (midi prep). The following day the cultures were spun at 4000 rpm for 10 min. Supernatants were discarded and the bacterial pellet was resuspended in either 400 µl TSM (mini prep) or 4 ml...
TSM (midi prep) and 20 µl (mini prep) or 200 µl (midi prep) lysostaphin (2 mg/ml in 0.5M Tris, pH 8) was added. This was then incubated at 37°C for 10 (mini prep) or 30 min (midi prep). After incubation, the bacteria were pelleted by centrifugation according to the appropriate protocol. The remaining steps of the mini and midi prep were performed according to the QIAGEN protocol. All polymerase chain reactions were performed using Phusion High-Fidelity DNA polymerase and the corresponding protocol.

**E. coli Competent Cell Preparation**

*E. coli* cells were grown in 3ml of LB, overnight at 37°C shaking. Cultures were then subcultured in LB at a 1:55 dilution in a final volume of 112 ml of LB. The subculture was then grown for 2.5 hours or until an OD600 of 0.3 was reached. The culture was chilled on ice for 10 minutes and then centrifuged for 10 minutes at 4000 rpm. The supernatant was discarded, and the pellet was resuspended in 10 ml of TBF1 (30 nM KOAc, 100 mM RbCl₂, 10 mM CaCl₂, 50 mM MnCl₂, 15% glycerol, pH 5.8 with 0.2 M acetic acid), and chilled on ice for 10 minutes. The bacteria were pelleted again by centrifugation at 4000 rpm for 5 minutes. The pellet was resuspended in 1 ml of TBF2 (10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl₂, 15% glycerol, pH 6.5 with KOH). The competent bacteria were then aliquoted, 100 µl per tube, and stored at -80°C.

**E. coli Heat Transformation**

*E. coli* competent cells were thawed at room temperature for 5 minutes. 50 µL of the competent cells were then combined with 5 µl (approximately 500 ng) of plasmid and set on ice for 30 minutes. Cells were placed in a water bath at 42°C for 45 seconds, and then returned to ice for 2 minutes. 250 µL of Super Optimal broth with Catabolite repression (SOC) medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM
glucose) was added to the cells and the culture was allowed to recover at 37°C shaking for two hours. 100 µL of the bacterial suspension was plated on LB plates containing the appropriate antibiotic for selection. Plates were then placed in the incubator at 37°C overnight to allow for transformants to grow.

**S. aureus Electrocompetent Cell Preparation**

*S. aureus* cells were grown in 5 ml of TSB, overnight at 37°C with shaking. Overnights were then subcultured in 30 ml TSB at a 1:100 dilution. Subcultures were grown to an OD600 of 0.5 (approximately 3 hours depending on the growth rate of the strain) at 37°C. The bacteria were then pelleted at 8000 rpm for 10 minutes. Resulting pellets were resuspended in 30 ml of 10% glycerol. The bacteria were pelleted by centrifugation at 8000 rpm for 10 minutes and resuspended in 30 ml of 10% glycerol. This process was repeated once more with resuspension of the bacterial pellet in 15ml of 10% glycerol and a final centrifugation step. Finally, the pellet was resuspended in 3 ml of 10% glycerol and 100 µL aliquots were stored at -80°C.

**S. aureus Transformation by Electroporation**

Competent cells were thawed at room temperature for 5 minutes. 50 µL of competent cells were then combined with 5 µl (approximately 1-2 µg) of plasmid DNA followed by incubation at room temperature for 30 minutes. Cells were transferred to an electroporation cuvette and were pulsed under the following conditions: 1800 V, 10 µF, 600 Ω, in a 2 mm cuvette. Immediately after pulsing, 750 µL of TSB was added to the cuvette. Cells were then placed at 30°C or 37°C depending on the requirement of the plasmid and allowed to recover for 1.5 hours. 100 µL of the bacterial suspension was plated on TSB plates containing the correct antibiotic for selection of transformants. Cultures were then spun down in a microcentrifuge tube at 1300 rpm for 2 minutes. Supernatant was removed except for 100 µL, in which the pellet was
resuspended. The remaining 100 µL were then plated. Plates were incubated overnight to allow for the growth of transformants.

**Generation of In-Frame Deletion Mutants**

Primers amplifying 500 base pairs upstream and downstream of the pta gene were generated. Each region was amplified by PCR using wild type *S. aureus* genomic DNA as a template and the ptaSOE1/ptaSOE2, ptaSOE3/ptaSOE4 primer pairs. After amplification, fragments were spliced together using a Splicing by overhang extension (SOEing) reaction yielding a final product of approximately 1000 base pairs. The amplicon was then digested using KpnI and SacI in parallel with the digestion of the pIMAY plasmid. The digested amplicon and plasmid were then ligated together. pIMAY containing the amplicon was transformed into DC10B *E. coli* and transformants were selected based upon their resistance to chloramphenicol. PCR amplification was used to confirm successful ligation. Plasmids were purified from DC10B and electroporated into wildtype *S. aureus* followed by selection on TSA+ CM plates grown at 30°C due to the temperature sensitivity of the plasmid in *S. aureus*. For mutagenesis, plasmid recombination was induced by diluting colonies in TSB and plating on TSB+ CM plates followed by incubation at 37°C. A second recombination event and subsequent plasmid loss was induced by growth at 30°C in liquid TSA lacking antibiotic. Finally, cultures were diluted 1:1000 and plated on TSB+ AnTet to counter-select against any bacteria that still contained the plasmid. Strains with successful gene mutations were first identified by screening for AnTet resistance and CM sensitivity followed by PCR.

**Bacteriophage Mediated Transduction**

Phage transduction was used to generate complementation strains Δpta+pta, Δpta + lipL, Δpta+ pta-lipL and ΔlipL + exlipL. This process was also used to generate the mutant strains
\( \Delta lplA1 \Delta lplA2 \Delta gcvH::kan \) and \( \Delta lplA1 \Delta lplA2 \Delta lipA \Delta lipM::erm \Delta lipL \) The plasmid used for complementation was the pJC1111 integrative plasmid\(^5\). This plasmid contains the \textit{S. aureus} pathogenicity island 1 phage attachment site that allows for the integration into SapI1 site\(^5\). This allows for single copy, stable complementation. All genes were expressed under the control of \( P_{HELP} \) promoter to ensure constitutive expression of the gene\(^5\). Primers were designed to allow for the amplification of the \( P_{HELP} \) promoter as well as the gene for complementation (NewlipL comp SOE2, NewlipLcompSOE3/NewlipLcompSOE4). The templates for these sequences were the pIMAY plasmid and \textit{S. aureus} chromosomal DNA respectively. After PCR to amplify these fragments, a SOEing reaction was used to splice the sequences together. The amplicon was then digested and ligated into pJC1111. The resulting plasmid was transformed into DH5\( \alpha \) and plated on LB+ AMP plates. The plasmid was purified from successful transformants and subsequently electroporated into RN9011. This strain allows for the integration into the chromosome at the SapI1 site. The RN9011 strain was then used as a donor strain to package phage with DNA. To accomplish this, the RN9011 strain was used to inoculate a 3 ml overnight culture in TSB/LB (1:1) with the addition of 5mM CaCl\(_2\) and 5mM MgSO\(_4\). The following day, the overnight was subcultured 1:100 in the same medium and incubated for 2.5 hours or until an OD\(600\) of 0.3. 500 \( \mu \)l of culture was incubated with \( \phi 11 \) phage in TMG (10 mM Tris pH 7.5, 5 mM MgCl\(_2\), 0.01% gelatin) for 30 minutes. Bacteria and phage were mixed with 3 ml of CY top agar (casamino acids 3 g/L, yeast extract 3 g/L, NaCl 6 g/L, agar 7.5 g/L, and BCFA if needed), which was then added to TSA plates. Plates were incubated at 30°C overnight, and the following day top agar containing confluent plaques was removed by scraping with a spatula and resuspended in 2 ml TMG. Phage-agar suspensions were extensively vortexed and centrifuged at 13,000 rpm for 15 minutes. Supernatant containing bacteriophage was filtered using a 0.2 \( \mu \)m filter, three times.
Phage were stored at 4°C. Recipient strains were grown overnight in 20 ml of TSB/LB with 5 mM CaCl₂. Overnights were then pelleted by spinning at 13,000 rpm for 15 minutes. Pellets were resuspended in 3 ml TSB/LB with 5 mM CaCl₂. 500 µL of bacterial suspension were diluted in a 10-fold series and 100 µL of phage was added to each dilution. Bacteria-phage mixtures were incubated at room temperature for 30 minutes, inverting the tubes every 10 minutes. 40 mM sodium citrate was then added to each tube and the mixture was incubated for 30 minutes, again inverting the tubes every 10 minutes. Bacteria-phage mixtures were washed twice by spinning at 13,000 rpm for 3 min and resuspending in 500 µl TSB/LB with 10 mM sodium citrate. Bacteria were then resuspended in 250 µl, of which 200 µl were plated on TSB/LB + 10 mM sodium citrate + 0.1mM cadmium chloride and incubated at 37°C. Selection of integrants was based on growth on selective medium after incubation for 24-28 hours and confirmed by PCR.

**Growth Curves**

A 96-well plate was filled with 200 µL of RPMI supplemented with BCFA per well. Wells were inoculated with each strain of bacteria (in triplicate) and grown overnight at 37°C shaking. The following day the 96 well plate was centrifuged at 3700 rpm for 10 minutes to pellet bacteria. The cells were washed in RPMI medium three times. After the final wash, pellets were resuspended in 200 µL of RPMI and 2 µL of washed cells were used to inoculate 200 µl of medium for the growth curve (RPMI or RPMI+ BCFA. OD at 550nm from each well was then measured every hour for a minimum of 9 hours.
**Generation of 6x- Histidine Tagged Protein Expression Plasmids**

Most constructs were previously generated by Irina for use in her thesis\textsuperscript{29,38}. Primers were designed for the amplification of $pta$ and $lipL$ with the addition of a 6x-histidine tag at the N terminus (Nthis-LipLlongF/Nthis-LpLlongR and ptaN/C-F/ptaN-R). PCR was used to amplify these constructs, which were then digested and ligated into the pET-15b plasmid. Following the ligation, the plasmid was transformed into the LysY $E. coli$ strain. However, if the plasmid contained the gene for an E2 or H subunit, the plasmid was transformed into a $\Delta lipA$ LysY $E. coli$ strain to prevent the lipoylation of the protein once expressed. To confirm protein expression, trial inductions were performed. Cultures of the pET-15b containing strains were grown overnight in LB+CM at 37°C shaking. The following day overnights were subcultured 1:1000 in a total volume of 4 ml and incubated again for 3 hours at 37°C shaking. Cultures were then divided, 3ml and 1ml. 1 mM IPTG was added to the 3 ml culture to induce protein expression, while the remaining 1 ml was left uninduced. All cultures were then incubated for an additional 3 hours. From each culture 500 µL was removed and centrifuged at 13,000 rpm for 10 minutes. The supernatant was removed, and the remaining pellet was resuspended in 100 µL of 2x SDS PAGE sample buffer + BME (1.11mL 0.9M Tris-HCl pH6.8, 0.16g SDS, 2ml 100% glycerol, 0.2mL 14.7M BME, 0.5mL 0.5M EDTA, 4mg Bromophenol blue, H$_2$O to 10ml) . Samples were then boiled for 10 minutes and 20 µL was run on a 12% SDS PAGE gel.

Uninduced and induced lysates were compared to determine the colony with the highest protein expression. This colony was then cultured and frozen and used for future protein purification.
Protein Purification of Lipoic Acid Biosynthesis and Salvage Enzymes

LysY I^Q strains that had confirmed protein expression were used to inoculate overnight cultures of 20 ml in LB + AMP. The following day overnights were used to subculture 2 L of LB+AMP at a dilution of 1:100, followed by incubation for 3 hours at 37°C or until reaching an OD600 of 0.3. Protein expression was induced by the addition of 0.1 mM IPTG and cultures were incubated overnight at 16°C with shaking. The following day, cultures were centrifuged at 8,500 rpm for 10 minutes. Supernatant was discarded, and the remaining pellet was stored overnight at -80°C. Pellets were then thawed at 37°C and resuspended in lysis buffer (50 mM Tris HCl, 300 mM NaCl) with the addition of 25 mM imidazole, 1 mM dithiothreitol (DTT), and 1 mM phenylmethane sulfonyl fluoride (PMSF). Bacteria were sonicated on ice for 30 minutes in 20 second intervals. The sonication was done using a Branson S-450A large tip sonicator set at a constant rate of 0.8 seconds per pulse and an output of 340W. The debris from lysed bacteria pelleted by centrifugation at 11,000 rpm for 30 minutes. Supernatant was filtered using a 0.45 µm filter. 1ml of nickel-NTA resin was washed and equilibrated in lysis buffer containing 25 mM imidazole and 1mM DTT. Resin was then added to the filtered supernatant and the mixture was incubated for 1 hour at 4°C with rocking and manual agitation every 15 minutes to ensure adequate mixing. Following the incubation, resin was allowed to settle into a column, where it was washed with 50 ml of lysis buffer containing 100 mM imidazole and 1mM DTT. Protein was then eluted in lysis buffer containing 500 mM imidazole and 1mM DTT. Elutions were combined and sequential dialysis was performed using 10kDa MWCO snakeskin dialysis tubing to remove all imidazole. The protein was first dialyzed at 4°C in 300 ml of lysis buffer containing 100 mM imidazole and 1 mM DTT for 3 hours, followed by lysis buffer containing 25 mM imidazole and 1 mM DTT for 3 hours, and then lysis buffer containing 1 mM DTT.
overnight. The following day, buffer was replaced again with lysis buffer containing only 1 mM DTT for a minimum of 3 hours. Proteins were concentrated by spinning in an Amicon Ultra centrifugal filter column with a 30000 MW cutoff membrane. Protein concentrations were determined with the use of a bicinchoninic acid kit (BCA) with the addition of iodoacetamide to reduce DTT interference in the assay. Proteins were stored at -80°C.

Protein Purification of apo E2 and H Subunits of Lipoylated Enzymes Complexes

ΔlipA LysY I° stains with confirmed protein expression were used to inoculate 20 ml overnight cultures in LB+AMP. The following day the overnight cultures were then used to start subcultures in 2L of LB+AMP at a 1:100 dilution. Subcultures were incubated at 37°C shaking for 15 hours or until reaching an OD600 of 0.3. 0.5 mM IPTG was added to the culture and incubated for 3 hours at 37°C to induce protein expression. Cultures were centrifuged at 8500 rpm for 10 minutes at 4°C to pellet the bacteria, which were then stored at -80°C overnight. The remaining steps used to purify H and E2 subunits were the same as those described above to purify the salvage and biosynthesis enzymes.

Generation of Whole Cell Lysates

Strains were grown overnight in 5 ml of RPMI + BCFA at 37°C shaking. The following day overnights were subcultured 1:1000 (60 µL into 6 ml) into RPMI + BCFA or other supplemented medium as indicated. Cultures were grown for 9 hours at 37°C with shaking. The OD600 of each culture was measured, and cultures were normalized to the culture with the lowest OD. Cultures were pelleted and resuspended in 250 µl of PBS followed by transfer to tubes compatible with the bead beater containing 250 µl of glass beads. Tubes were placed in the bead beater and run at 5.0 m/sec for 20 seconds followed by incubation on ice for 5 minutes and run again in the bead beater at 4.5 m/sec for 20 seconds. Lysates were centrifuged at 13,000 rpm
at 4°C for 15 minutes. 90 µl of the supernatant from each culture were removed and mixed with 45 µl of 2x SDS sample buffer with BME.

**Immunoblot Procedure**

Samples were boiled for 10 minutes at 100°C and centrifuged for 1 minute at 13000 RPM. 10 µl of sample was loaded onto two separate 12% SDS PAGE gels and each gel was run at 120 V for approximately 3 hours. One gel was washed 3 times for 5 minutes in water and stained with Coomassie blue stain for 1 hour followed by destaining overnight in water. The second gel was transferred to an Immobilon-PSQ (pore size 0.2µm) membrane for 1 hour at 1 Amp followed by blocking in 5% BSA in TBST overnight at 4°C. The following day, primary antibody (rabbit anti lipoic acid antibody) was used at a 1:7500 dilution and added to the membrane for 1 hour at room temperature. The membrane was then washed in TBST 3 times for 15 minutes each. Secondary antibody (goat anti rabbit, AP conjugated) was used at a 1:5000 dilution and added to the membrane for 1 hour at room temperature. The membrane was washed again 3 times for 15 minutes each in TBST. Finally, the membrane was developed using NBT/BCIP in AP buffer to detect lipoylated proteins.

**Lipoylation Assays**

E2 or H subunits were added to reactions at 20 µM concentration while salvage or de novo biosynthesis pathway enzymes were added at a concentration of 1 µM. Proteins were combined with 6 mM ATP, 1 mM DTT, 1 mM MgCl₂, 2.4 mM lipoyl source (lipoamide, DK¹-A, or free lipoic acid), and assay buffer (50 mM Tris- HCl, 300 mM NaCl, pH 8) in a final reaction volume of 50 µl. Reactions were incubated in a thermomixer at 37°C with shaking at 600rpm for two hours. 500 ng of the lipoyl protein from each reaction was resolved on two separate 12%
SDS PAGE gels. One gel was stained with Coomassie blue dye and the other was used in an immunoblot as described for the cell lysate procedure.

**Pta-LipL Complex Formation Assays**

10 µM of each protein (PDH, GcvH, LipL, and LplA1) were combined into a microcentrifuge tube in a final volume of 100 µl. These protein combinations were incubated for one hour at 37°C in a thermomixer. Each individual protein was run at the same concentration on the FPLC prior to running the combined protein reactions. The proteins were run on the FPLC using the Superdex 3.2/300 column at a flow rate of 0.04 mL/min in assay buffer (50 mM Tris-HCl, 300 mM NaCl, pH 8). UV sketches from each run were collected and overlaid to determine any differences between peaks generated from individual protein runs and those of the combined proteins.

**Animal Infections**

Overnight cultures in TSB+ BCFA, were subcultured 1:100 in 15 ml of TSB+ BCFA and grown at 37°C to an OD of 1.0. Cultures were then centrifuged for 5 minutes at 3700 rpm and washed two times in PBS. 2 ml of culture was added to 8 ml of PBS and normalized to an OD600 of 0.32 (1x10^8 CFU/ml). 4-week-old female Swiss Webster mice were used in these experiments. For systemic infections, mice were anesthetized with 2,2,2- tribromoethanol at 250 mg/kg and then infected with 100 µL PBS containing 1x10^7 CFU of bacteria by injection into the retro-orbital sinus. 96 hours post infection, mice were euthanized, and kidneys and hearts were isolated. In the skin infection model, PBS containing 1x10^7 CFU of bacteria was mixed 1:1 with Cytodex beads. After mice were anesthetized, they were shaved and 200µl of the bacterial and bead mixture was injected into each flank. After 120 hours, mice were euthanized, and abscesses
were isolated. Tissue was homogenized and plated onto a TSA+BCFA plates and incubated overnight at 37°C. The following day CFU was enumerated.
CHAPTER THREE

EXPERIMENTAL RESULTS

Introduction

*Staphylococcus aureus* is a commensal bacterium as well as an opportunistic pathogen\(^2\). As a pathogen, *S. aureus* causes disease that varies in severity from skin and soft tissue infections to sepsis. *S. aureus* pathogenesis is often attributed to the variety of virulence factors that it produces; however, there is also evidence to suggest that metabolism of the bacterium plays a significant role in infection\(^{12}\). The focus of my thesis is on the vital metabolic cofactor lipoic acid, and the processes by which *S. aureus* is able to acquire it. It is known that *S. aureus* uses two pathways to lipoylate its proteins\(^{25}\). The first is the salvage pathway, where free lipoic acid from the environment is directly attached to *S. aureus* E2 subunits. This pathway is composed of two ligases, LplA1 and LplA2 that are able to lipoylate different E2 and H subunits targets\(^{38}\). The second lipoic acid acquisition pathway is the *de novo* biosynthesis pathway, by which lipoic acid is generated from octanoic acid\(^{25}\). This process starts with an octanoic acid moiety attached to an acyl carrier protein of fatty acid biosynthesis. The octanoic acid is then transferred to GcvH where it is converted to lipoic acid. Finally, lipoic acid is transferred to E2 subunits by the amidotransferase LipL. While these pathways are now well understood due to the work of Azul Zorzoli and Irina Laczhovich, a few questions regarding lipoic acid biosynthesis and salvage remain unanswered.

My thesis focuses on LplA1, LplA2, and LipL, and seeks to determine how *S. aureus* uses substrates other than free lipoic acid to lipoylate its metabolic enzyme complexes. Within
the environment of the host, free lipoic acid is a limited nutrient while lipoylpeptides are more prevalent\textsuperscript{28}. It would therefore be extremely beneficial for \textit{S. aureus} to have a mechanism to extract lipoic acid from these lipoylpeptides. I first tested if LplA1 and LplA2 can use lipoylpeptides as a lipoic acid substrate. I also aimed to study the function of LipL. While former members of the lab had previously attempted to study LipL, they were unable to identify a function for the enzyme. My studies aimed to test why this was the case.

**Utilization of Lipoylpeptides by Salvage Pathway Enzymes LplA1 and LplA2**

Previously, Irina Laczkovich determined that the LplA1 and LplA2 ligases are able to use free lipoic acid to differentially target E2 and H subunits of \textit{S. aureus} for lipoylation \textsuperscript{38}. Both LplA1 and LplA2 were also shown to ligate octanoic acid to H subunits. Together, these studies provided a great deal of insight into the function of the \textit{S. aureus} salvage enzymes; however, they did not determine whether or not LplA1 or LplA2 have the capacity to use alternative lipoyl substrates for protein lipoylation. During an infection, \textit{S. aureus} is unlikely to encounter free lipoic acid since the free acid is limiting in the host environment. However, amide-bound lipoic acid is likely to be in great abundance. Therefore, to determine if LplA1 and LplA2 can use alternative lipoyl substrates, lipoylation assays were conducted with lipoamide as the source of lipoic acid and GcvH-L as the target of lipoylation. Lipoylation of GcvH-L was assessed by immunoblot as well as Coomassie staining of SDS-PAGE gels. We detected lipoylation of GcvH-L by immunoblot in reactions containing LplA1, but not LplA2 (Figure 12).
Figure 12. LplA1 is Able to Lipoylate GcvH-L in the Presence of Lipoamide. Lipoylation reactions performed to evaluate the use of lipoamide by LplA1 and LplA2 to lipoylate GcvH-L. Reactions were run on a 12% SDS-PAGE gel and stained with Coomassie blue (bottom panel) or used in an immunoblot (top panel). Lipoylated proteins were probed with rabbit anti lipoic acid primary antibody in the immunoblot. The first lane in the gel corresponds to the ladder, and the subsequent lanes contain the reactions described in the chart directly above.

It has previously been observed that lipoylation of a lysine on GcvH or GcvH-L can be detected on a Coomassie stained SDS-PAGE gel, as a shift downward in apparent molecular weight, due to a loss of the positive charge on the lipoyl domain lysine. However, this band shift was not observed in lipoylation reaction with LplA1 despite detectable lipoylation by immunoblot (Figure 12). Together, these data initially suggested that LplA1 uses lipoamide to target GcvH-L for lipoylation and that attachment is unlikely to be on the lipoyl domain lysine.

The conserved lysine in GcvH-L is flanked by a serine and a threonine, two amino acids that contain a free hydroxyl group (Figure 13A). We wondered if the lipoamide was being attached to one of these amino acids given that the attachment of lipoamide directly to a lysine is not biochemically feasible. To explore this possibility, reactions mixtures were sent for mass spectrometry analyses at the University of Illinois at Chicago. We attempted to detect a mass shift of 188, which is indicative of lipoic acid attachment, on a lysine, serine or threonine. The
mass spectrometry analysis identified GcvH-L with good confidence; however, it was noticed that the peptide that contained the conserved lysine was detected at a much lower rate in the lipoylated reaction and a mass shift of 188 was not identified. It was therefore suggested that we resubmit a larger quantity of reaction to be analyzed as it was assumed that the modified peptide was not being detected.

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Matched peptides shown in bold red.

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Figure 13. Lipoylated GcvH-L was Not Detected by Mass Spectrometry. Results from mass spectrometry preformed at University of Illinois at Chicago. A) Detected peptides (in red) from lipoyl GcvH-L. B) Detected peptides from a non-lipoylated GcvH-L control. C) Frequency at which each peptide was detected in each sample (S1 corresponds to the lipoylated GcvH-L, sequence shown in A, and S2 corresponds to the non-lipoylated control sequence shown in B).

During the preparation of the samples for an additional round of mass spectrometry analysis, the LplA1 reaction was run on an SDS-PAGE gel and stained with Coomassie blue (Figure 14A). Unlike the first run (Figure 12), the reaction now had a double band corresponding to a fraction of GcvH-L that appeared to lipoylated on the conserved lysine (lower MW band
shift), and a fraction that was un-lipoylated. Reactions were repeated and analyzed by SDS-PAGE and Coomassie blue staining. The repeated reactions now showed full lipoylation of GcvH-L on the lysine, by LplA1, as indicated by complete band shift (Figure 14B). This observation lead us to wonder if our lipoamide stock was contaminated with free lipoic acid. To test this notion, new highly pure (>98%) lipoamide was purchased and lipoylation assays were repeated. With pure lipoamide, we no longer observed significant lipoylation of GcvH-L by LplA1 or LplA2, indicating that these enzymes likely are not using lipoamide as a substrate (Figure 14C). This was further confirmed in biochemical assays containing an additional source of lipoamide, DK\(^\alpha\)-A. DK\(^\alpha\)-A is a tripeptide that contains lipoic acid attached to a lysine through an amide bond (Figure 15). We tested the ability of LplA1 and LplA2 to use DK\(^\alpha\)-A to lipoylate GcvH and GcvH-L and found that neither LplA1 nor LplA2 could lipoylate either GcvH or GcvH-L, further supporting the conclusion that the salvage pathway enzymes do not have lipoamidase or amidotransferase functions.
Figure 14. Lipoylation with Lipoamide is a Result of Free Lipoic Acid Contamination. Lipoylation reactions containing LpIA1, LpIA2, GevH-L and lipoamide. A) shows reactions run at a higher volume (10µl). B) shows reactions repeated with old lipoamide stock, and C) shows reactions with new highly pure lipoamide stock. Reactions were resolved on a 12% SDS PAGE gel and either stained with Coomassie blue (A, B and lower panel of C) or used in an immunoblot with rabbit anti lipoic acid antibody. Each gel has a ladder in the first lane and subsequent lanes are reactions.

Figure 15. DK\(^{\alpha}\)A Cannot be Directly Used by LpIA1 or LpIA2 to Lipoylate GevH and or GevH-L. Lipoylation reactions containing DK\(^{\alpha}\)A and LpIA1 and LpIA2. Reactions were resolved on a 12% SDS PAGE gels and either stained with Coomassie blue (lower panel) or used in an immunoblot with rabbit anti lipoic acid primary antibody (top panel). No ladder is shown, each lane is a reaction containing the proteins in the chart above.
**S. aureus Produces a Lipoamidase, that Allows for the Use of Lipoylpeptides by LplA1 and LplA2**

While we have shown that LplA1 or LplA2 are unable to directly use lipoylpeptides as a source of lipoic acid, we reasoned that *S. aureus* must still possess a means to use alternative lipoyl substrates. This is because in growth curve assays performed by Irina, we observed that lipoic acid auxotrophs are able to grow in medium supplemented with DK\(^{-}\)A\(^{38}\). Because DK\(^{-}\)A was the only possible source of lipoic acid in these assays, the bacterium must be able to use it in some way to allow for growth. To further examine this possibility, using a biochemical approach, we generated cell lysates from \(\Delta{lipA}\ \Delta{lipM}\ \Delta{lipL}\ \Delta{lplA1}\ \Delta{lplA2}\), a strain that is unable to lipoylate any of its proteins, and added it to lipoylation reactions containing LplA1 or LplA2 and DK\(^{-}\)A. We then assessed protein lipoylation of GcvH and GcvH-L. We found that in the presence of cell lysate, LplA1 and LplA2 are able to lipoylate GcvH and GcvH-L respectively (Figure 16). Thus, while LplA1 and LplA2 alone do not have lipoamidase or amidotransferase activity, a lipoamidase produced by *S. aureus*, is able to liberate free lipoic acid from lipoylpeptide sources for attachment by LplA1 and LplA2.

![Figure 16. In the Presence of Crude Cell Lysate, LplA1 and LplA2 are Able to Utilize DK\(^{-}\)A.](image-url)

Lipoylation assays in the presence of crude lysate from a \(\Delta{lipA}\ \Delta{lipM}\ \Delta{lipL}\ \Delta{lplA1}\ \Delta{lplA2}\) strain as well as DK\(^{-}\)A. Reactions were run out on a 12% SDS PAGE gel and either stained with Coomassie blue (lower panel) or used in an immunoblot with mouse anti lipoic acid primary antibody (top panel). No ladder is shown, each lane is a reaction containing the proteins as shown directly above in the chart.\(^{38}\)
*lipL* is Incorrectly Annotated in NCBI

Previously in the lab, the function of *lipL* was examined; however, several problems were encountered. The first was that recombinant LipL protein seemed to be nonfunctional and, therefore, LipL could not be studied in lipoylation assays. Also, in the evaluation of *lipL* with respect to bacterial growth in broth, we showed that a ∆*lipL* mutant could only be complemented by *lipL* when its neighboring gene *pta* was included in the complementation plasmid (Figure 9). This complementation phenotype is interesting because it sheds light on the unusual orientation of *pta* directly upstream of *lipL*. Furthermore, we identified a secondary start codon for *lipL* two base pairs downstream of the *pta* open reading frame (Figure 10). Together, this information led us to hypothesize that the *lipL* is currently incorrectly annotated in NCBI.

To test if *lipL* was incorrectly annotated, we first generated an extended *lipL* construct, which began at the upstream start codon. This construct was then used to complement a ∆*lipL* mutant, followed by assessment of growth and protein lipoylation. In growth assays, we found that the ∆*lipL*+ex*lipL* complementation strain grew equivalently to wild type *S. aureus* and that lipoylation was fully restored (Figure 17). These results support the idea that ex*lipL* is the correct gene sequence for LipL and that we had previously been using a truncated gene, and that the N terminal sequence is critical for protein function.
Figure 17. An Extended lipL Construct Restores Wildtype Levels of Growth to a ΔlipL Mutant.
Evaluation of growth of lipL mutants and complement strains in RPMI medium, which lacks free lipoic acid and therefore eliminates function of the salvage pathway.

The effective complementation of a ΔlipL mutant using extended lipL in growth curves indicated the exlipL is likely the correct open reading frame that encodes functional LipL protein. This was further confirmed by purification of exLipL and use in lipoylation assays. exlipL was used to generate recombinant 6x-Histidine tagged exLipL, which was subsequently purified. A reaction including LplA1 and GcvH with free lipoic acid was performed in order to generate lipoyl-GcvH, which was then directly used to evaluate exLipL amidotransferase activity. These reactions evaluated the ability of exLipL to transfer lipoic acid from lipoyl-GcvH to PDH (Figure 18). Immunoblots showed that addition of exLipL led to lipoylation of PDH and therefore the enzyme is functional. From this set of experiments, we can conclude that exlipL is the correct coding sequence of the lipL gene due to its ability to complement a ΔlipL mutant and its production of functional protein with apparent amidotransferase activity. For the rest of the study, lipL will refer to the functional sequence including the upstream start codon.
Figure 18. Ex-LipL is Functional and Allows for the Lipoylation of PDH. Lipoylation assays used to confirm the correct length of LipL. Reactions containing either truncated LipL used previously in the lab or newly purified extended LipL (ex-LipL) were resolved on a 12% SDS PAGE gel and used in an immunoblot with rabbit anti lipoic acid primary antibody (top panel). The first lane corresponds to the ladder, each subsequent lane is a reaction containing the proteins a shown directly above in the chart.

The Role of *pta* in *S. aureus* Growth and Lipoylation Profile

*S. aureus* has an uncommon gene arrangement with *lipL* wherein *pta*, encoding Pta, a component of the Pta/AckA pathway, is encoded in the same operon. Because there are only two base pairs between these two genes, it is likely that they are co-transcribed and their proteins are co-translated (Figure 10). This genetic architecture may imply some relationship between the two proteins. While *pta* has previously been investigated in *S. aureus* (Figure 8), I continued to evaluate the role of Pta in the context of lipoylation and its relationship to LipL. To do this, a Δ*pta* mutant and its complementation strain were generated by Wei Ping Teoh, a postdoc in the lab; and these strains were used to assess growth and lipoylation profiles. It has previously been shown that a Δ*pta* mutant in *S. aureus* has a significant growth defect (Figure 8). This defect in
growth rate is also observed for the mutant that was generated for this study and was restored in the \( \Delta pta + pta \) mutant, suggesting that it was in fact due to the mutation in the \( pta \) gene (Figure 19). Of interest, a \( \Delta pta + lipL \) strain still shows a growth defect; however, less so than that of the \( \Delta pta \) mutant. This indicates some functions of LipL may also be disrupted in a \( \Delta pta \) mutant.

Figure 19. Deletion of \( pta \) Results in a Growth Rate Defect. Evaluation of growth of \( pta \) mutants and complement strains in RPMI medium, which lacks free lipoic acid and therefore eliminates function of the salvage pathway.

Indeed, there is a decrease in the amount of lipoylation observed in a \( \Delta pta \) mutant when compared to wild type and \( \Delta pta + pta \) is unable to complement this defect. Only when a \( \Delta pta \) mutant was complemented with \( lipL \) were lipoylation levels restored to wild type levels. This conclusively demonstrates that the mutation in \( pta \) secondarily disrupts the function of \( lipL \) (Figure 20).
Figure 20. \( \Delta pta + pta \) Does Not Complement Lipoylation Defects. Lipoylation profiles of strains that were either grown in A) RPMI+BCFA or B) RPMI+BCFA+LA for 9 hours. Cell lysates were collected and 10 µl were resolved on a 12% SDS PAGE gel, which was then used for an immunoblot with rabbit anti lipoic primary antibody. In A and B, the first lane corresponds to the ladder and the following lanes correspond to the strains as listed above.

Lipoylation Ability of LipL

LipL Function in Conjunction With LplA1

In my previous studies of LipL amidotransferase activity, I used lipoyl-GcvH directly from a reaction that contains LplA1. To control for the presence of this additional enzyme in LipL reactions, I set up a more extensive set of lipoylation reactions to confirm whether or not the LplA1 was contributing to LipL lipoylation of PDH. Any reaction containing LplA1, free lipoic acid and GcvH were expected to show lipoylation of GcvH based on the known function of LplA1 as a ligase with specificity for GcvH. Lipoylation of PDH was only anticipated in a reaction containing LplA1, GcvH, LipL and PDH, as LplA1 would lipoylate GcvH, then allow LipL to transfer the lipoyl moiety from GcvH to PDH. Interestingly, this was not the case as additional reactions showed the lipoylation of PDH (Figure 21). Reactions containing LplA1, LipL and PDH, showed lipoylation of PDH regardless of whether or not GcvH was present. To
further validate this observation, I set up another set lipoylation reactions containing free lipoic acid, PDH, LipL, and LplA1. When LplA1 and LipL were included together we observed lipoylation of PDH, whereas LplA1 and LipL individually were unable to perform the lipoylation reaction (Figure 22).

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**Figure 21. PDH is Lipoylated in the Presence of LplA1 and LipL.** Lipoylation assays were resolved on a 12% SDS PAGE gel, which was then used in an immunoblot with rabbit anti lipoic acid primary antibody. The first lane corresponds to a ladder and subsequent lanes correspond to reactions contains proteins as listed above in the chart. * mark each band on the immunoblot indicating lipoylated protein.
Figure 22. LplA1 and LipL Together Can Use Free Lipoic Acid to Lipoylate PDH. Lipoylation reactions to evaluate the function of LplA1 and LipL together in the presence of free lipoic acid. Reactions were resolved on a 12% SDS PAGE gel, which was stained with Coomassie blue (lower panel) or used an immunoblot (upper panel) with rabbit anti lipoic acid primary antibody. The first lane contains a ladder and subsequent lanes contain reactions with the proteins as listed above in the chart.

LipL Ability to Ligate Free Lipoic Acid

With the new knowledge that LplA1 and LipL can act together to attach free lipoic acid to E2-PDH, we wondered whether or not LipL alone could use free lipoic acid to lipoylate any of the other E2 or H subunits. To address this question, we used genetic and biochemical approaches. First, we generated a ΔlplA1ΔlplA2ΔgcvH strain. This strain has a nonfunctional salvage pathway as well as an inhibited de novo biosynthesis pathway, because it is unable to generate lipoyl-GcvH for subsequent lipoyl transfer to E2 subunits. This strain allowed me to ask if LipL used free lipoic acid directly to lipoylate any E2 proteins. Wildtype, ΔlplA1, ΔlplA2, ΔlplA1 ΔlplA2, ΔgcvH, ΔlplA1 ΔlplA2 ΔgcvH, ΔlipL, and ΔlipL ΔlplA1 were grown for 9 hours with or without free lipoic acid, cell lysates were collected, and lipoylation profiles were assessed by immunoblot. In the absence of free lipoic acid, the ΔlplA1 ΔlplA2 ΔgcvH strain shows no detectable lipoylation on any proteins (Figure 23). However, in the presence of free
lipoic acid, OGDH is lipoylated in this same strain. In a ΔlipL strain, we see that OGDH is lipoylated, presumably due to the function of LplA1. This lipoylation is lost in a ΔlipL ΔlplA1 indicating that either of these enzymes is able to use free lipoic acid to lipoylated OGDH.

**Figure 23. LipL is Able to Use Free Lipoic Acid to Lipoylate OGDH.** Strains as listed above, were grown in either A) RPMI+BCFA or B) RPMI+BCFA+LA for 9 hours and cell lysates were collected. 10 µl of cell lysates were resolved on a 12% SDS PAGE gel and lipoylated proteins were analyzed by immunoblot with rabbit anti lipoic acid primary antibody. The first lane in A and B corresponds to a ladder and each lane following corresponds to the cell lysate from the strain listed above. * indicates the band indicating the lipoylation of OGDH by LipL.

**Role of LipL in Infection**

As previously mentioned, lipoic acid is a limiting source in the host environment. One might anticipate that *S. aureus* is able to combat this problem by relying on the *de novo* biosynthesis pathway. However, we know that this is not the case in all tissues. The final step of de novo biosynthesis is the transfer of the lipoyl domain from an H subunit of GcvH to the E2 subunit of metabolic enzymes presumably by LipL. It is therefore predicted that LipL would play a significant role in an infection in tissues where de novo biosynthesis is required. To test this hypothesis, we compared wildtype, ΔlipL, and ΔlipL +lipL strains in a systemic and skin infection model. A ΔlipL mutant was significantly attenuated in the kidneys of systemically infected mice (Figure 24). The bacterial load in the kidney is restored with the ΔlipL + lipL complementation strain. In skin and soft tissue infection, this significant attenuation was
markedly reduced with modest, but significant differences in CFU isolated from the skin abscess (Figure 24). From these experiments, we can conclude that lipL plays a major role in the ability of S. aureus to cause systemic infection, while a role in skin infection is far less pronounced.

Figure 24. lipL is Required for Infection of the Kidneys but Not for Infection of the Skin. 4-week-old Swiss Webster mice were infected with either wildtype, ΔlipL, or ΔlipL + lipL strains A) retro-orbitally to create a systemic infection for 4 days, or B) under the skin after shaving the mouse to cause a skin infection for 5 days. Resulting bacteria were plated and CFU/mL was enumerated to evaluate the bacterial load and therefore the severity of infection.

Exploring Pta and LipL Complex Formation

It has been shown in T. acidophilum that LplA, the enzyme that is responsible for the transfer of lipoic acid, requires a specific conformation to function \(^{37}\). This conformation requires complex formation between LplA and a secondary protein, LplB. While we currently have little insight into the significance of pta and lipL genetic coupling, the knowledge that an amidotransferase in another organism forms a complex with a secondary protein lead us to investigate if this was also true for LipL and Pta. Pta and LipL were purified and mixed at a 1:1 ratio for an hour, followed by running on the FPLC, and UV peaks were compared to that of the individual proteins. The UV peaks from the mixed protein samples exactly match those of
individual Pta and LipL samples; therefore, we can conclude no complex was formed under this condition (Figure 25A). I also set up additional conditions where I mixed PDH, GcvH, Pta, and LipL at a 1:1:1:1 ratio. Again, under these conditions the UV peak from the mixed protein directly traces the UV peaks of the individual proteins (Figure 25B). From this we can conclude that, under these conditions, Pta and LipL do not form a detectable complex.

Figure 25. No Detection of Complex Formation Between Pt and LipL. Proteins mixed in a 1:1 ratio for an hour at 37°C that were run on the FPLC. Resulting UV peaks were then compared to those of individual proteins run on the FPLC.
CHAPTER FOUR
DISCUSSION

Introduction

Similar to a variety of other species, *S. aureus* requires the metabolic cofactor lipoic acid for growth\(^\text{25}\). *S. aureus* has evolved two different pathways to acquire lipoic acid. The first is the salvage pathway by which lipoic acid from the environment is used by either LplA1 or LplA2 to directly lipoylate E2 or H subunits\(^\text{38}\). The second pathway is the *de novo* biosynthesis pathway in which lipoic acid is generated from octanoic acid\(^\text{25}\). The dependency of *S. aureus* on this cofactor poses a problem for the bacterium due to the limited availability of free lipoic acid in the environment\(^\text{28}\). Lipoylated proteins are significantly more prevalent in the environment than free lipoic acid, so use of these substrates would be extremely beneficial, if not critical for the survival of the bacteria especially in an infected host\(^\text{27}\). This leads us to the overarching question: How does *S. aureus* use substrates other than free lipoic acid. In the context of the salvage pathway, we analyzed the ability of LplA1 and LplA2 to use lipoylpeptides from the environment as a source of lipoic acid. In terms of the *de novo* biosynthesis pathway, we focused our attention on the function of the critical enzyme LipL, an amidotransferase, as well as its possible connection to another critical enzyme Pta, which had not been previously assessed.
LplA1 and LplA2 require a bacterial encoded lipoamidase to utilize lipoylpeptides as a source of lipoic acid

The use of free lipoic acid by the salvage enzymes in *S. aureus* has been well-studied in the lab using genetic and biochemical approaches. Our data clearly indicate that these enzymes have ligase function in the presence of free lipoic acid. However, it was unclear if these enzymes possess the lipoamidase or amidotransferase activities required to use lipoylpeptides directly. To test if this were possible, I set up a lipoylation assay including lipoamide, either LplA1 or LplA2, and GcvH-L. We originally saw lipoylation of GcvH-L by LpA1, but this did not correspond with the shift in apparent molecular weight that is typically seen with lipoylation of lysine on GcvH-L (Figure 12). Therefore, we wondered if LplA1 might attach lipoamide to an alternate amino acid in the lipoyl domain of GcvH-L. Because lipoamide lacks a free hydroxyl, which is required for the attachment to lysine by an amide bond (Figure 2), we hypothesized that the attachment was to a different amino acid. The lysine that is normally lipoylated in GcvH-L is amino acid 56. The amino acids directly flanking this lysine are a serine and a threonine at amino acids 55 and 57 respectively (Figure 13A). Both amino acids have a terminal hydroxyl group that could allow for attachment of lipoamide. Mass spectrometry was used to verify the location of the lipoic acid attachment; however, no specific attachment could be detected in this analysis. While preparing additional samples for follow-up mass spectrometry studies, I observed a shift in band size when the GcvH-L was run on an SDS PAGE gel and stained with Coomassie blue (Figure 14A). This band shift suggested to me that a change in protein charge was occurring, as is the case when free lipoic acid is attached to lysine. These observations led us to suspect that, rather than a novel ligation reaction between lipoamide and GcvH-L, our observations of lipoylation in the presence of lipoamide seemed to be a
consequence of contaminating free lipoic acid in our lipoamide stock. This hypothesis was validated by determining that GcvH-L was not efficiently lipoylated when fresh lipoamide stocks were used (Figure 14C). The inability of the salvage enzymes to use lipoylpeptides was confirmed in a biochemical assay containing DK\(^{1}\)A, lipoic acid that is bound to a lysine through an amide bond (Figure 15). Due to the lack of a free hydroxyl group, DK\(^{1}\)A cannot be directly attached to the lysine on GcvH-L. With this experiment we saw that these reactions do not lead to the lipoylation of GcvH-L. From the experiments with lipoamide and DK\(^{1}\)A, we conclude that LplA1 and LplA2 are unable to directly use lipoylpeptides and therefore lack amidotransferase or lipoamidase activity.

Bacteria of the gut are an example of a microbe that can use host lipoylpeptides as a nutrient source\(^{20}\). These bacteria rely on an intestinal amidase that frees the lipoic acid from other proteins found in the gut environment. Bacteria are then able to use this liberated lipoic acid by their ligase to lipoylate bacterial enzymes. There are also examples of bacteria, such as \(L.\) monocytogenes, that contain their own lipoamidase\(^{24}\). My studies suggest that \(S.\) aureus may also contain a lipoamidase for liberation of free lipoic acid from lipoyl-peptides. Previously Irina grew mutants of the \(de\)\(^{\text{ novo}}\) biosynthesis pathway in the presence of DK\(^{1}\)A, and observed that strains containing LplA1 show growth comparable to wildtype \(^{38}\). This growth indicates that \(S.\) aureus is able to use DK\(^{1}\)A as a source of lipoic acid. In this thesis, I further interrogated this observation by using DK\(^{1}\)A in lipoylation assays with LplA1 or LplA2, cell lysate from a \(\Delta lipA \Delta lipM \Delta lipL \Delta pla1 \Delta pla2\) mutant that is unable to lipoylate any of its own proteins, and either GcvH or GcvH-L. This experiment tested if some undefined enzyme is produced by \(S.\) aureus that allows for the use of DK\(^{1}\)A by LplA1 and or LplA2. From the results of this experiment, I concluded that this is true, due to the fact that under these conditions, GcvH and GcvH-L are
lipoylated by LplA1 and GcvH-L is lipoylated by LplA2 (Figure 16). The knowledge that *S. aureus* has inherent lipoamidase activity further reiterates that this bacterium is well-adapted to survive in a variety of environments such as those with limited free lipoic acid availability (Figure 28).

While we can conclude that *S. aureus* produces an enzyme with lipoamidase activity, we do not yet know the identity of this enzyme. Before 2017, the only known bacterial lipoamidase was Lpa from *Enterococcus faecalis* \(^{60}\). Recently a study by Rowland et al. showed that the sirtuins CobB, SrtN, and SIRT4 in *E. coli*, *B. subtilis*, and mammalian cells, respectively, harbor lipoamidase activity\(^ {21,60}\). The homologous sirtuin in *S. aureus* was identified as SAUSA300_215759; however, deletion of this gene led to no effect on lipoylation when tested by Wei Ping Teoh (data not shown). Identifying the lipoamidase in *S. aureus* may be difficult as this may be a previously uncharacterized enzyme in the bacterium or, the lipoamidase activity may comprise a secondary function of a protein with other activities, as was the case with the sirtuins in *E. coli* and *B. subtilis*\(^ {60}\). To begin the process of attempting to identify this lipoamidase, I would first see if I could identify other proteins with predicted amidase or sirtuin activities. For example, SAUSA300_0327 is predicted to function as a SIR2 deacetylase (BLAST). This class of protein is normally responsible for the deacetylation\(^ {61}\). However, SIR2 proteins interact with lysines to modify them and therefore may have a secondary function of interacting with lysines on other proteins to allow for the removal of lipoic acid. The SIR2 function as a lipoamidase could be tested by repeating the lipoylation assay containing DK^A and cell lysate in addition to LplA1 or LplA2 and GcvH or GcvH-L. However, the cell lysate would now be from a ΔlipA ΔlipM ΔlipL ΔlplA1 ΔlplA2 Δ0327 strain. If lipoylation no longer occurs, lipoamidase activity can be attributed to the function of SAUSA300_0327. However, it should be noted that sirtuin’s
function as a lipoamidase was detected by mass spectrometry, evaluating the presence or absence of lipoylated protein\textsuperscript{60}. There was no detection of the lipoic acid after is was removed from the proteins. Therefore, it is unknown if the freed lipoic acid has a free hydroxyl group. It is possible that a hydrolase is required, to provide free lipoic acid that can then be utilized by LplA1 and LplA2.

**Function of Extended LipL**

Previous attempts in the lab to study the function of LipL were met with limited success due to unsuccessful complementation of a $\Delta$lipL strain with lipL, and purification of non-functional recombinant LipL. Upon further investigation of the lipL sequence, we noticed that upstream of the annotated start site was another in frame ATG (Figure 10). This led us to hypothesize that the annotation of lipL in NCBI was incorrect and that this upstream start site might lead to generation of a full-length functional gene product. To test this hypothesis, we made a construct containing this upstream start codon, which allowed us to evaluate its effectiveness in the complementation of the $\Delta$lipL mutant for growth and lipoylation defects. In an evaluation of the growth of a $\Delta$lipL+exlipL strain in medium lacking free lipoic acid, we saw that this strain grows equivalently to wildtype (Figure 17). This indicated to us that the NCBI annotation and that we used in our early complementation vectors in the lab was likely generating a non-functional truncated protein.

We also tested the function of extended LipL by in vitro lipoylation assays. While we observed that exlipL complementation of a $\Delta$lipL strain allowed for wildtype levels of growth, it was also important to directly test the function of the extended LipL protein. To do this, I purified 6x-His tagged ex-LipL and compared its function to that of our previously purified truncated LipL in its ability to lipoylate PDH from lipoyl-GcvH. We observed that the previously
purified LipL was unable to lipoylate PDH while our new ex-LipL proved to be functional (Figure 18). This further confirmed that the upstream ATG is the true start site of LipL and that we were using a truncated gene and protein in our previous experiments.

**Connection Between LipL and Pta**

With the reevaluation of the *lipL* start codon, we now know that open reading frames of *pta* and *lipL* are just two base pairs apart (Figure 10). Through a BLAST search we determined that this sequence is unique to staphylococcal species, whereas more commonly, *pta* is located in the genome next to its partner in the Pta/AckA pathway, *ackA*43. The significance of these genes’ proximity needs to be further evaluated. The proximity of these two genes indicates that they are co-transcribed and their proteins potentially co-translated as there is a potential Shine-Dalgarno sequence upstream of *pta* but not in between *pta* and *lipL*. To determine if these genes are co-transcribed, q-PCR to detect the levels of *lipL* could be performed on the ∆pta strain that we have already made. If no *lipL* transcript is produced, this supports the idea that *pta* is critical in the regulation of *lipL*. This would also further explain the lipoylation profile of the ∆pta strain, in which lipoylation was decreased compared to the wildtype, and restoration of lipoylation only occurred with the complementation of *lipL* (Figure 20). This would suggest that the deletion of *pta* leads to the disruption of *lipL*, and could be confirmed by q-PCR.

It was initially proposed that Pta could be required for the function of LipL, as we had not been able to detect the function of LipL in either our growth curves in terms of complementation or in our biochemical assays. It was thought that if Pta could aid in the function of LipL, that these proteins would form a complex. This is based on information from *T. acidophilum*, in which the ligase LplA requires a secondary protein LplB for function. LplA and LplB have been shown to form a complex to allow for the lipoylation of enzymes within *T. acidophilum*20. Initial
testing to determine if Pta and LipL form a complex using size exclusion chromatography showed no complex formation. To further explore possible complex formation between Pta and LipL, I would propose two additional experiments. The first is to evaluate complex formation under conditions that are more similar to that in which LipL would be functional. Lipoylated GcvH could be incubated in the presence of PDH, LipL, Pta and ATP, better simulating the conditions in which LipL would transfer lipoic acid from GcvH to PDH. This reaction could then be run on the FPLC and UV peaks could be compared to those of individual proteins. This could also be done in the presence of a cross linker to better stabilize interactions between Pta and LipL that may be short lived. If this does not lead to an indication that a complex is forming, I would then propose performing a pull-down assay using histidine and Myc tagged proteins. These methods could also be used to better determine any relationship between LipL and LplA1.

Another area that was not addressed in this thesis is whether or not Pta effects LipL function. We have now been able to observe LipL function in vivo and in biochemical assays, so Pta is not essential for function as previously thought. However, this is not to say that the presence of Pta does not increase the efficiency of LipL activity. To test this possibility, I would perform LipL lipoylation assays with or without the addition of Pta and compare the efficiency of lipoylation monitoring the intensity of the band on an immunoblot over time.

**Use of Free Lipoic Acid by the Combination of LipL and LplA1**

The original lipoylation assay testing the function of our purified extended LipL protein led to our investigation into the relationship between LipL and LplA1 for protein lipoylation. While this experiment was successful in showing that the ex-LipL protein was functional, it uncovered an unexpected observation. As mentioned before, the lipoylated GcvH that was added to this reaction came from a reaction that still contained free lipoic acid and LplA1. As we
further discovered in subsequent lipoylation reactions, LipL and LplA1 were able to act together to attach free lipoic acid to E2-PDH (Figure 21 and 22). This is the first indication that these two enzymes are able to work together. To further explore this functional dependency, it would be important to test whether LipL and LplA1 act in connection to lipoylate E2-PDH in vivo. To do this, the lipoylation profile of a ΔlplA2 ΔgcvH mutant strain could be evaluated with or without the addition of free lipoic acid. This strain contains LplA1 and LipL; however, because gcvH is deleted, the de novo biosynthesis pathway is inhibited. Therefore, we can conclude that free lipoic acid supplemented in the medium is the only source of lipoic acid. The ΔlplA2 ΔgcvH strain’s profile could then be compared to that of a ΔlplA2 ΔgcvH ΔlplA1 and a ΔlplA2 ΔgcvH ΔlipL strain. These triple mutant strains are also lacking lplA1 or lipL, which would show function of these enzymes independently. Any protein that is lipoylated in the ΔlplA2 ΔgcvH strain that is not lipoylated in the triple mutant strains, would have been lipoylated due to the presence of both LipL and LplA1. We would expect that this would be true for PDH, as this would be consistent with the biochemical data shown.

Learning that LplA1 and LipL are able to function together leads us to question the mechanics of this activity. Lipoic acid ligases are able to utilize free lipoic acid by an enzymatic attachment mechanism that occurs in two steps. The first is the formation of an intermediate of lipoyl-adenylate, lipoyl-AMP\textsuperscript{14}. The lipoyl moiety from lipoyl-AMP is then transferred to the lipoyl domain of an E2 subunit or H protein (Figure 26). We know through previous biochemical and genetic analysis that LplA1 only lipoylates the H subunits of GcvH and GcvH-L, as well as one E2 subunit of OGDH\textsuperscript{38}.
Figure 26. Function of Lipoic Acid Ligases. The two-step process by which lipoic acid is attached to an E2 or H subunit by a lipoic acid ligase. First the free lipoic acid is attached to AMP in the generation of the lipoyl-AMP intermediate. From this intermediate, the lipoyl domain is then transferred to the E2 or H subunit where it is attached by an amide bond.
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Conversely, we know that LipL is critical for the lipoylation of E2 subunits, as indicated in lipoylation profiles of ΔlipL mutant, which show lipoylation only of E2-OGDH. This is presumably due to the amidotransferase function of LipL. This process again is carried out in two steps. First, the lipoyl group is removed from GcvH, by attack from the active site of LipL. This active site contains a thiol in which the lipoic acid is attached to LipL, forming an intermediate structure. From here, the lysine on the lipoyl domain of the E2 subunit attacks the intermediate and, through an amide bond formation, the E2 subunit is lipoylated (Figure 27).
Figure 27. Amidotransferase Activity of LipL. Depicted is the function of LipL in *B. subtilis*. Octanoic acid is attached to GcvH, where it is then transferred to the active site if LipL. Finally the octanoic acid is transferred from LipL to the E2 subunit.
(Reprinted with permission from Cronan, 201620)

While we have observed that these enzymes independently are unable to use free lipoic acid to lipoylate PDH, I predict that these two enzymes are working together to aid in the formation of the LipL acyl enzyme intermediate to allow for the lipoylation. This process could happen in three stages. The first is that LplA1 is able to use free lipoic acid and ATP to form the lipoyl-adenylate intermediate. Then either the function of LplA1 and/or LipL allows for the transfer of the lipoyl domain to LipL to form the acyl enzyme intermediate. Finally, the lipoyl domain could be transferred from LipL to PDH. The formation of this lipoylated LipL intermediate could be biochemically tested for and detected by immunoblot using our anti-lipoic acid antibody24.

**LipL Use of Free Lipoic Acid Alone**

From this study, we also unveil the ability of LipL alone to use free lipoic acid. We asked if LipL could utilize free lipoic acid, after finding that LipL and LplA1 together were able to lipoylate E2-PDH with free lipoic acid. To assess this, we observed the lipoylation profile of the
ΔlplA1 ΔlplA2 ΔgcvH mutant strain. This strain still contains LipL, but has no salvage pathway, and with no GcvH, LipL cannot use lipoyl-GcvH as a source of lipoic acid. This strain tests the ability of LipL to use free lipoic acid that was supplemented into the medium. We see that under these conditions, OGDH is lipoylated, as indicated by immunoblot. This could then be further supported in biochemical lipoylation assays in which LipL and free lipoic acid were combined in reactions with either PDH, OGDH, GcvH or GcvH-L. We would expect to see that the only positive reaction is that containing OGDH. This is an unpredicted function of LipL. It was previously thought that LipL could exclusively use lipoic acid that was attached to GcvH. While this is a unique finding and allows us to better understand the capabilities of LipL, this may not be physiologically relevant due to the lack of free lipoic acid in the environment. There seems to be a high amount of redundancy in the lipoylation of OGDH and it is unclear why this is specific to OGDH, or why LipL is unable to use free lipoic acid to lipoylate other E2 or H subunits.

**LipL Amidotransferase Function**

While we have investigated some previously unknown functions of LipL, we have yet to view the amidotransferase function of LipL biochemically. To do this, I would first lipoylate GcvH in a reaction with LplA1 and free lipoic acid. The lipoylated GcvH would be purified away from LplA1 by running the reaction on the FPLC using size exclusion chromatography. Pure lipoylated GcvH would then be added to a reaction containing PDH and LipL; in this reaction, the only source of lipoic acid is lipoylated GcvH, meaning that if lipoylation of PDH is observed, it must be due to the ability of LipL to transfer the lipoyl moiety from GcvH to LipL. This experiment could also be repeated with lipoylated GcvH-L as the source of lipoic acid, as well as different E2 subunits such as OGDH. These experiments would definitively show the amidotransferase function of LipL.
LipL Role in Infection

Previously in the lab, Swiss Webster mice were infected with either wild type or ∆lipL S. aureus to induce a systemic infection. From this experiment, it was observed that mice infected with the ∆lipL strain developed significantly less disease, resulting in less bacterial burden in the heart and kidneys (Figure 6). I repeated this experiment in order to include our successfully complemented ∆lipL+ lipL strain. I also examined these strains in the context of a skin infection, as we predicted that the ∆lipL would show attenuation in all infection types due to the bacterium’s inability to generate lipoic acid in the de novo biosynthesis pathway. The results from the systemic infection were consistent with what we had previously seen, with the ∆lipL strain infection resulting in a significantly decreased bacterial load in the kidneys. The bacterial burden was restored to wildtype levels in the ∆lipL+ lipL strain, as we had predicted. However, unexpectedly, we did not see substantial reduction in bacterial CFU in the skin infection model. This experiment was performed twice and would need to be repeated in order to confirm these results. However, if these results do repeat, it may indicate the lack of dependency on de novo biosynthesis of lipoic acid for survival in skin, or a lack of dependency on lipoic acid altogether. The availability of lipoic acid is known to be low but the exact concentration of lipoylated proteins in the body is not well documented. It is thought that the concentration of lipoic acid can increase due to dietary supplementation, but these increases are described as minimal, transient, and located in the plasma. The results of this skin and systemic infection may indicate the prevalence of lipoylated proteins in the skin, while it is lacking in organs such as the kidneys. If this were true, it would make sense that in the skin, this de novo biosynthesis mutant would be able to salvage lipoic acid from the environment where it is relatively available. By contrast, in the kidneys, S. aureus would need to rely on the synthesis of lipoic acid and then transfer to the
E2 subunits, but is unable to do so with the lipL deletion. However, this does not entirely agree with what we have previously seen in the lab. In an experiment examining the role of LplA1 and LplA2 in the lab, it was seen that the salvage pathway was also essential for infection in the kidneys\textsuperscript{25}. It is possible that, in the case of the kidney infection, both the salvage and the \textit{de novo} biosynthesis pathways are critical.

\textbf{Concluding Remarks}

In this work, I have shown in biochemical assays that LplA1 and LplA2 are ligases, unable to use lipoylpeptides directly as a source of lipoic acid. I have also shown that \textit{S. aureus} has lipoamidase activity that enables the use of lipoylpeptides by these salvage enzymes. This further increases our knowledge of how \textit{S. aureus} is able to utilize a variety of resources to enable survival in different environmental niches.

This thesis also explores a component of the \textit{de novo} biosynthesis pathway, LipL. Through a genetic and biochemical approach, I have concluded that the lipL annotated in NCBI is truncated, and that the true start site is upstream. This new genetic annotation indicates that lipL and its upstream neighbor pta are in an operon together; however, the significance of this has yet to be understood. With this information on the sequence of lipL, I have also now been able to start addressing the function of LipL. In biochemical assays, I have determined that LipL and LplA1 are able to function together and utilize free lipoic acid to lipoylated PDH. I also showed through lipoylation profiles that LipL has a ligase function and is able to independently lipoylate OGDH in the presence of free lipoic acid. Overall, this thesis clarifies how \textit{S. aureus} is able to satisfy its requirement for lipoic acid by illuminating new functions of LipL and the production of a lipoamidase.
Figure 28. Lipoic Acid Biosynthesis and Salvage Pathway in \textit{S. aureus}. Pathway highlighting the function of A) LplA1, B) LplA2, C) LipL in the salvage pathway and their ability to use lipoic acid, liberated from lipoamide, to lipoylate E2 and H subunits of \textit{S. aureus} enzymes.
APENDIX A

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Figure 29. Authorization to Republish.

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BACK CLOSE WINDOW
Supplemental Figure 1. ∆pta and ∆lipL Growth is Comparable to Wildtype in RPMI+BCFA After 9 Hours. Evaluation of growth of pta and lipL mutants along with complemented strains in RPMI + BCFA medium


VITA

Sarah Flury was born on August 12, 1993 in Highland Park, Illinois to Jim and Carol Flury. She is the eldest child, with a brother Henry. Sarah attended Bradley University from 2011-2015. Starting her sophomore year, she worked in the lab of Dr. Melinda Faulkner studying the uncharacterized peroxide scavenging enzyme AhpA. Sarah graduated cum laude from Bradley with a Bachelor of Science in 2015.

In 2016 Sarah entered the Masters program in the Department of Microbiology and Immunology at Loyola University Chicago. Here she joined the lab of Dr. Francis Alonzo, studying Staphylococcus aureus pathogenesis and evasion of the host immune response. Sarah’s work focused on understanding the dynamics of protein lipoylation in examining the function of lipoic acid ligases and amidotrasnferase. After finishing the Masters program, Sarah will continue her education pursuing a PhD at Loyola University.