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Defining the Roles of the Lipoic Acid Ligases in Promoting Staphylococcus Aureus Metabolic Homeostasis and Virulence

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DEFINING THE ROLES OF LIPOIC ACID LIGASES IN PROMOTING *STAPHYLOCOCCUS AUREUS* METABOLIC HOMEOSTASIS AND VIRULENCE

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PDH	Pyruvate Dehydrogenase
OGDH	2-Oxoglutarate Dehydrogenase
GcvH	H subunit of the Glycine cleavage system
GcvH-L	H subunit of the Glycine cleavage-like system
BCODH	Branched Chain 2-Oxoacid Dehydrogenase
BCFA	Branched Chain Fatty Acid
CFU	Colony Forming Units
LA	Lipoic acid
MRSA	Methicillin Resistant *Staphylococcus aureus*
OA	Octanoic acid
PCR	Polymerase Chain Reaction
RPMI	Roswell Park Memorial Institute
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
WT	Wild type
SSTI	Skin and Soft Tissue Infection
CDC	Center for Disease Control and Prevention
Isd	Iron-regulated Surface Determinant

AoDH	Acetoin Dehydrogenase
Gcs	Glycine Cleavage System
PBST	Phosphate Buffered Saline and Tween
TBST	Tris Buffered Saline and Tween
BSA	Bovine Serum Albumen
IPTG	Isopropyl β-D-1-thiogalactopyranoside
PMSF	Phenylmethane Sulfonyl Fluoride
DTT	Dithiothreitol
MWCO	Molecular Weight Cut-off
FPLC	Fast Protein Liquid Chromatography
CHIPS	Chemotaxis Inhibitory Protein of Staphylococci
SCIN	Staphylococcal Complement Inhibitor
PSM	Phenol-soluble Modulins
KOR	α-Ketoglutarate Oxidoreductase
POR	Pyruvate:flavodoxin Oxireductase
ECM	Extracellular Matrix

ABSTRACT

Staphylococcus aureus is a major human pathogen known to cause disease in a wide range of tissues. In order to thrive in such diverse environments, *S. aureus* uses multiple adaptive traits such as trace metal/nutrient acquisition, shifts in metabolic activity, and expression of detoxification systems, all of which allow the bacterium to proliferate and survive in nutritionally deficient and inhospitable environments.

One essential metabolite used by *S. aureus* is lipoic acid, a cofactor of enzyme complexes used in aerobic metabolism, fatty acid biosynthesis, glycine detoxification, and maintenance of redox homeostasis. Prior studies in the lab used a genetic approach to define the lipoic acid biosynthesis and salvage pathways of *S. aureus*. These studies determined that *S. aureus* synthesizes lipoic acid from an octanoic acid precursor, or through salvage mechanisms, where lipoic acid is acquired from the environment by the action of lipoic acid ligases LplA1 and LplA2. In addition, it was demonstrated that LplA1, but not LplA2, is necessary for the salvage of lipoic acid in vitro, whereas both ligases are sufficient to promote infection of tissues in vivo. Because the LplA2 ligase does not have a discernable function in vitro, its exact role in lipoic acid salvage is unknown.

Based on this information, I hypothesized that LplA1 and LplA2 may stimulate growth by using alternate lipoylated substrates. To determine if the ligases use alternate sources of lipoic acid, I evaluated growth phenotypes by supplementing media with derivatives of lipoic acid. I

found that only LplA1, and not LplA2, can use free lipoic acid and peptide bound lipoic acid to stimulate bacterial growth in vitro. In order to further elucidate the functional differences and substrate usage of the ligases, I conducted lipoylation assays with purified recombinant ligases in the presence of lipoic acid, lipoamide, $DK^{L}A$, and octanoic acid. My results indicated that LplA1 can directly use lipoic acid to lipoylate GcvH, GcvH-L and E2-OGDH, whereas LplA2 can directly use lipoic acid to lipoylate E2-PDH, E2-OGDH, E2-BCODH, as well as GcvH-L. These data suggest that both lipoic acid ligases in *S. aureus* have preferred targets for lipoylation and that they can act independently from one another.

Together, these studies highlight the importance of the divergent functions of LplA1 and LplA2 and may explain why *S. aureus* thrives so well when faced with low levels of free lipoic acid during host infection.

CHAPTER ONE

LITERATURE REVIEW

Introduction

Staphylococcus aureus is a Major Human Pathogen.

Staphylococcus aureus is a Gram-positive commensal bacterium commonly found on the skin, anterior nares, and in the gastrointestinal tract. It is estimated that up to 30% of the world's population is asymptomatically colonized with *S. aureus* (1; 2). However, upon a breach in physical barriers such as the skin, *S. aureus* can become a major human pathogen and is known to cause a wide range of infections, including mild skin and soft tissue infections (SSTI), bacteremia, sepsis, and osteomyelitis (1; 2; 3). Numerous factors can make a person more susceptible to *S. aureus* infections such as the presence of foreign bodies including catheters, pace makers, and prostheses. In addition, patients who have recently undergone surgery or are immunocompromised are also at an increased risk of *S. aureus* infections (4). Due to a rise in antibiotic resistant strains known as methicillin resistant *S. aureus* (MRSA), it has become increasingly difficult to treat *S. aureus* infections, leading to greater morbidity and mortality (1). Traditionally, MRSA infections were commonly found in healthcare settings, however in recent years there has been an increase in community-associated methicillin resistant *S. aureus* (CA-MRSA) infections, leading to the spread of MRSA among healthy individuals (4; 5; 6; 7). Studies suggest that CA-MRSA strains exhibit increased virulence due, in part, to a greater production of *S. aureus* peptides that recruit and lyse human neutrophils (8). However, virulent

S. aureus strains also contain a significant amount of genetic diversity, a trait that likely has allowed *S. aureus* to acquire additional virulence mechanisms (8; 9; 10). This constant acquisition of new traits, allows *S. aureus* to evade modern therapeutics such as antibiotics, making it increasingly difficult to treat MRSA infections (8; 9; 10). Due to the increased prevalence of *S. aureus* infections in the community, it is imperative for us to deepen our knowledge of the pathways *S. aureus* uses to colonize and proliferate in the host. These new findings may lead to the development of novel therapeutics that can specifically target the essential pathways that *S. aureus* requires for survival.

S. aureus Expresses a Multitude of Virulence Factors that Facilitate Colonization and Survival in the Host Environment.

S. aureus infects a wide array of tissues such as the skin, bones, heart, kidney, and joints (2). Its ability to colonize and proliferate in such diverse environments is directly linked to the production of virulence factors that allow the bacterium to adhere to surfaces, evade and suppress the immune system, release toxins, and take up trace nutrients (11; 12; 13; 14; 15).

In order to initiate colonization, *S. aureus* uses surface proteins to adhere to plasma or extracellular matrix (ECM) components (13; 16). The largest class of surface proteins in *S. aureus* are called microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) (17). One class of MSCRAMMs, the fibronectin-binding proteins A and B (FnbpA and FnbpB), allows *S. aureus* to bind to osteoblasts resulting in the formation of biofilms (18). Not only is this class of surface proteins vital for *S. aureus* to adhere to different surfaces, they are also important for immune evasion (12). Protein A, which is also part of the MSCRAMM family, binds to the Fcγ domain of immunoglobins resulting in the impairment of

phagocytic leukocytes and inhibits the activation of the complement cascade (13; 19). The ability to adhere to a wide range of host tissues is one of the traits that sets *S. aureus* apart from other pathogens.

In addition to producing surface adhesion molecules, *S. aureus* also secretes toxins, which target the membrane of the host cells resulting in the efflux of metabolites and other molecules (20). Some well-known *S. aureus* pore-forming toxins include the α-toxin and bicomponent leukocidins. α-toxin is a cytotoxic molecule and was one of the first secreted toxins identified in *S. aureus*. This membrane damaging toxin binds to receptors on host cells, triggering lysis and inducing a host-inflammatory response (13; 21). However, this toxin can have different effects depending on the amount released by *S. aureus*. High amounts of the α-toxin results in the formation of Ca^{2+}-permissive pores, which leads to massive necrosis, while sublytic amounts result in DNA fragmentation and eventual cell death via apoptosis (13; 22; 23). Another group of *S. aureus* secreted proteins that form pores are the bicomponent leukocidins, which include Panton-Valentine leucocidin (PVL), LukED, HlgAB, HlgCB, LukMF, and LukAB/HG (24). The lytic activity of the bicomponent leukocidins results in the induction of inflammation, host tissue damage, immune cell killing, and further prevents phagocytosis of *S. aureus* (24; 25; 26; 27). Overall, the ability of *S. aureus* to release toxins further distinguishes it as a prominent pathogen.

Another important component of *S. aureus* virulence is the ability of the bacterium to release immunomodulatory proteins that further perturb host immune responses. For instance, the release of the superoxide dismutases SodA and SodM results in the inactivation of reactive oxygen species (28). Further, the release of the small secreted protein chemotaxis inhibitory

protein of staphylococci (CHIPS) is important for preventing recruitment of neutrophils to the site of infection, while the staphylococcal complement inhibitor (SCIN) is vital for inhibiting phagocytosis of the bacteria (29; 30). Lastly, phenol-soluble modulins (PSMs), which are toxins important for targeting red and white blood cells, are responsible for increased inflammation and innate immune cell recruitment during infection (31). Taken together, the secretion of immunomodulatory proteins allows *S. aureus* to counteract the host immune response.

A final adaptive trait that distinguishes *S. aureus* as a major pathogen is its ability to use metabolites from the host for growth and survival (15). One defining example is the ability to acquire iron from the environment (32). The majority of iron in the host is stored intracellularly, making it inaccessible to extracellular bacteria such as *S. aureus* (33). Trace extracellular host iron is not freely available and is usually found bound to high-affinity iron binding glycoproteins such as transferrin and lactoferrin (34). In order to circumvent iron sequestration by the host, *S. aureus* secretes siderophores, small molecules with even higher binding affinity for iron than the glycoproteins produced by the host (33). As a result, *S. aureus* is effectively able to steal iron from these host iron-binding proteins. The preferred iron source for *S. aureus* is host heme, however, siderophores are unable to extract heme from this iron source (33). In order to acquire iron from heme, *S. aureus* encodes an iron-regulated surface determinant (Isd) system, allowing it to effectively extract iron-bound heme (35; 36; 37). Without siderophores or the Isd system to promote iron acquisition, *S. aureus* would not be able to successfully infect and proliferate in the host (36; 38)

The ability of *S. aureus* to suppress and evade the host immune response using a wide array of surface adhesion proteins, toxins, and immunomodulatory proteins has certainly allowed

it to become a successful pathogen in hospitals and the communities. Many of these virulence factors are well-defined. However, aside from mechanisms of iron acquisition, the pathways *S. aureus* uses to acquire other trace nutrients and vital cofactors in the host remains comparatively understudied. In addition to needing trace metals for successful colonization and metabolism, *S. aureus* also requires the cofactor lipoic acid, a short-chain fatty acid derivative used in the function of multi-enzyme metabolic complexes and for maintaining redox homeostasis (15; 39). The biosynthesis and salvage of lipoic acid is crucial for obtaining this vital cofactor and will be the focus of this thesis.

Lipoic Acid Metabolism

Lipoic acid and Lipoylated Enzyme Complexes.

Lipoic acid, a derivative of the medium-chain fatty acid octanoic acid, is a sulfur-containing cofactor that is covalently attached to subunits of multi-enzyme complexes needed for one carbon metabolism (Fig 1A) (15). It is a conserved molecule and is used to maintain metabolic flux in all domains of life. Currently, five different lipoylated enzyme complexes have been identified in bacteria, although they are not necessarily present in all species: pyruvate dehydrogenase (PDH), which catalyzes the oxidative decarboxylation of pyruvate to acetyl CoA; 2-oxoglutarate dehydrogenase (OGDH), which converts α-ketoglutarate to succinyl-CoA; branched-chain 2-oxoacid dehydrogenase (BCODH), which degrades branched chain amino acids to make a branched chain CoA intermediate needed for fatty acid biosynthesis; acetoin dehydrogenase (AoDH), which is similar to the PDH complex and also catalyzes the conversion of pyruvate to acetyl CoA; and the glycine cleavage system (Gcs), which catalyzes the reversible decarboxylation of glycine (15; 39; 40). The α-ketoacid dehydrogenases are comprised of

multiple copies of three different subunits referred to as E1, E2, and E3, whereas the glycine cleavage system uses subunits referred to as P protein (pyridoxal phosphate-containing protein), H protein (hydrogen carrier protein), T protein (tetrahydrofolate-containing protein), and L protein (lipoamide dehydrogenase). The lipoic acid cofactor is attached through an amide bond to a conserved lysine residue on the E2 subunits and acts as a swinging arm channeling substrates through the different active sites (Fig 1B). Unlike the α-ketoacid dehydrogenase complexes where the lipoyl group is attached to the E2 subunit, in Gcs, lipoic acid is covalently attached to the H protein (Fig 1C) (15; 40; 41)

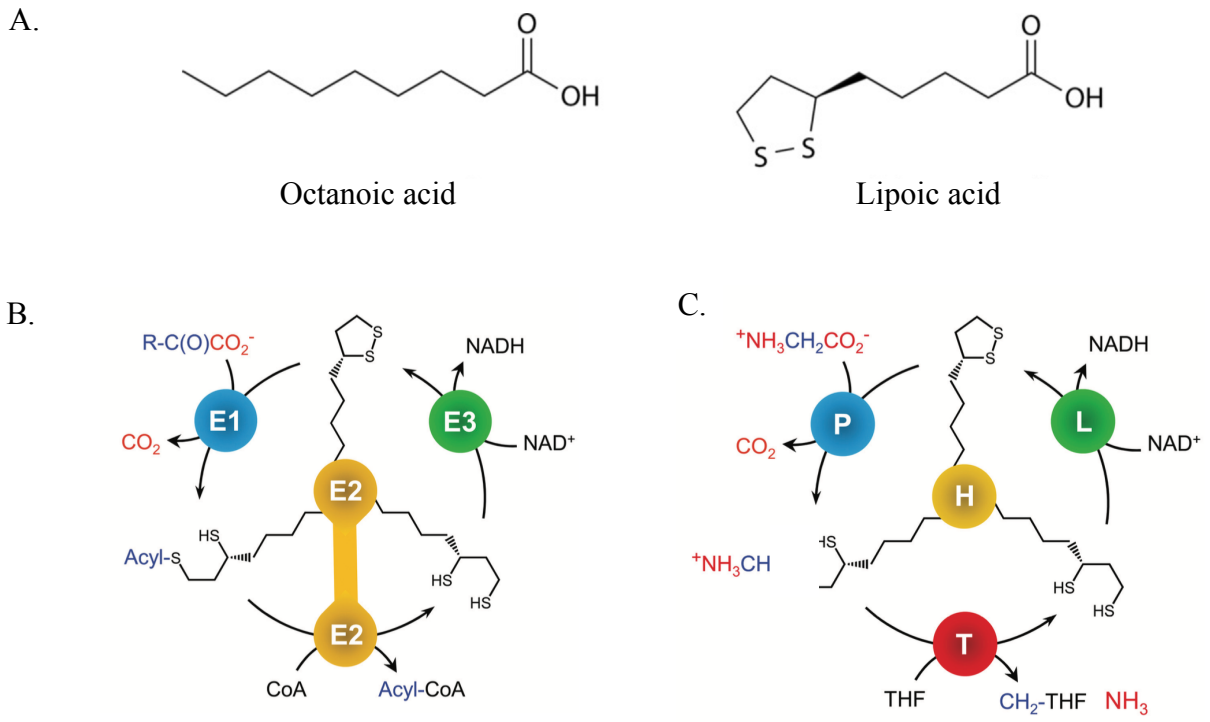

Figure 1. Structure and function of lipoic acid in lipoylated enzyme complexes
A. Lipoic acid is derived from octanoic acid. Once sulfur atoms are inserted at carbons 6 and 8, the two thiols form disulfide bonds which results in the formation of lipoic acid. B. In the Pyruvate Dehydrogenase complex, lipoic acid is attached to the E2-subunit, which has catalytic activity and acts as a swinging arm channeling substrates to their different active sites on E1 and E3. C. In the glycine cleavage complex, lipoic acid is attached to the H-subunit. However, unlike the E2 subunit, the H subunit does not have catalytic activity but is able to transfer substrates to the different active sites on the P, L, and T subunits.
Reprinted with permission from Spalding & Prigge (15) and Cronan (39) (Figure 28 – Appendix I)

Lipoic Acid Biosynthesis and Salvage in Microbes.

The processes used to acquire lipoic acid in microbes are diverse (15; 39). Microbes such as bacteria, fungi, and protozoa can potentially use two independent pathways to acquire lipoic acid (39). They either generate lipoic acid through *de novo* biosynthesis mechanisms where the cofactor is synthesized from an octanoic acid precursor by a lipoic acid synthetase, or by scavenging free lipoic acid from the environment through lipoic acid salvage (42).

The first lipoic acid biosynthesis and salvage pathway was discovered in the Gram-negative bacterium *Escherichia coli* (Fig 2). In *E. coli,* the *de novo* biosynthesis pathway requires two proteins: an octanoyl transferase, LipB, and a lipoic acid synthetase, LipA. Since LipB is not very efficient at transferring free octanoic acid, it relies on the type two fatty acid synthase to generate an octanoylated acyl carrier protein (ACP) from which it can then transfer the octanoyl moiety onto an apo E2 subunit or H subunit (43). LipA then converts the octanoyl domain to lipoic acid by inserting two sulfur atoms to form a dithiolane ring (44). During the salvage pathway, *E. coli* uses a lipoic acid ligase, LplA, to scavenge free octanoic acid and lipoic acid from the environment (Fig 2). The lipoic acid ligase reaction proceeds in a two-step manner: first a tightly bound lipoyl-adenylate intermediate is formed, which is then followed by the transfer of the lipoyl moiety onto either an apo E2 subunit such as OGDH or H subunit such as GcvH (42; 43; 44; 45; 46; 47).

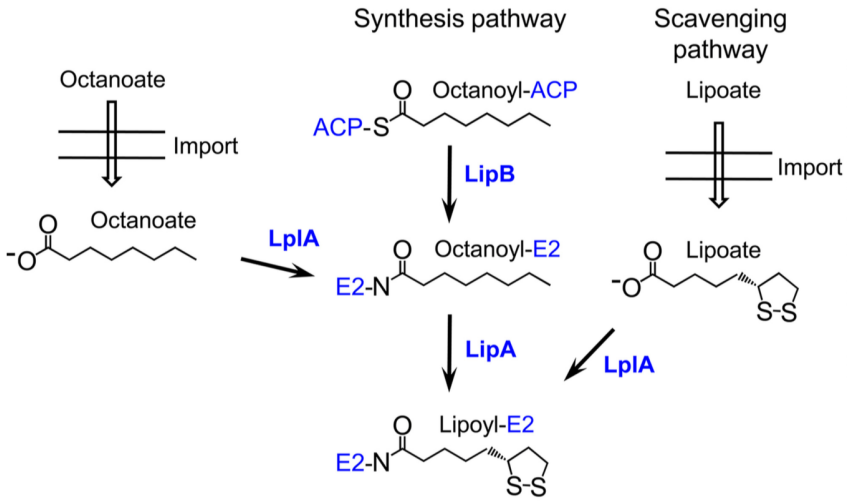

Figure 2. Lipoic acid biosynthesis and salvage pathway of *E. coli*
E. coli encodes two independent pathways to acquire lipoic acid. During the *de novo* biosynthesis pathway, LipB, an octanoyl transferase, transfers the octanoyl domain from an acyl carrier protein (ACP) onto the E2 subunit of α-ketoacid dehydrogenase complexes or the H subunit of GcvH. This octanoyl moiety is then used as a substrate for LipA, a lipoic acid synthetase, to form a lipoyl domain. During the salvage pathway, *E. coli* uses the lipoic acid ligase, LplA, to scavenge lipoic acid from the environment and ligate it onto enzyme complexes.
Reprinted with permission from Spalding & Prigge (15) (Figure 28 – Appendix I)

A more complex lipoic acid biosynthesis and salvage pathway compared to *E. coli* was first discovered in the Gram-positive bacterium, *Bacillus subtilis.* In *B. subtilis*, four proteins are required for *de novo* biosynthesis and salvage of lipoic acid: the octanoyl transferase, LipM; lipoic acid synthetase, LipA; lipoyl transferase, LipL; and the lipoic acid ligase, LplJ (Fig 3) (48). During *de novo* biosynthesis, *B. subtilis* uses LipM, which is functionally similar to *E. coli* LipB, to transfer octanoic acid from an acyl carrier protein onto the H subunit of GcvH. Just like in *E. coli*, LipA is then used to catalyze the formation of lipoic acid from the octanoyl moiety. Even though LipL has the most sequence similarity with LipB, it has a very different enzymatic activity. In *B. subtilis*, LipL is responsible for transferring the lipoyl moiety from the H subunit onto additional lipoyl domains such as apo E2-PDH, apo E2-OGDH, and apo E2-BCODH (48). During this transfer, LipL attacks the amide linkage whereas LipM and LipB attack the thioester

bond when transferring the octanoyl moiety. In addition, the LipL reaction is completely reversible indicating that the lipoyl moiety can be transferred among different E2 subunits (39). Just like *E. coli*, *B. subtilis* encodes a single lipoic acid ligase, LplJ, responsible for scavenging lipoic acid from the environment and lipoylating various enzyme complexes such as PDH, OGDH, and BCODH (48).

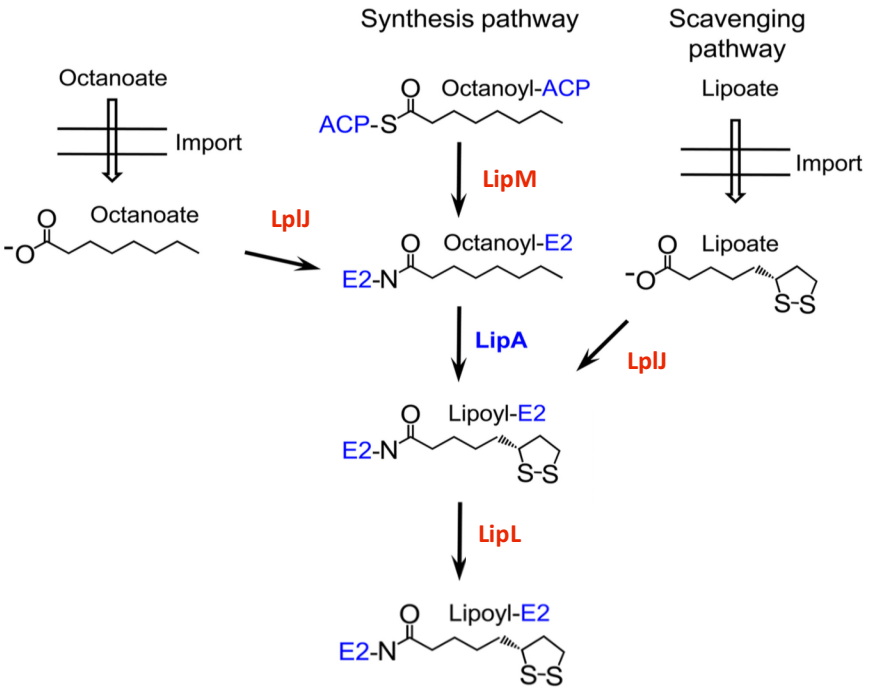

Figure 3. Lipoic acid biosynthesis and salvage pathway of *B. subtilis*
Similar to *E. coli, B. subtilis* is able to synthesize and scavenge lipoic acid. However, *B. subtilis* uses a octanoyltransferase, LipM, to transfer the octanoyl moiety to the E2 subunit. From there, LipA converts the octanoyl moiety to lipoic acid. Then, LipL transfers the lipoyl moiety onto different enzyme complexes. In the salvage pathway, *B. subtilis* uses the LplJ ligase to scavenge lipoic acid from the environment.
Reprinted with permission from Spalding & Prigge (15). (Figure 28 – Appendix I)

The lipoic acid biosynthesis and salvage pathways of *E. coli* and *B. subtilis* are the most characterized and well-understood pathways to date. However, lipoic acid metabolism can be found in numerous organisms with varying degrees of complexity. The comparison of *Helicobacter pylori* and *Pseudomonas aeruginosa* is one example in Gram-negative bacteria that

highlights the diversity of lipoic acid acquisition strategies in microorganisms. Unlike many other bacteria, *H. pylori* does not encode the lipoylated enzyme complexes nor the enzymes needed for lipoic acid biosynthesis and salvage. Instead, *H. pylori* uses anaerobic and microaerophilic alternatives such as α-ketoglutarate oxidoreductase (KOR) as an alternative to OGDH and pyruvate:flavodoxin oxireductase (POR) as an alternative to PDH to maintain a functional TCA cycle (49; 50; 51; 52). *P. aeruginosa* on the other hand, has both the *de novo* biosynthesis and salvage enzymes and encodes all five known lipoylated enzyme complexes (15).

Gram-positive bacteria, which include the *Firmicutes* phylum, display a similar level of diversity in lipoic acid metabolism. In contrast to other bacteria, members of the *Firmicutes* phylum often encode multiple ligases needed to scavenge lipoic acid, however they do not always encode the enzymes necessary for *de novo* biosynthesis (15). *Listeria monocytogenes* for instance, does not encode the enzymes needed for *de novo* biosynthesis, rather it uses two lipoic acid ligases, LplA1 and LplA2, and a lipoylamidotransferase, LipL, to scavenge lipoic acid from environmental sources (53; 54). Recent studies have shown that LplA1 is largely responsible for scavenging lipoic acid and modifying the glycine cleavage system subunit, GcvH. In addition, *L. monocytogenes* was found to have lipoamidase activity, which may be responsible for cleaving the amide bond linking lipoic acid to its conserved lysine residue, subsequently allowing LplA1 to acquire the lipoyl domain from host derived lipoyl peptides (55) (Fig 4). Unlike *L. monocytogenes*, bacteria belonging to *Bacillales* often encode multiple ligases, but may also encode enzymes required for the *de novo* biosynthesis pathway such as LipA, the lipoic acid synthetase described earlier for *B. subtilis* (15).

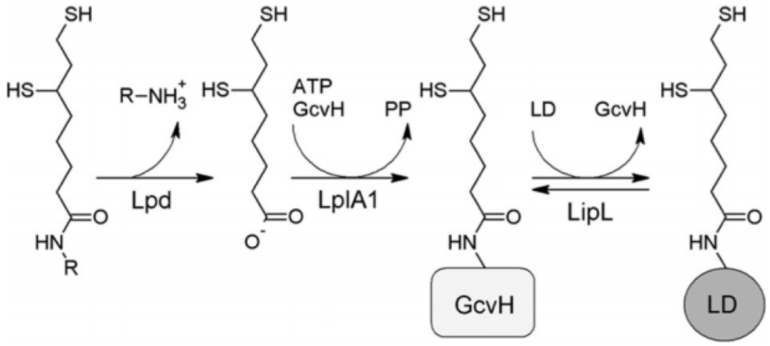

Figure 4. Model of lipoic acid ligase activity in *L. monocytogenes*
In *L. monocytogenes*, the lipoamidase (Lpd) is important for cleaving lipoic acid off of a lipoylated peptide allowing LplA1 to scavenge the lipoyl domain and attach it to the H-subunit of the Gcs. Subsequently, LipL is able to transfer the lipoyl moiety to different E2 subunits.
Reprinted with permission from Christensen *et al*. (55) (Figure 28 – Appendix I)

In contrast to bacteria, where lipoic acid biosynthesis occurs in the cytosol, lipoic acid metabolism in fungi is localized in the mitochondria. Yeasts such as *Saccharomyces cerevisiae* and *Candida albicans* only encode three enzymes (Lip2, Lip3, Lip5) that are used to lipoylate OGDH, GcvH, and PDH (56; 57; 58). Lip2 and Lip5 are orthologs of the *E. coli* LipB and LipA enzymes respectively, and are responsible for *de novo* biosynthesis. Even though the yeast encodes a lipoic acid ligase, Lip3, it is not able to scavenge lipoic acid. Current literature suggests that disruption of both *lip2* and *lip5* in the presence of a functional *lip3* renders the yeast incapable of growing in medium supplemented with lipoic acid (59; 60). These data imply that yeast solely depend upon the *de novo* biosynthesis pathway for the acquisition of lipoic acid, or that lip3 may not be expressed under the experimental conditions used in prior studies.

In protozoans, specifically apicomplexans, lipoic acid metabolism can be found in both the mitochondria and apicoplasts (61; 62). To date, the acquisition of lipoic acid in protozoans has been best characterized in the pathogenic *Plasmodium falciparum* and *Toxoplama gondii*. Both *P. falciparum* and *T. gondii* are capable of lipoylating four of the five known lipoylated

enzyme complexes: PDH, OGDH, BCODH, and GcvH. Interestingly, the lipoylated complexes OGDH, BCODH, and GcvH are localized in the mitochondria, whereas PDH is found in the apicoplast. Furthermore, lipoylation of the enzyme complexes occurs strictly during the blood stage of the parasitic lifecycle and is divided into the two different organelles (63). Lipoic acid *de novo* biosynthesis occurs in the apicoplast with the help of the *E. coli* orthologs LipA and LipB, whereas lipoic acid salvage occurs in the mitochondria with the help of two lipoic acid ligases (62; 64; 65). Both of the apicomplexans are known to cause severe disease in humans and it has been suggested that lipoic acid metabolism may play a role in promoting pathogenicity.

In summary, lipoic acid is a conserved molecule, however the acquisition of lipoic acid is very diverse among bacteria, fungi, and protozoans. Many of these organisms encode either a *de novo* biosynthesis pathway, salvage pathway, or both thereby conferring a range of complexities that may have evolved to satisfy the unique nutrient requirements of that particular organism. Lipoic acid metabolism can be found in several organelles of eukaryotes, further implying that each organism has adapted the pathway that best suits its lifestyle. Important to the work in this thesis, these adaptations appear to be beneficial to the pathogenic lifestyle of some bacteria.

Lipoic Acid Biosynthesis and Salvage in Pathogenic Bacteria and Parasitic Microbes.

A small body of literature exists that suggests lipoic acid metabolism can have a major role in facilitating optimal pathogenesis in microorganisms (15; 66; 67; 68; 69; 70). It has been shown that lipoic acid salvage in parasites such as *P. falciparum*, the causative agent of malaria, is crucial for the survival and growth of the parasite at the blood-stage (15; 67). In addition, disruption of the lipoic acid biosynthesis and salvage pathway can lead to attenuation of *Burkholderia pseudomallei* virulence in an intranasal mouse infection model. In *P. aeruginosa*, a

functional lipoylated PDH enzyme complex has been shown to be important for the expression of the type three secretion system (T3SS) (15; 66; 68). Furthermore, disruption of *dlaT*, a gene encoding the E2 PDH subunit in *M. tuberculosis*, results in increased susceptibility to macrophage killing and oxidative stress (15; 69; 70).

As mentioned previously, *L. monocytogenes,* a prominent pathogen responsible for foodborne illnesses, has two lipoic acid ligases, LplA1 and LplA2. It has been demonstrated that both ligases have ligase activity in vitro. However, LplA2 activity could only be demonstrated in vitro when the medium was supplemented with a surplus of free lipoic acid. LplA1, on the other hand, stimulated bacterial growth with low concentrations of peptide bound lipoic acid, but not when the medium was supplemented with free lipoic acid. During in vivo mouse infections, only LplA1 contributed to bacterial replication, indicating LplA2 activity is dispensable during intracellular growth. This is yet another example of how divergent functions of lipoic acid acquisition can promote survival within a nutrient limited niche. Overall, the ability of pathogens such as *L. monocytogenes*, to acquire lipoic acid through biosynthesis and/or salvage is a defining characteristic required for survival during host infection.

Lipoic Acid *De Novo* Biosynthesis and Salvage in *S. aureus.*

S. aureus, another prominent pathogen and member of the *Firmicutes* phylum like *B. subtilis* and *L. monocytogenes*, also harbors genes for lipoic acid metabolism. *S. aureus* has one of the most complicated lipoic acid biosynthesis and salvage pathways. It encodes the three enzymes of *de novo* biosynthesis LipM, LipA, and LipL along with two lipoic acid ligases LplA1 and LplA2. In addition, *S. aureus* encodes two GcvH proteins, which may be used to transfer lipoic acid onto different E2 subunits (15; 48; 71; 72). Just like *B. subtilis, S. aureus*

encodes an octanoyl transferase, LipM, which transfers an octanoyl moiety from the ACP to GcvH; and a lipoyl synthetase, LipA, which then converts the octanoyl moiety to lipoic acid. In the last step of the *de novo* biosynthesis pathway, it is hypothesized that LipL then transfers the lipoyl domain from GcvH onto PDH, BCODH, and OGDH. Like *L. monocytogenes*, *S. aureus* also encodes two lipoic acid ligases, LplA1 and LplA2, responsible for scavenging lipoic acid from the environment (Fig 5).

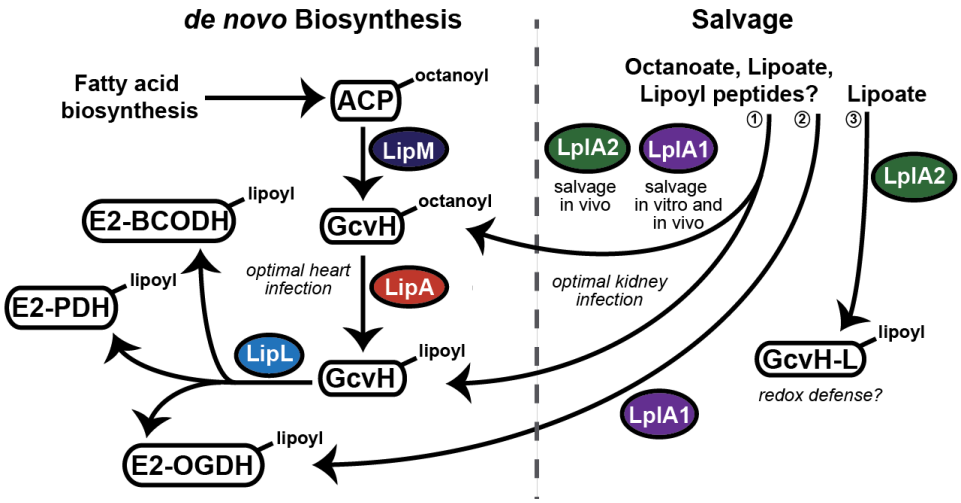

Figure 5. Lipoic acid biosynthesis and salvage pathway of *S. aureus*
S. aureus can acquire lipoic acid through the lipoic acid biosynthesis and salvage pathway. LipM, LipA, and LipL are enzymes involved in lipoic acid biosynthesis. LipM, an octanoyl transferase, transfers octanoic acid from an acyl carrier protein to GcvH. LipA, a lipoic acid synthetase, converts octanoic acid to lipoic acid. LipL, a transferase, transfers the lipoic acid onto the E2 subunits of other enzyme complexes such as OGDH, PDH, and BCODH. In the lipoic acid salvage pathway, LplA1, a lipoic acid ligase, salvages lipoic acid from the environment and attaches it to the E2 subunits of OGDH or GcvH. Currently the function of LplA2 is not well characterized but it is thought that LplA2 attaches lipoic acid to GcvH-L during oxidative stress.
Reprinted with permission from Zorzoli *et al.* (71)

LplA1, a Lipoic Acid Ligase in *S. aureus*, is Crucial for Lipoic Acid Salvage In Vitro.

A unique feature of the *S. aureus de novo* biosynthesis and salvage pathways is that, unlike *B. subtilis* and many of the other pathogenic *Firmicutes, S. aureus* encodes two lipoic acid ligases, LplA1 and LplA2 in addition to its *de novo* biosynthesis enzymes (15; 48; 72). Just like

S. aureus, *L. monocytogenes* also encodes two lipoic acid ligases, where it was found that LplA1 uses host derived-lipoyl peptides whereas LplA2 uses free lipoic acid. Based on this information, it was hypothesized that LplA1 and LplA2 in *S. aureus* have distinct functions that facilitate important acquisition activities in vitro and in vivo. To test this hypothesis, a former member of the Alonzo laboratory, Azul Zorzoli, generated a set of lipoic acid ligase mutants in a Δ*lipA* mutant background in order to block *de novo* biosynthesis of lipoic acid and directly assess salvage activity without confounding outcomes associated with *de novo* biosynthesis. Azul conducted growth curves in Roswell Park Memorial Institute medium (RPMI), RPMI supplemented with branched chain fatty acids (BCFA) to bypass the requirement of lipoic acid, or either base medium (RPMI or RPMI+BCFA) supplemented with lipoic acid or octanoic acid. She found that all strains with a Δ*lipA* mutation were unable to grow in the absence of supplements (Fig 6A). The same growth pattern was observed when RPMI was supplemented with octanoic acid (Fig 6B). However, when supplemented with lipoic acid, all strains grew similar to the WT strain, except the Δ*lipA*Δ*lplA1* double mutant, which was unable to replicate (Fig 6C) (71). Azul's data suggested that only LplA1 is required for lipoic acid salvage in vitro, while the role of LplA2 in lipoic acid salvage remained unknown.

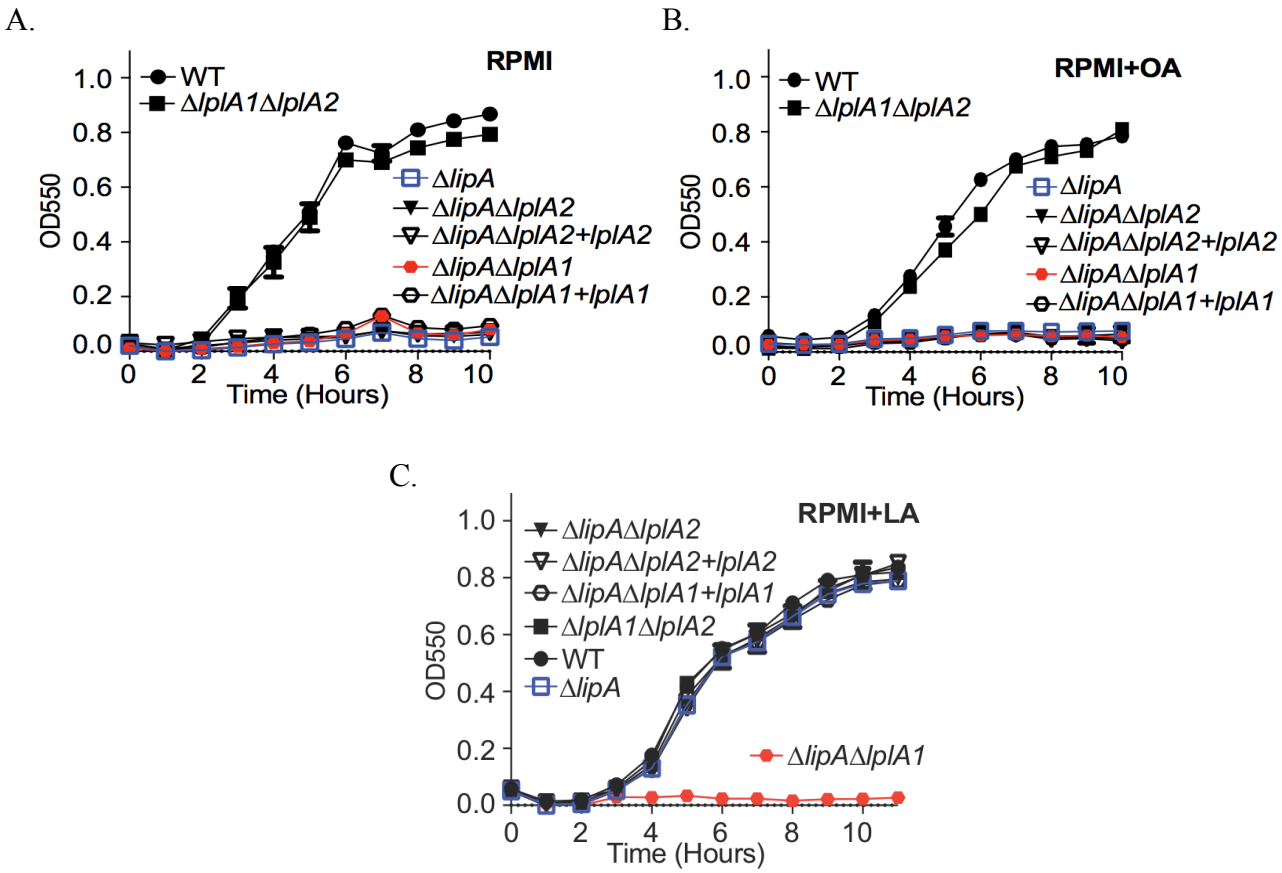

Figure 6. LplA1 facilitates lipoic acid salvage in vitro
Growth assessment of lipoic acid ligase in-frame deletion mutants in the background of a Δ*lipA* mutant. A. Growth curve in RPMI with no supplementation (RPMI). B. Growth curve in RPMI supplemented with octanoic acid (RPMI+OA). C. Growth curve in RPMI supplemented with lipoic acid (RPMI+LA).
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Either of the Two Lipoic Acid Ligases, LplA1 and LplA2, are Sufficient to Promote Infection of the Kidneys During Murine Systemic Infection.

Since LplA2 did not have an apparent role in *S. aureus* lipoic acid salvage in vitro, it was hypothesized that it might not be functional under the conditions tested. It was reasoned that LplA2 might play a role in facilitating lipoic acid use in alternative environments where free lipoic acid is restricted, such as in mammalian tissues. In order to evaluate the ability of LplA1 and LplA2 to promote bacterial survival in mammalian tissues, mice were infected with WT,

Δ*lipA*Δ*lplA1*Δ*lplA2,* Δ*lipA*Δ*lplA1*Δ*lplA2+lplA1,* and Δ*lipA*Δ*lplA1*Δ*lplA2+lplA2* strains. In animals infected with a Δ*lipA*Δ*lplA1*Δ*lplA2* mutant, which lacks a functional *de novo* biosynthesis and salvage pathway, a severe decrease in bacterial burden was observed. However, when infected with strains expressing either LplA1 or LplA2 in single copy from constitutive promoters, Δ*lipA*Δ*lplA1*Δ*lplA2+lplA1* and Δ*lipA*Δ*lplA1*Δ*lplA2+lplA2*, mice had similar colony forming units (CFU) in the kidney when compared to the WT strain. These data imply that the salvage pathway is crucial for bacterial replication in the kidneys and that either LplA1 or LplA2 is sufficient to promote lipoic acid acquisition in the kidneys.

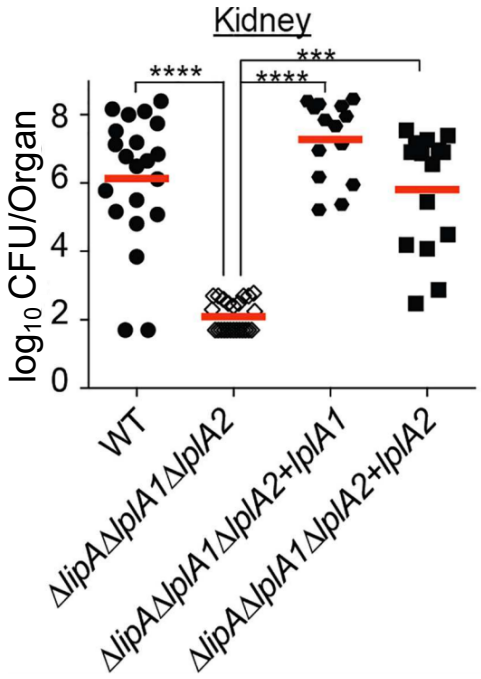

Figure 7. LplA1 and LplA2 are important for lipoic acid salvage in vivo
In a murine *S. aureus* bloodstream infection model, bacterial burden (CFU/organ) was determined in the kidney 96 hours post infection after infecting mice with $1X10^7$ CFU of WT (N=21), Δ*lipA*Δ*lplA1*Δ*lplA2* (N=20), Δ*lipA*Δ*lplA1*Δ*lplA2+lplA1* (N=14), and Δ*lipA*Δ*lplA1*Δ*lplA2+lplA2* (N=15) strains. Compared to WT, Δ*lipA*Δ*lplA1*Δ*lplA2+lplA1,* and Δ*lipA*Δ*lplA1*Δ*lplA2+lplA2,* a Δ*lipA*Δ*lplA1*Δ*lplA2* strain had significantly decreased bacterial burden. Log_{10}CFU/organ is displayed for each mouse infected, along with the median as a measure of central tendency – red line. Statistics were determined using nonparametric 1-way ANOVA with Kruskal-Wallis multiple comparisons post-test to evaluate statistical significance. Statistical significant differences are indicated by ****, $P<0.0001$; and ***, $P<0.001$.
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Concluding Remarks

S. aureus is a prominent pathogen that uses myriad virulence factors to establish itself in the host. One of the lesser studied traits that enhances *S. aureus* colonization in the host is the ability to acquire lipoic acid from nutrient deficient environments. Our lab's previous studies have characterized the lipoic acid biosynthesis and salvage pathway of *S. aureus* using a genetic approach and murine systemic infection models. It was determined that only LplA1 is sufficient for bacterial growth in vitro, whereas both ligases are sufficient for lipoic acid acquisition during infection. However, the exact activities of the two lipoic acid ligases, LplA1 and LplA2, have yet to be fully elucidated. Though limited information exists in the literature, work in *L. monocytogenes* suggests that bacteria with multiple ligases likely use these enzymes to acquire the cofactor in distinct ways. In addition, the *lplA2* gene in *S. aureus* is encoded in an operon that is upregulated under oxidative stress conditions, suggesting that *lplA2* expression may be upregulated in vivo where oxidative stress to the bacterium is presumed to be high (72). Based on this information, I hypothesized that LplA1 and LplA2 in *S. aureus* stimulate growth by using alternative lipoylated substrates and/or that *lplA2* gene expression is induced during infection, providing *S. aureus* with tremendous opportunity to adapt in the face of nutrient paucity. To test this hypothesis, I (i) conducted growth curves to evaluate the ability of the ligases to use alternative lipoyl substrates and (ii) purified the proteins of the *de novo* biosynthesis and salvage pathway along with apo E2/H subunits of lipoylated enzyme complexes to directly assess the ability of either ligase to lipoylate the known lipoylated enzyme complexes of *S. aureus*. Characterizing the mechanisms of lipoic acid salvage in *S. aureus* will be instrumental to understanding the ability of this bacterium to colonize and proliferate in diverse nutrient limited

environments, including host tissue, and may help in the development of new therapeutics to combat *S. aureus* infections.

CHAPTER TWO

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

All bacterial strains used in this manuscript are listed in Table 1. *E. coli* strains were routinely grown in Lysogeny Broth (LB) (Amresco) with antibiotics added as necessary. *S. aureus* strains were grown in either rich medium, Tryptic Soy Broth (TSB) (Criterion), or in defined medium, Roswell Park Memorial Institute medium (RPMI) (Corning) supplemented with 1% casamino acids (Amresco). All strains were grown overnight at 37°C at a 45° angle, shaking at 220 rpm unless stated otherwise. For growth curves, *S. aureus* overnight cultures were grown in RPMI containing branched chain carboxylic acids (10.8 mM isobutyric acid, 9.2 mM 2-methylbutyric acid, 9 mM isovaleric acid, and 10 mM sodium acetate) (Sigma) in order to bypass the requirement of lipoic acid or octanoic acid. When needed, cultures were supplemented with the following concentrations of antibiotics; 100 μg/ml of ampicillin (AMP), 3 μg/ml of erythromycin (ERM), 10 μg/ml of chloramphenicol (CM), and 1 μg/ml of anhydrous tetracycline (ANTET).

Table 1. List of Strains

Designation	Description	Strain
WT LAC	*S. aureus* USA300 Strain LAC. Plasmid cured.	USA300 LAC
DH5α	*E. coli* strain used for propagating pIMAY in *S. aureus*	
RN4220	Restriction deficient *S. aureus* for plasmid propagation	RN4220
Δ*lipA*	LAC with in-frame deletion of *lipA*	FA-S831
Δ*lipA*Δ*lplA1*	LAC with in-frame deletion of *lipA* and *lplA1*	FA-S1249
Δ*lipA*Δ*lplA2*	LAC with in-frame deletion of *lipA* and *lplA2*	FA-S1180
Δ*lipA*Δ*lplA1*Δ*lplA2*	LAC with in-frame deletion of *lipA, lplA1* and *lplA2*	FA-S1178
Δ*lipA*Δ*lplA1*Δ*lplA2* + *lplA1*	LAC with in-frame deletion of *lipA, lplA1* and *lplA2,* complemented with *pJC1111-lplA1*	FA-S1200
Δ*lipA*Δ*lplA1*Δ*lplA2* + *lplA2*	LAC with in-frame deletion of *lipA, lplA1* and *lplA2,* complemented with *pJC1111-lplA1*	FA-S1212
Δ*lipA*Δ*lplA1*Δ*lplA2*Δ*lipL*	LAC with in-frame deletion of *lipA, lipL, lplA1,* and *lplA2*	FA-S1319
Δ*lipA*Δ*lplA1* + pOS1-$P_{lplA2(L)}$-*gfp*	pOS1-$P_{lplA2(L)}$-*gfp* - *gfp* promoter fusion (long - 300 bp) plasmid transformed into Δ*lipA*Δ*lplA1*	FA-S1369
Δ*lipM*Δ*lplA1* + pOS1-$P_{lplA2(L)}$-*gfp*	pOS1-$P_{lplA2(L)}$-*gfp* - *gfp* promoter fusion (long - 300 bp) plasmid transformed into Δ*lipM*Δ*lplA1*	FA-S1391
Δ*lipA*Δ*lplA1* + pOS1-$P_{lplA2(S)}$-*gfp*	pOS1-$P_{lplA2(S)}$-*gfp* - *gfp* promoter fusion (short - 100bp) plasmid transformed into Δ*lipA*Δ*lplA1*	FA-S1393
Δ*lipM*Δ*lplA1* + pOS1-$P_{lplA2(S)}$-*gfp*	pOS1-$P_{lplA2(L)}$-*gfp* - *gfp* promoter fusion (short - 100 bp) plasmid transformed into Δ*lipM*Δ*lplA1*	FA-S1395
6x-His-GcvH	pET15b encoding 6x-His-GcvH transformed into Δ*lipA*::kan LysY I^Q *E. coli*	FA-S1357
6x-His-GcvH-L	pET15b encoding 6x-His-GcvH-L transformed into Δ*lipA*::kan LysY I^Q *E. coli*	FA-S1383
6x-His-OGDH	pET15b encoding 6x-His-OGDH transformed into Δ*lipA*::kan LysY I^Q *E. coli*	FA-S1363
6x-His-PDH	pET15b encoding 6x-His-PDH transformed into Δ*lipA*::kan LysY I^Q *E. coli*	FA-S1359
6x-His-BCODH	pET15b encoding 6x-His-BCODH transformed into Δ*lipA*::kan LysY I^Q *E. coli*	FA-S1367
6x-His-LipM	pET15b encoding 6x-His-LipM transformed into LysY I^Q *E. coli*	FA-S1276
6x-His-LipA	pET15b encoding 6x-His-LipA transformed into LysY I^Q *E. coli*	FA-S1283
6x-His-LipL	pET15b encoding 6x-His-LipL transformed into LysY I^Q *E. coli*	FA-S1277
6x-His-LplA1	pET15b encoding 6x-His-LplA1 transformed into LysY I^Q *E. coli*	FA-S1284
6x-His-LplA2	pET15b encoding 6x-His-LplA2 transformed into LysY I^Q *E. coli*	FA-S1278

Molecular Genetic Techniques

Chromosomal DNA was isolated from *S. aureus* using the Wizard Genomic DNA purification kit (Promega) following the manufacturers protocol with minor modifications. Overnight cultures were started in 5 ml TSB and 1.5 ml was spun down the next day at 15,000 rpm for 3 min. Bacterial pellets were resuspended in 200 µl of TSM (50 mM Tris, 0.5 M Sucrose, 10 mM $MgCl_2$, pH 7.5). In order to disrupt the cell wall, 2.5 µl of lysostaphin (2 mg/ml in 0.5 Tris, pH 8.0) was added to the resuspended cell pellet and incubated for 15 min at 37°C. Following incubation, the bacteria were pelleted at 15,000 rpm for 3 min and the supernatant was discarded. The remaining steps to purify genomic DNA from *S. aureus* were completed using the manufacturers protocol. Recombinant plasmids were extracted using QIAGEN mini and midi prep kits with the following modifications for plasmid isolation from *S. aureus.* An overnight culture of *S. aureus* was grown in 5 ml TSB and pelleted at 4000 rpm for 10 min the following day. The bacterial pellet was resuspended in 400 µl TSM (50 mM Tris, pH 7.5, 0.5 Sucrose, 10 mM $MgCl_2$) followed by the addition of 20 µl of lysostaphin. This mixture was incubated for 10 min at 37°C to break down the cell wall and then spun down at 13,000 rpm for 2 min after which the supernatant was discarded. The following steps were completed as suggested by the manufactures protocol. Polymerase chain reaction (PCR) products were either gel extracted or purified using QIAGEN QIAquick gel extraction and PCR purification kits. All PCRs were conducted using Phusion High-Fidelity DNA polymerase (New England Biolabs).

E. coli Competent Cell Preparation

An overnight culture of *E. coli* was grown in 3 ml LB at 37°C with shaking at 180 rpm. The next day, the bacteria were subcultured 1:55 into a 250 ml flask and grown for an additional

~2.5 hours at 37°C shaking at 180 rpm until the culture reached an optical density at 600 nm (OD600) of 0.3-0.4. The following steps were all completed on ice. Cultures were aliquoted into 50 ml tubes and chilled on ice for 10 min. Afterwards they were spun down at 4000 rpm for 10 min and the bacterial pellet was resuspended in TFB-1 (20 mM KOAc, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol, adjusted to pH 5.8 using 0.2 M Acetic acid) and incubated on ice for an additional 10 min. Bacteria were then pelleted at 4000 rpm for 10 min and the bacterial pellet was resuspended in 1/25 of the original culture volume in TFB-2 (10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% glycerol, adjusted to pH 6.5 using KOH). 100 µl of the competent cells were aliquoted into Eppendorf tubes and stored at -80°C.

E. coli Heat Transformation

In order to transform competent *E. coli,* 5 µl of ligation mix or 1 µl of purified plasmid was added to 50 ul of competent *E. coli.* This reaction mixture was incubated on ice for 30 min, heat shocked at 42°C for 45 seconds, and then incubated for 2 min on ice. Following the incubation, 250 µl of SOC medium was added and the bacteria were incubated for 2 hours at 37°C shaking at 220 rpm. Subsequently, 100 µl of the bacteria was plated on LB agar plates containing the antibiotic needed to select for plasmid transformants.

Preparation of *S. aureus* Electrocompetent Cells

An overnight culture of *S. aureus* was grown in 5 ml TSB at 37°C shaking at 220 rpm. The following day the bacteria were subcultured 1:100 in 30 ml TSB and incubated for an additional 3 hours at 37°C until the culture reached an OD600 of 0.5. The bacterial culture was spun down for 10 min at 8000 rpm to pellet the bacteria. All subsequent steps from here were carried out on ice. After the bacteria were spun down, the pellet was washed by resuspending it

in 30 ml ice cold 10% glycerol and spun down again for 10 min at 8000 rpm. These wash steps were repeated three times. After the last wash, the bacteria were resuspended in 3 ml 10% glycerol, aliquoted into Eppendorf tubes, and stored at -80°C.

S. aureus Transformation via Electroporation

Frozen competent cells were thawed at room temperature for 5 min. 2 µl of plasmid DNA was then added to 50 µl of *S. aureus* RN4220 or LAC competent cells and incubated at room temperature for 30 min. The competent cell mixture was transferred to sterile 2 mm electroporation cuvettes and pulsed at 1800 V, 10 µF, and 600 Ω. After electroporation, the bacteria were resuspended in 750 µl of TSB or TSB+BCFA and incubated at 37°C or 30°C for 1.5 hours to allow the bacteria to recover. After the incubation, the bacteria were pelleted at 10000 rpm for 2 min and resuspended in 100 µl of TSB or TSB+BCFA, plated on TSA/TSA+BCFA plates containing antibiotic and incubated at 37°C or 30°C for 1-2 days.

Generation of In-Frame Deletion Mutants

Regions of homology corresponding 500 bp upstream and 500 bp downstream of genes *lplA1* and *lipA* were amplified using primer pairs 0930 SOE1/SOE2 and 0930 SOE3/SOE4 and 0829 SOE1/SOE2 and 0829 SOE3/SOE4 (Table 2) and PCR purified using the QIAGEN PCR clean-up kit. The upstream and downstream amplicons from these PCRs were used as template in a splicing by overlap extension (SOEing) reaction with primers 0930 SOE1/0930 SOE4 and 0829 SOE1/0829 SOE4 to generate the amplicon used in the subsequent mutagenesis. The two amplicons of 0829 and 0930 were then cloned individually into the allelic replacement plasmid, pIMAY, which contains a chloramphenicol resistance marker using KpnI and SacI restriction endonucleases. The pIMAY-*lplA1*(0930) mutagenesis plasmid was introduced into a Δ*lipM*

lplA1::erm S. aureus mutant and the pIMAY-*lipA* (0829) mutagenesis plasmid was introduced into a Δ*lplA1 lipA::erm S. aureus* mutant strain by electroporation and grown overnight at 30°C in the presence of chloramphenicol. The Δ*lipM lplA1::erm* and Δ*lplA1 lipA::erm S. aureus* mutants containing the pIMAY-*lplA1* and pIMAY-*lipA* mutagenesis plasmid respectively were cultured at 37°C in the presence of chloramphenicol. pIMAY is unable to replicate at 37°C and as a result it is forced to integrate into the *S. aureus* genome at the region of homology upstream or downstream of *lplA1* and *lipA*. To facilitate a second homologous recombination event, cultures were grown at 28°C without chloramphenicol to allow for plasmid replication resulting in the excision of the plasmid from the *S. aureus* genome and generation of a clean deletion. To cure mutant strains of the pIMAY plasmid, bacteria were plated on agar plates containing anhydrous tetracycline (AnTet). Lastly, colonies were screened for chloramphenicol-sensitivity and clean deletion mutants Δ*lipA* Δ*lplA1* and Δ*lipM* Δ*lplA1* were confirmed via PCR amplification of the desired target region using primers 0829 SOE1/4, 0930 SOE1/4, and 1494 SOE1/4 (Table 2).

Bacteriophage Mediated Transduction

All transductions in *S. aureus* were conducted with phage ϕ11. In order to package the phage with donor DNA, a 3 ml overnight culture of the marked donor strain (Δ*lipL*::kan) was started in TSB/LB (1:1) supplemented with 5 mM $CaCl_2$ and 5 mM $MgSO_4$ and grown overnight shaking at 37°C. The following day, the overnight strain was subcultured 1:100 into 10 ml TSB/LB (1:1) supplemented with 5 mM $CaCl_2$ and 5 mM $MgSO_4$ and grown for ~2.5 hours shaking at 37°C until the culture reached an OD 600 of 0.3 to 0.9. 500 µl of the bacterial culture was incubated with 10-fold serial dilutions of ϕ11 phage stock in TMG (10 mM Tris pH 7.5, 5

mM $MgCl_2$, 0.01% gelatin (v/v)), vortexed gently, and incubated at room temperature for 30 minutes. After 30 minutes, tubes containing the bacteria and phage dilutions were mixed with 3 ml CY Top agar (Casamino acids 3g/L, Yeast Extract 3g/L, NaCl 6g/L, 7.5 g/L agar, +/- BCFA as needed) supplemented with 5 mM $CaCl_2$ and 5 mM $MgSO_4$, cooled to 55°C, and poured onto TSA plates. After the top agar solidified, plates were incubated at 30°C overnight. The next day the top agar from 2-3 plates with confluent plaques was scraped off the plate using a sterile scoopula and resuspended in 2ml of TMG buffer per plate followed by extensive vortexing. The tubes were then spun down at 13,000 rpm for 15 minutes. The supernatant was filtered twice using a 0.2 μm filter and then an additional two times with a 0.45 μm filter. All packaged phage stocks are kept at 4°C.

To transduce marked mutations, the recipient strain (Δ*lipA* Δ*lplA1* Δ*lplA2*) was grown overnight with shaking at 37°C in 20 ml TSB+BCFA/LB (1:1) supplemented with 5 mM $CaCl_2$. The following day, the recipient strain was spun down at 13,000 rpm for 15 min and resuspended in 3 ml of TSB+BCFA/LB (1:1) supplemented with 5 mM $CaCl_2$. 500 μl of the recipient bacteria were serial diluted and incubated with 100 μl of the packaged ϕ11 phage (10^8-10^9 PFU) or 100 μl of TMG buffer as an uninfected control for 30 min at room temperature, inverting the tubes every 10 min. After 30 min the bacterial/phage suspension was supplemented with 40 mM NaCitrate and incubated for an additional 30 min, inverting the tubes every 10 min. The tubes were spun down at 13,000 rpm for 3 min and washed twice with 500 μl TSB+BCFA/LB (1:1) supplemented with 10 mM NaCitrate. Washed bacterial pellets were resuspended with 250 μl of TSB+BCFA/LB (1:1) supplemented with 10 mM NaCitrate and 200 μl was plated out on TSB+BCFA containing 10 mM NaCitrate and the respective antibiotic of interest. Plates were

incubated at 37°C for 24-48 hours until bacterial colonies were detected. All mutants were verified using PCR and their respective primers.

Table 2. List of Primers

Name	Sequence
0328NC-F	ATAT-CATATG(NdeI)-TACTTAATAGAACCGATTAG
0328N-R	ATAT-GGATCC(BamHI)-TTAACTTAAAATCATATCCAC
0571NC-F	ATAT-CATATG(NdeI)-CAATCTTTCGCATTCGATG
0571N-R	ATAT-GGATCC(BamHI)-CTATTGCATTTGATCTATCAT
0930NC-F	ATAT-CATATG(NdeI)-AAATTCATTAGTAATAATAATATT
0930N-R	ATAT-GGATCC(BamHI)-TTATGACATTAATCTAATTAATT
0829NC-F	ATAT-CATATG(NdeI)-GCGACAAAAAACGAGGAAA
0829N-R	ATAT-GGATCC(BamHI)-TTAACTATTTAACTGTGCCT
1494NC-F	ATAT-CATATG(NdeI)-ACTGAAACTTGGAATTTTATT
1494N-R	ATAT-GGATCC(BamHI)-CTACTTTCTAAACATCCATT
GcvH-L NC-F	ATAT- CATATG (NdeI)-AAAAAGTTAGCCAATTATTTAT
GcvH-L N-R	ATAT- GGATCC(BamHI)-TTAAGCCTCCGGTAATGC
gfp1720-25F short	ATAT-CTGCAG(PstI)-AAGTTAATTGAAAAACGTTATC
gfp1715/20-25R short/long	ATAT-GGTACC(KpnI)-TCCGTCATCTCCAAACTTA
gfp1715-25F long	ATAT-CTGCAG(PstI)-ACTATGATTCCTTTTCTATTC
791hisN/C-F-	ATAT-CATATG(NdeI)-GCAGTACCAAATGAATTGAA
791hisN-R	ATAT-GGATCC(BamHI)-TTATTCACCAATCATTTCTGA
995hisN/C-F	ATAT-CATATG(NdeI)-GCATTTGAATTTAGATTACCC
995hisN-R-	ATAT-GGATCC(BamHI)-TTACCCCTCCATTAATAATAA
1305hisN/C-F-	ATAT-CATATG(NdeI)-CCAGAGGTTAAAGTTCCAG
1305hisN-R	ATAT-GGATCC(BamHI)-TTAAGATTCTAATAATAAGTCTT
1464hisN/C-F	ATAT-CATATG(NdeI)-GAAATAACAATGCCTAAGTTA
1464hisN-R	ATAT-GGATCC(BamHI)-CTAATATATATTTGTATTTTCTAA
0930 SOE1	CCC-GGTACC(KpnI)-GCATTATACCTGTATAAATAC
0930 SOE2	ATAGTCCTTTAATCGTTTATGA-GGCGCC(KasI)-TTTCATTACAATCTCTCCCTT
0930 SOE3	AAGGGAGAGATTGTAATGAAA-GGCGCC(KasI)-TCATAAACGATTAAAGGACTAT
0930 SOE4	CCC-GAGCTC(SacI)-ATAAGCAAAACCTCGCTTTAT
0829 SOE1	CCC-GGTACC(KpnI)-GCACAATGTGCCATCATCAA
0829 SOE2	CCTTATTAATGGTTAAATATTAACT-GGCGCC(KasI)-CGCCATAACAACACATACCC
0829 SOE3	GGGTATGTGTTGTTATGGCG-GGCGCC(KasI)-AGTTAATATTTAACCATTAATAAGG
0829 SOE4	CCC-GAGCTC(SacI)-ATTAATGTTCAGTATCTTGAATG
1494 SOE1	CCC-GGTACC(KpnI)-TTATAGCCGCCTTTTAACATA
1494 SOE4	CCC-GAGCTC(SacI)-ATATTCAAAGTGCTCACACTT

Preparation of Proteinase K Agarose Beads and Digestion of OGDH and PDH from Porcine Heart

Proteinase K agarose beads, pyruvate dehydrogenase (PDH), and 2-oxoglutarate dehydrogenase (OGDH) were purchased from Sigma. The Proteinase K beads were activated by resuspending 40 mg beads in 1 ml activation buffer (20 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.4) and incubating for 2 hours at room temperature. Before protein digestion, the beads were spun down for 3 min at 2000 rpm and resuspended in 800 µl 20 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.4. This step was repeated three times. Stocks of both PDH (15.9 mg/ml) and OGDH (7 mg/ml) were buffer exchanged into 20 mM Tris-HCl, 1 mM $CaCl_2$ pH 7.4 by taking 2 ml of the stock solutions and diluting them into 100 ml of 20 mM Tris-HCl, 1 mM $CaCl_2$ pH 7.4 followed by concentration with 10 kDa cut-off Amicon Ultra-15 spin columns (Milipore). Subsequently, 2.5 mg/ml PDH and 1.16 mg/ml OGDH were digested overnight with 400 µl of the Proteinase K agarose beads. The following morning, the digestion reaction was spun down at 13,000 rpm for 10 min to pellet the agarose beads and the supernatant was used for subsequent growth curves.

Growth Curves

Overnight cultures were grown in 200 µl of RPMI + BCFA in a 96-well plate shaking at 220 rpm at 37°C. The next day, the strains were pelleted for 10 min at 3700 rpm at 4°C. The strains were washed three times with 200 µl of RPMI alone in order to remove any remaining BCFA that might stimulate growth. Each strain was grown in RPMI supplemented with 48 µM lipoic acid (Sigma), 48 µM lipoamide (Sigma), 1.16 mg/ml OGDH (Sigma) or proteinase K digested OGDH, 2.5 mg/ml PDH (Sigma) or proteinase K digested PDH, 48 µM octanoic acid (Sigma), and 100 µM DK^LA or 100 µM DKA tripeptides (Anaspec). All growth curves were

conducted in a 96-well plate at 37°C over a 10-hour period to allow the bacterial strains to reach stationary phase. Bacterial replication was monitored every hour by measuring OD at 550 nm on a BioTek plate reader.

Generation of P_{lplA2}-*gfp* Transcriptional Reporter Fusions

Based on annotations from the genome sequence of *S. aureus* FPR3757 USA300, *lplA2* is the last gene in an operon that encodes four additional genes; *LLM*, *gcvH-L*, *macro*, and *sirTM*. The predicted promoter region of *lplA2* lies approximately four genes upstream of the *lplA2* open reading frame. Since the predicted *lplA2* promoter region and its regulatory elements are unknown and a small putative gene (*SAUSA300_0323*) exists approximately 100 bp upstream of *LLM*, regions 100 bp and 300 bp upstream of *LLM* were amplified in order to generate transcriptional reporters that fuse the predicted promoter region of *lplA2* to *gfp*. Primers gfp 1720-25F, and gfp 1715/20-25R were used to amplify the 100 bp regions, whereas primers gfp 1715-25F and gfp 1715/20-25R were used to amplify the 300 bp region (Table 2). Both the 100 bp and 300 bp predicted promoter regions were subcloned upstream of the *gfp* gene in plasmid pOS1 using PstI and KpnI restriction endonucleases and subsequently transformed into DH5α *E. coli.* Both reporter constructs were then transformed via electroporation into Δ*lipA*Δ*lplA1* and Δ*lipM*Δ*lplA1 S. aureus* strains.

Generation of 6x-Histidine Tagged Protein Expression Plasmids

In order to purify proteins of the lipoic acid biosynthesis and salvage pathway (LipM, LipA, LipL, LplA1, and LplA2*)* as well as non-lipoylated E2-subunits of PDH, OGDH, BCODH, and non-lipoylated H subunits GcvH, and GcvH-L, primers were designed to amplify each gene of interest (Table 2). *lipM* was amplified using primers 1494NC-F/1494N-R, *lipA* was

amplified using primers 0829NC-F/0829-R, *lipL* was amplified using primers 0571NC-F/0571-R, *lplA1* was amplified using primers 0930NC-F/0930-R, *lplA2* was amplified using primers 0328NC-F/0328-R, *e2-PDH* was amplified using primers 995hisNC-F/995his-R, *e2-OGDH* was amplified using primers 1305hisNC-F/1305his-R, *e2-BCODH* was amplified using primers 1464hisNC-F/1464his-R, *GcvH* was amplified using primers 791hisNC-F/791his-R, and *GcvH-L* was amplified using primers GcvH-LNC-F/GcvH-L-R. The resulting amplicon from each PCR reaction was sub-cloned into pET-21a to generate a 6x-Histidine C-terminal tag and into pET-15b to generate a 6x-Histidine N-terminal tag using NdeI and BamHI restriction endonucleases. Plasmids containing genes of the lipoic acid biosynthesis and salvage pathway were transformed into LysY I^Q *E. coli* and plasmids containing the genes of the E2-subunits were transformed into Δ*lipA*::*kan* LysY I^Q *E. coli* to ensure each subunit was non-lipoylated. Both pET-21a and pET-15b encode a *lac* operator upstream of the inserted gene that is induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG). After generation of plasmids capable of expressing 6x-His-LipM, 6x-His-LipA, 6x-His-LipL, 6x-His-LplA1, 6x-His-LplA2, 6x-His-PDH, 6x-His-OGDH, 6x-His-BCODH, 6x-His-GcvH, and 6x-His-GcvH-L, induction of protein expression was assessed by growing the strains in LB for 3 hours at 37°C and then adding 1 mM IPTG to the culture for an additional 3 hours to induce protein expression. Bacterial cell lysates were resolved on 12% sodium dodecyl sulfate polyacrylamide gels at 120 V for 3 hours and total protein was visualized via Coomassie staining using Gel Code Blue (Thermo Scientific). Strains that did not show obvious induction of the protein of interest on a Coomassie stained gel were re-run on an SDS-PAGE gel and transferred onto a 0.2 μM PVDF membrane at 1000 mA for 1 hour. The membrane was incubated overnight in phosphate buffered saline + 0.1% Tween (PBST)

containing 5% Bovine serum albumin (BSA) at 4°C. The membrane was then probed with a 1:3000 dilution of mouse anti-6x-Histidine antibody in PBST+ 5% BSA for 1 hour at room temperature with rocking followed by three 15 min washes in PBST. Goat anti-mouse IgG HRP conjugate was then added to the membrane at a 1:400 dilution for 1 hour followed by an additional three 15 min washes in PBST. 6x-His tagged protein was visualized on an auto-processor after addition of SuperSignal West Pico Chemiluminescent Substrate to the membrane (Thermo).

Protein Purification of Lipoic Acid Biosynthesis and Salvage Enzymes

Enzymes of the lipoic acid biosynthesis and salvage pathway were purified using Ni^{2+} affinity chromatography. LysY I^Q *E. coli* strains containing 6x-Histidine protein expression plasmids for each of the lipoic acid biosynthesis and salvage enzymes were grown in 5 ml LB with 100 μg/ml ampicillin at 37°C with shaking at 220 rpm overnight. The following day, the bacteria were subcultured 1:100 and allowed to grow for 3 hours at 37°C until reaching an OD 600 of 0.25-0.3. Expression of the 6x-Histidine tagged proteins was induced with 0.1 mM IPTG followed by incubation overnight at 16°C with shaking at 220 rpm. The next day, cultures were spun down at 8500 rpm for 10 min at 4°C to collect the cell pellet followed by storage at -80°C. In order to purify the recombinant proteins, bacterial pellets were thawed at 37°C and resuspended in lysis buffer (25 mM imidazole, 50 mM Tris-HCL, 300 mM NaCl, pH 8) supplemented with 1 mM dithiothreitol (DTT) and 1 mM phenylmethane sulfonyl fluoride (PMSF). Using a Branson S-450A large tip sonicator, the bacteria were lysed at a constant rate of 0.8 seconds per pulse and an output of 340 W for 15 min on ice for 20 seconds at a time. The lysed bacteria were then spun down for 30 min at 11,000 rpm followed by filtering the lysate

using a 0.45 μm filter. The supernatant was then incubated with 1 ml nickel-NTA resin (Qiagen) while rocking for 1 hour at 4°C. The resin was washed with 50 mM imidazole, 1 mM DTT, 50 mM Tris-HCL, 300 mM NaCl (pH 8) followed by elution of the bound protein using the same buffer containing 500 mM Imidazole. In order to remove the imidazole, 10 kDa molecular weight cut-off (MWCO) snakeskin dialysis tubing (Thermo Scientific) was used to dialyze the purified protein into 100 mM imidazole + 50 mM Tris-HCL, 300 mM NaCl (pH 8) for 3 hours, then 25 mM imidazole + 50 mM Tris-HCL, 300 mM NaCl (pH 8) overnight, and an additional 3 hours the following day in 50 mM Tris-HCL, 300 mM NaCl (pH 8). The concentration of the purified protein was measured using a bicinchoninic acid (BCA) kit (Thermo Fisher) and stored at -80°C. Protein purity was confirmed by loading 1 μg of purified protein on an SDS-PAGE gel followed by Coomassie staining. Where necessary, proteins were further purified to homogeneity using fast protein liquid chromatography (FPLC).

Protein Purification of the apo E2 and H Subunits of Lipoylated Enzyme Complexes

The E2-subunits of PDH, BCODH, OGDH, GcvH, and GcvH-L were purified from a Δ*lipA*::*kan* LysY I^Q *E. coli* strain using Ni^{2+} affinity chromatography. Δ*lipA*::*kan* LysY I^Q *E. coli* strains containing the 6x-Histidine expression plasmids for each of the apo E2 and H-subunits of *S. aureus* lipoylated enzyme complexes were grown overnight in 30 ml LB with 100 μg/ml ampicillin at 37°C with shaking at 220 rpm. The following day, the strains were subcultured 1:100 into LB with 100 μg/ml ampicillin and grown for 20 hours at 37°C with shaking at 220 rpm. The next day, cultures were induced with 0.5 mM IPTG for an additional 4 hours at 37°C with shaking at 220 rpm. After induction, the bacterial cultures were spun down at 8500 rpm for

10 min at 4°C and stored at -80°C overnight. The remaining steps of the purification of E2 subunits are the same as for the lipoic acid biosynthesis and salvage enzymes.

Lipoylation and Octanoylation Assays

Lipoylation assays were set up as described by Martin *et al*. (16). Assays were conducted in 50 µl reaction volumes in a 50 mM Tris-HCl, 300 mM NaCl buffer (pH 8.0) containing 6 mM ATP, 1 mM DTT, 1 mM $MgCl_2$, 2 µM purified LplA1 or LplA2, and 10 µM substrate (apo E2-PDH, apo E2-OGDH, apo E2-BCODH, apo GcvH, or apo GcvH-L). The reactions were incubated with or without lipoic acid (2.4 mM), octanoic acid (2.4 mM), lipoamide (2.4 mM), or DK^LA (2.4 mM) for 2 hours at 37°C shaking at 600 rpm. After incubation, the reaction mixtures were run out on 12% SDS-PAGE gels at 120 V for approximately 3 hours. SDS-PAGE gels were stained with Gel Code Blue (Thermo Scientific) as a loading control and to visualize octanoylation/lipoylation via a shift in band size. In addition, SDS-PAGE gels were transferred to 0.2 µM PVDF membrane at 1000 mA for 1 hour. After transfer, the membrane was incubated overnight in Tris-buffered saline + 0.1% Tween (TBST) containing 5% BSA at 4°C. The following day, the membrane was probed with a 1:7500 dilution of rabbit anti-lipoic acid antibody in TBST + 5% BSA for 1 hour at room temperature with rocking. Goat anti-rabbit IgG AP conjugate was then added to the membrane at a 1:5000 dilution in TBST + 5% BSA for 1 hour at room temperature, followed by an additional three 15 min washes in TBST. Lipoylated proteins were visualized using a colorimetric detection method by adding 66 µl of nitro-blue tetrazolium (NBT) (50 mg NBT in 1 ml 70% dimethylformamide (DMF)/30% H_2O) and 35 µl of 5-bromo-4-chloro-3'-indolyphosphate (BCIP) (50 mg BCIP in 1 ml DMF) to 10 ml AP Buffer (100 mM Tris, 100 mM NaCL, 5 mM $MgCl_2$, pH 9.5) and incubating the membrane with this

solution for ~2 min rocking at room temperature. Development of the blot was stopped by washing the membrane with water and allowing it to dry at 37°C for 15 min.

CHAPTER THREE

EXPERIMENTAL RESULTS

Introduction

Staphylococcus aureus is a Gram-positive commensal bacterium commonly found on the skin, nasal passages, and in the gastrointestinal tract. In addition, *S. aureus* can also be a major human pathogen in both hospital and community settings, where it is known to cause a wide range of conditions such as mild skin and soft tissue infections (SSTI), bacteremia, sepsis, and osteomyelitis (4; 9; 11). The success of *S. aureus* as a pathogen stems from its ability to infect a wide range of host tissues. In order to thrive in such diverse environments, *S. aureus* uses multiple adaptive traits such as trace metal/nutrient acquisition, shifts in metabolic activity, and expression of detoxification systems, all of which allow the bacterium to proliferate and survive in nutritionally deficient and inhospitable environments (8; 9; 11).

Instrumental to survival in nutrient restricted environments is the ability of *S. aureus* to acquire trace metals and other metabolic cofactors important for optimal metabolism. One essential metabolite used by *S. aureus* is lipoic acid. A former graduate student, Azul Zorzoli, used a genetic approach to define the lipoic acid biosynthesis and salvage pathways of *S. aureus*. Her studies determined that *S. aureus* synthesizes lipoic acid *de novo* from an octanoic acid precursor, or through salvage mechanisms, where free lipoic acid is acquired from the environment by the action of lipoic acid ligases, LplA1 and LplA2. In the *Firmicutes* phylum, *S. aureus* is the only pathogenic bacterium that encodes all *de novo* biosynthesis genes, as well as

two lipoic acid ligases involved in salvage (8). Azul's data demonstrated that LplA1, but not LplA2, is necessary for the salvage of lipoic acid in vitro, whereas both LplA1 and LplA2 are sufficient to promote infection of specific tissues in vivo. Furthermore, the work of Rack *et al.* suggests that *lplA2* gene expression is sub-optimal in vitro (72). Because the LplA2 ligase does not have a discernable function in vitro, its exact role in lipoic acid salvage is unknown. Based on these two pieces of information, I hypothesized that LplA1 and LplA2 stimulate growth by using alternative lipoylated substrates and/or that *lplA2* gene expression is induced in vivo compared to LplA1.

Construction of Lipoic Acid Ligase Mutants (Δ*lipA* Δ*lplA1* and Δ*lipM* Δ*lplA1*) to Test the Function of LplA2 In Vitro

Current literature states that *L. monocytogenes* encodes two lipoic acid ligases, LplA1 and LplA2, which have been shown to use alternative lipoylated substrates (54; 53). In addition, our preliminary data suggest that LplA2 in *S. aureus* has a functional role in vivo, however its activity could not be demonstrated in vitro. Based on this information, I hypothesized that LplA2 may use alternative lipoylated substrates in vivo to stimulate bacterial replication. I constructed Δ*lipA* Δ*lplA1* and Δ*lipM* Δ*lplA1* in-frame deletion mutants in order to ascertain the potential divergent activities of LplA2 and test its ability to use alternative sources of lipoic acid. Δ*lipA* Δ*lplA1* and Δ*lipM* Δ*lplA1* in-frame deletion mutants were verified by PCR amplification using primers that anneal 500 bp upstream and 500 bp downstream of genes *lipA, lplA1,* and *lipM.* PCR amplification resulted in a ~2 kb amplicon for all WT alleles (*lipA, lipM,* and *lipL* are each approximately 1 kb) and a ~1kb band for mutant Δ*lipA*, Δ*lipM*, and Δ*lplA1* alleles (Fig 8A and 8B). These data indicate successful construction of Δ*lipA* Δ*lplA1* and Δ*lipM* Δ*lplA1* mutants.

These mutants will be used in conjunction with already constructed Δ*lipA*, Δ*lipA* Δ*lplA2*, Δ*lipA* Δ*lplA1* Δ*lplA2*, Δ*lipA* Δ*lplA1* Δ*lplA2* + lplA1, Δ*lipA* Δ*lplA1* Δ*lplA2* + lplA2, and Δ*lipA* Δ*lplA1* Δ*lplA2* Δ*lipL::kan* mutants, to assess the ability of both LplA1 and LplA2 to use a variety of lipoylated substrates in vitro.

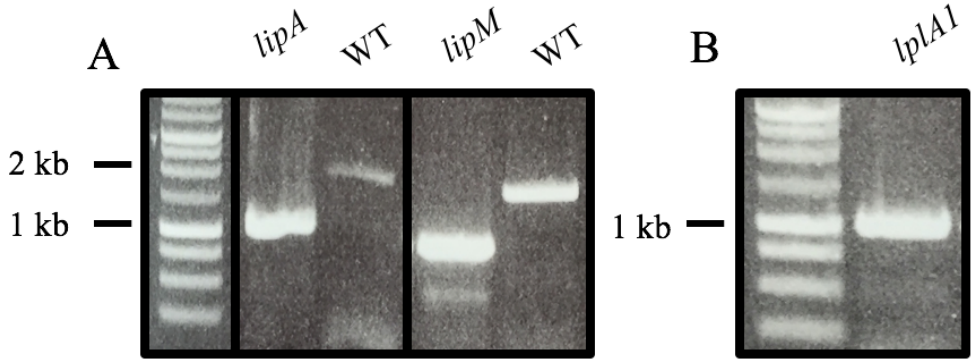


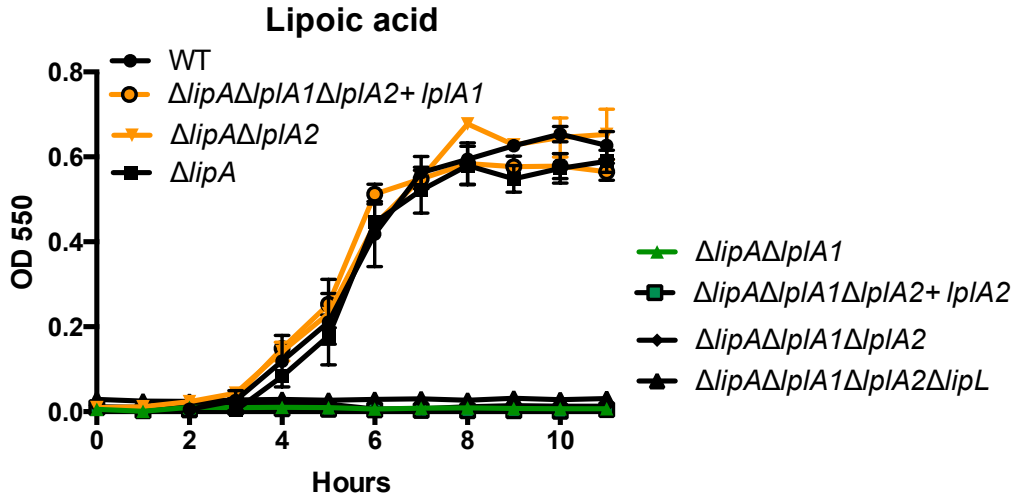
Figure 8. Verification of Δ*lipA*Δ*lplA1* and Δ*lipM*Δ*lplA1* mutants via PCR
PCR samples were run out on a 0.8% agarose gel to A. verify the deletion of Δ*lipA* and Δ*lipM* and B. Verify the deletion of Δ*lplA*.

Identify the Substrates Used by LplA1 and LplA2 by Assessing Growth Phenotypes after Supplementation with Different Lipoylated Substrates

Our preliminary data suggest that LplA1 uses free lipoic acid during in vitro growth whereas LplA2 does not. Nevertheless, it remains to be determined if either of the ligases can use alternative lipoylated substrates as a source of lipoic acid (71). As mentioned previously, *L. monocytogenes* encodes two lipoic acid ligases, LplA1 and LplA2, one of which uses alternative lipoylated substrates for growth (54; 53). From these data, I hypothesized that LplA2 uses lipoylated substrates other than free lipoic acid to stimulate growth in vitro. In order to assess the ability of LplA2 to use alternative lipoylated substrates, I conducted growth curves supplemented with lipoic acid, lipoamide, digested/undigested OGDH, digested/undigested PDH, and octanoic acid using WT, Δ*lipA,* Δ*lipA* Δ*lplA1*, Δ*lipA* Δ*lplA2*, Δ*lipA* Δ*lplA1* Δ*lplA2*, Δ*lipA* Δ*lplA1* Δ*lplA2* +

lplA1, Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2*, and Δ*lipA* Δ*lplA1* Δ*lplA2* Δ*lipL* strains. When the medium was supplemented with lipoic acid and lipoamide, Δ*lipA,* Δ*lipA* Δ*lplA2* and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1* mutants replicated, whereas Δ*lipA* Δ*lplA1* and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2* mutants were not able to grow (Fig 9A and 9B). As suspected, when the medium was supplemented with undigested PDH and OGDH only the WT strain replicated (Fig 10A and 10B). Contrary to my initial hypothesis, neither digested OGDH nor digested PDH could stimulate growth of the Δ*lipA* Δ*lplA1* and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2* strains (Fig 11A and 11B). Instead, Δ*lipA,* Δ*lipA* Δ*lplA2* and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1* mutants were able to replicate using digested PDH but not digested OGDH (Fig 11A and 11B). When the growth medium was supplemented with octanoic acid, the mutant strains were not able to grow due to a non-functional *de novo* biosynthesis pathway, similar to RPMI medium without supplements (Fig 12A and 12B). These data indicate that LplA1 may have substrate specificities that extend beyond free lipoic acid and that LplA2 appears to play no apparent role in using lipoic acid or alternative substrates of lipoic acid in vitro.

A.



B.

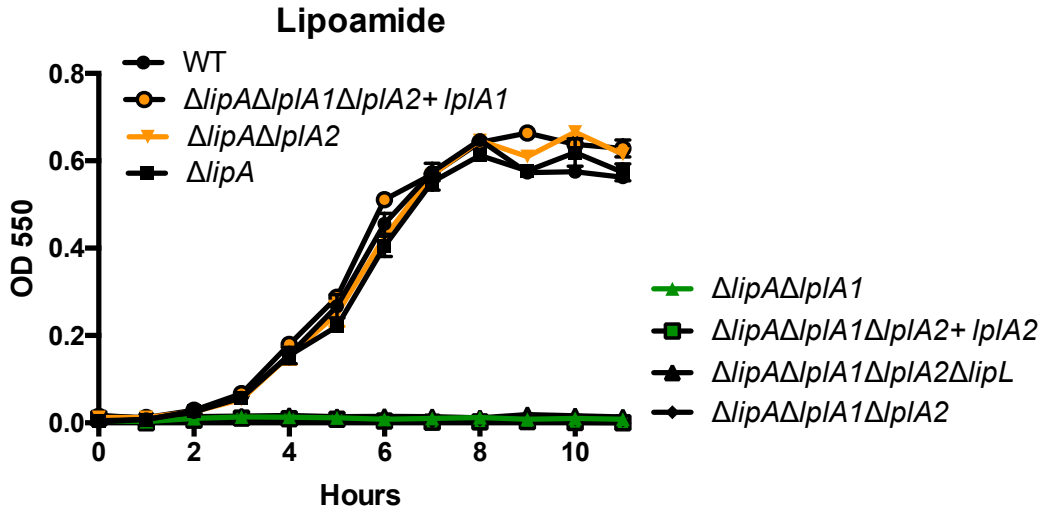
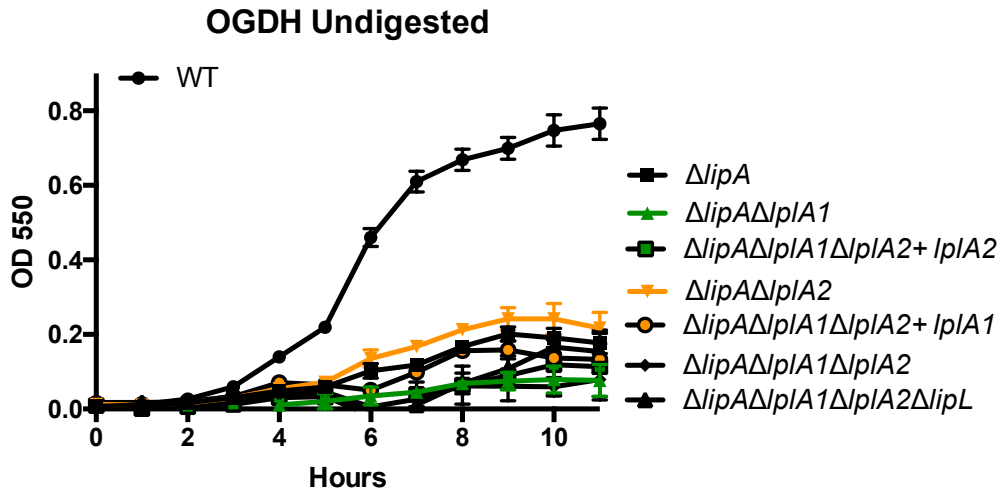


Figure 9. LplA1, but not LplA2, uses free lipoic acid and lipoamide as a lipoyl substrate
Growth assessment of lipoic acid ligase mutants in RPMI supplemented with A. Lipoic acid (10 μg/ml) and B. Lipoamide (10 μg/ml). Strains colored in orange test for the function of LplA1, whereas the strains colored in green test for the function of LplA2.

A.



B.

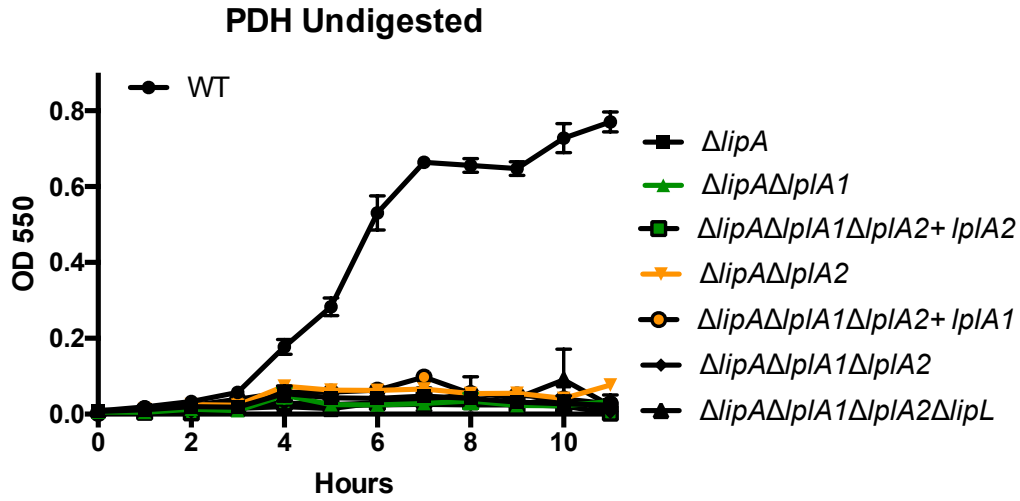
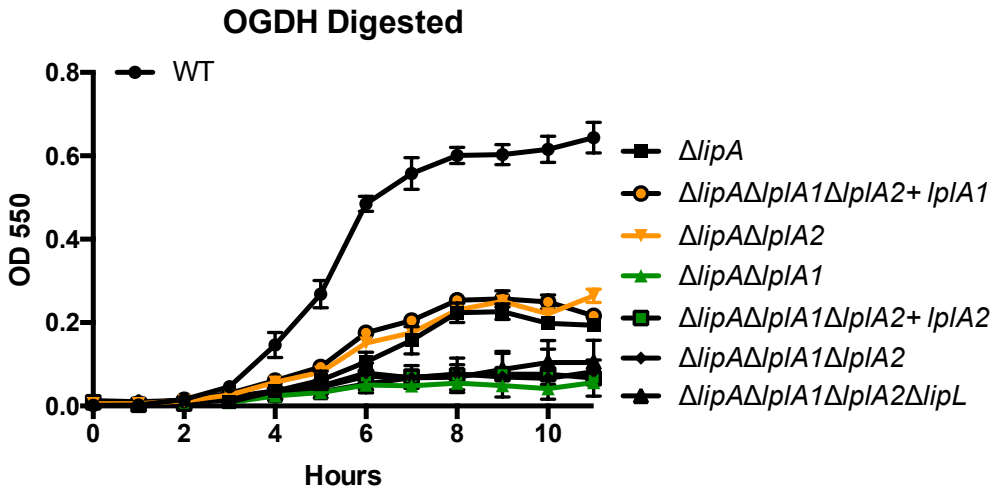


Figure 10. Neither LplA1 nor LplA2 use undigested OGDH and PDH to stimulate growth
Growth assessment of lipoic acid ligase mutants in RPMI supplemented with A. undigested OGDH (1.16 mg/ml) and B. undigested PDH (2.5 mg/ml). Strains colored in orange test for the function of LplA1, whereas the strains colored in green test for the function of LplA2.

A.



B.

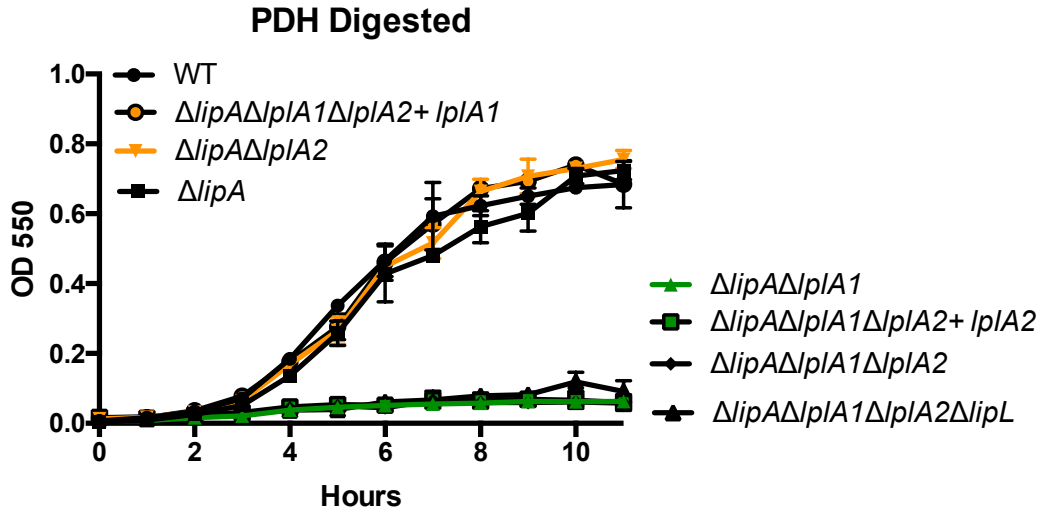
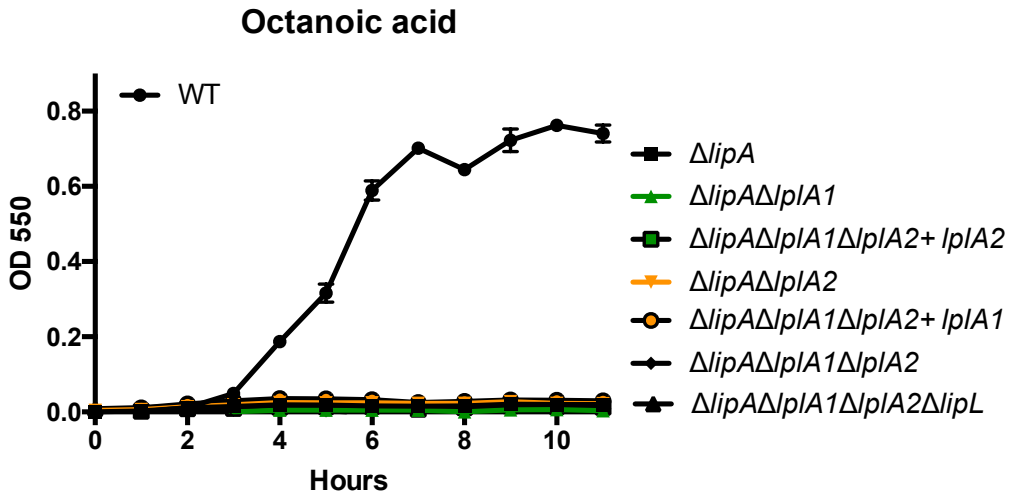


Figure 11. Only LplA1 uses digested PDH, but not digested OGDH, to stimulate growth
Growth assessment of lipoic acid ligase mutants in RPMI supplemented with A. digested OGDH (1.15 mg/ml) and B. digested PDH (2.5 mg/ml). Strains colored in orange test for the function of LplA1, whereas the strains colored in green test for the function of LplA2.

A.



B.

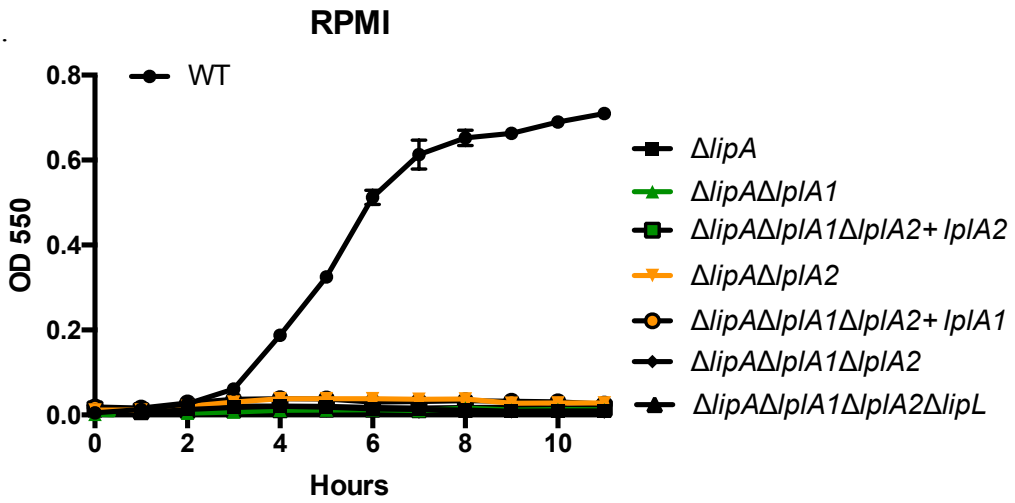


Figure 12. *S. aureus* lipoic acid biosynthesis and salvage mutants cannot grow in lipoic acid free medium
Growth assessment of lipoic acid ligase mutants in RPMI supplemented with A. octanoic acid (6.9 μg/ml) and B. RPMI alone. Strains colored in orange test for the function of LplA1, whereas the strains colored in green test for the function of LplA2.

Assessing the Ability of LplA1 and LplA2 to Use Synthetic Lipoylated ($DK^{L}A$) and Non-Lipoylated (DKA) Tripeptides

As mentioned previously, I know that lipoic acid ligases from at least one other bacterium have affinity for different lipoylated substrates and my data suggest that LplA1 can use lipoylated PDH peptides to stimulate growth (54; 53). A potential pitfall of these lipoyl-substrate addition studies is that they used entire PDH and OGDH complexes as sources of lipoic acid, even though it is known that only the E2 subunits contain lipoyl moieties. When porcine PDH and OGDH are digested with proteinase K the smallest lipoyl peptide achieved is $DK^{L}A$ and $DK^{L}T$ respectively (54). In addition, the PDH and OGDH tripeptide sequences share similarity with human, murine, and rat lipoyl domains (Fig 13A and 13B). Therefore, I repeated the growth curves using synthetic lipoylated and non-lipoylated DKA to ensure that other components of the PDH complex do not facilitate growth by a mechanism that does not depend on lipoic acid. Based on this information and previous observations, which indicated the ability of LplA1 to use PDH as a source of lipoic acid, I hypothesized that LplA1, but not LplA2 would stimulate bacterial growth when supplemented with $DK^{L}A$. In order to assess the ability of LplA1 and LplA2 to use the tripeptide $DK^{L}A$ and DKA, I conducted growth curves using WT, Δ*lipA,* Δ*lipA* Δ*lplA1*, Δ*lipA* Δ*lplA2*, Δ*lipA* Δ*lplA1* Δ*lplA2*, Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1*, Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2*, and Δ*lipA* Δ*lplA1* Δ*lplA2* Δ*lipL* strains. When the growth medium was supplemented with $DK^{L}A$, both Δ*lipA* Δ*lplA2* and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1* were able to grow, whereas Δ*lipA* Δ*lplA1* and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1* strains were not (Fig 14A). As expected, when RPMI medium was supplemented with DKA only the WT strain was able to replicate (Fig 14B). These data are consistent with my previous growth curves using digested

PDH complexes and suggest that only LplA1 is able to use peptide bound lipoic acid, or that LplA2 is not sufficiently expressed under these conditions.

A.

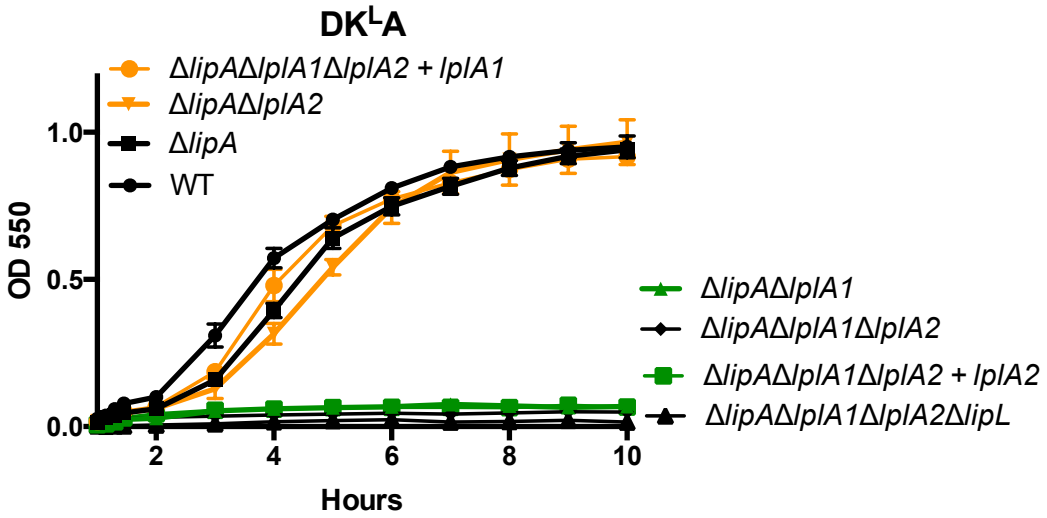
E2- PDH	
Homo sapien	VETDK^{L}ATVG IETDK^{L}ATIG
Sus scrofa	VETDK^{L}ATVG IETDK^{L}ATIG
Rattus norvegicus	VETDK^{L}ATVG IETDK^{L}ATIG
Mus musculus	VETDK^{L}ATVG IETDK^{L}ATIG

B.

E2- OGDH	
Homo sapien	IETDK^{L}TSVQ
Sus scrofa	IETDK^{L}TSVQ
Rattus norvegicus	IETDK^{L}TSVQ
Mus musculus	IETDK^{L}TSVQ

Figure 13. Amino acid sequences of the lipoyl domains from PDH and OGDH complexes
A. PDH lipoyl domain from *Homo sapien* (NP_001922.2), *Sus scrofa* (NP_999159.1), *Rattus norvegicus* (NP_112287.1), and *Mus musculus* (NP_663589.3) B. OGDH lipoyl domain from *Homo sapien* (NP_001231812.1), *Sus scrofa* (NP_999159.1), *Rattus norvegicus* (NP_001006982.2), and *Mus musculus* (NP_084501.1)

A.



B.

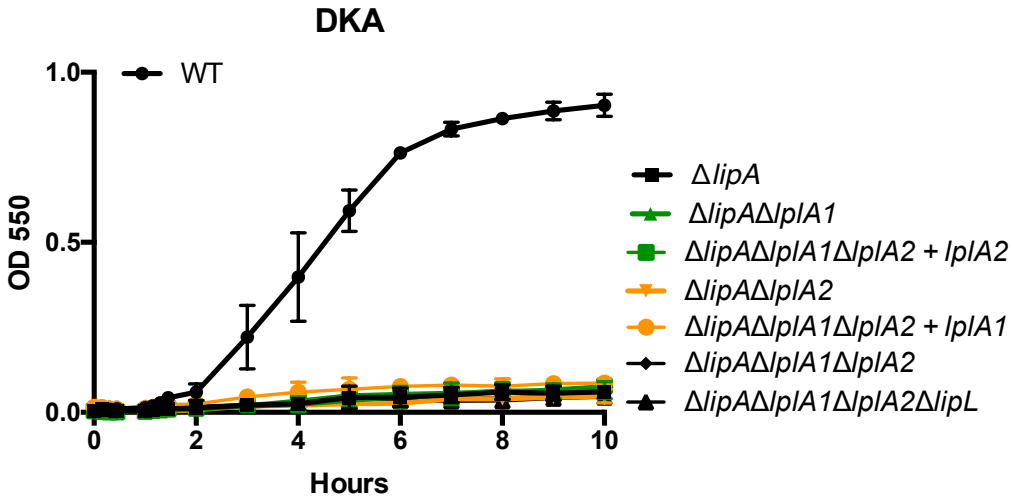


Figure 14. Only LplA1 uses DK^LA as a source of lipoyl substrate

Growth assessment of lipoic acid ligase mutants in RPMI supplemented with A. DK^LA (100 μM) and B. DKA (100 μM). Strains colored in orange test for the function of LplA1, whereas the strains colored in green test for the function of LplA2.

Assessing the Ability of LplA1 and LplA2 to use Synthetic Lipoylated ($DK^{L}T$) and Non-Lipoylated (DKT) Tripeptides

The above studies indicated that only LplA1 can use digested PDH, but not digested OGDH as a source of lipoic acid. To ensure that other components of digested PDH did not stimulate growth, I repeated the growth curves with the smallest known lipoyl moiety, $DK^{L}A$, when PDH is digested with proteinase K. Consistent with my previous data from growth curves supplemented with digested PDH, only LplA1 was able to use $DK^{L}A$ to stimulate bacterial replication. When OGDH is digested with proteinase K, the smallest known lipoyl moiety achieved is a $DK^{L}T$ tripeptide. Since digested OGDH and digested PDH have different amino acids flanking the lipoylated lysine and only digested PDH stimulated bacterial growth, I hypothesized that LplA1 can only recognize the lipoyl moiety in a specific context of these amino acids and will not be able to use $DK^{L}T$ to stimulate bacterial growth. To test this hypothesis and assess the ability of LplA1 and LplA2 to use the synthetic lipoylated and non-lipoylated DKT tripeptide, I conducted growth curves using WT, Δ*lipA,* Δ*lipA* Δ*lplA1*, Δ*lipA* Δ*lplA2*, Δ*lipA* Δ*lplA1* Δ*lplA2*, Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1*, Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2*, and Δ*lipA* Δ*lplA1* Δ*lplA2* Δ*lipL* strains. Contrary to my proposed hypothesis but consistent with my previous data, both Δ*lipA* Δ*lplA2* and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1* were able to grow, whereas Δ*lipA* Δ*lplA1* and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1* strains were not when RPMI medium was supplemented with $DK^{L}T$ and $DK^{L}A$ (Fig 15A and 15B). As expected, when I supplemented the RPMI medium with the non-lipoylated DKT and DKA tripeptide, only the WT strain was able to grow (Fig 15C and 15D). These data suggest that only LplA1, and not LplA2, can recognize the

lipoyl moiety on the conserved lysine in the context of different amino acids and that other components of digested OGDH may inhibit LplA1 to efficiently use the OGDH lipoyl moiety.

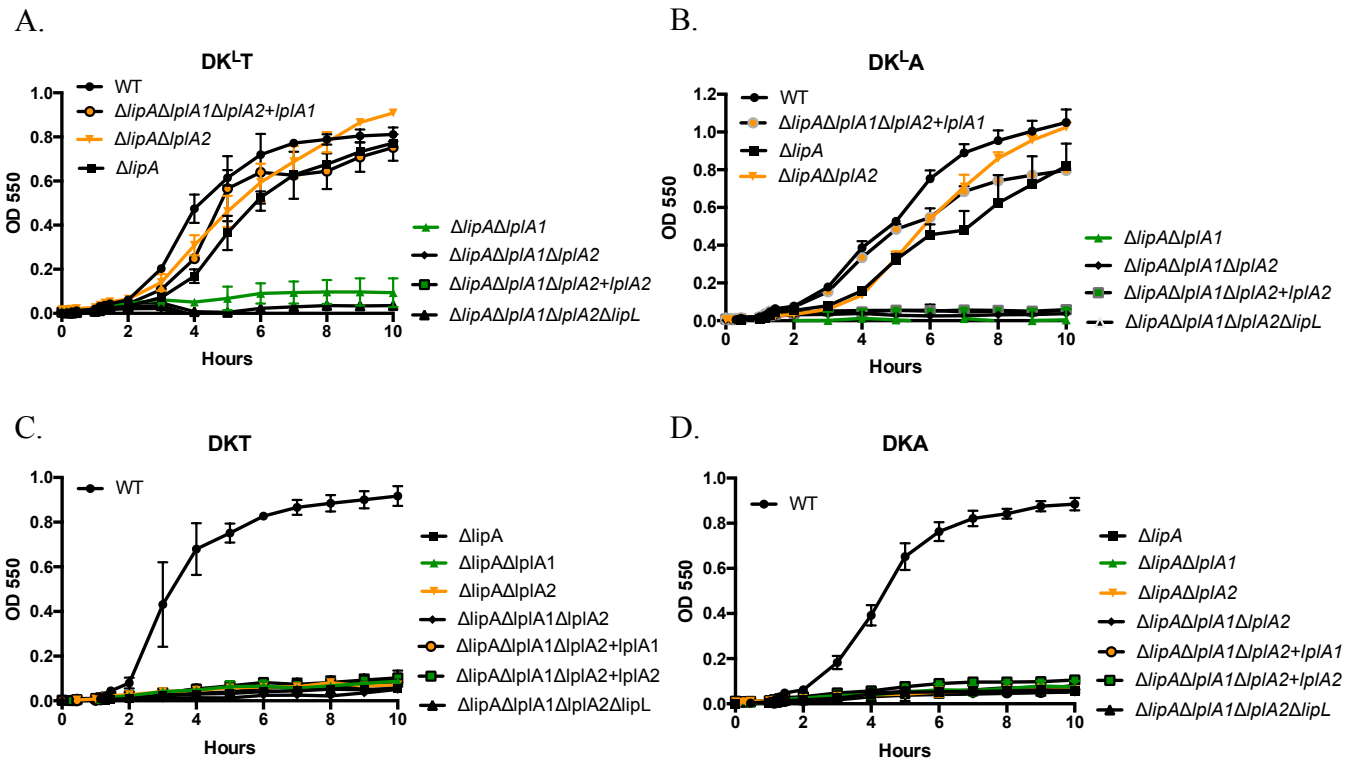


Figure 15. Both DK^{L}A and DK^{L}T stimulate bacterial replication in the presence of LplA1 but not LplA2
Growth assessment of lipoic acid ligase mutants in RPMI supplemented with A. DK^{L}T (100μM), B. DK^{L}A (100μM), C. DKT (100μM) and D. DKA (100μM). Strains colored in orange test for the function of LplA1, whereas the strains colored in green test for the function of LplA2.

Generation of a Transcriptional Reporter Fusion of the P_{lplA2} Promoter to *gfp*

In a recent study, Rack *et al.* suggested that LplA2 in *S. aureus* is responsible for lipoylating the GcvH-like protein, GcvH-L, using lipoic acid or lipoamide. This lipoylation allows for subsequent ADP-ribosylation of GcvH-L by a macrodomain-linked sirtuin (SirTM). These post-translational modifications were hypothesized to promote resistance to host oxidative stress responses and thereby permit bacterial survival during infection, however this activity has yet to be verified (72; 73). Interestingly, this study, as well as early transcriptome work, alludes

to the fact that the lack of LplA2 activity in vitro may be due to low levels of gene expression in the in vitro growth conditions used (73). Based on this information and my prior data which was unable to demonstrate LplA2 activity in vitro, I hypothesized that the induction of redox stress conditions may allow for increased *lplA2* gene expression and better incorporation of lipoic acid. In order to further elucidate the regulatory inputs that facilitate *lplA2* gene expression, I generated a transcriptional reporter by fusing the predicted promoter region of *lplA2* to *gfp*. With this promoter fusion, I will be able to use *gfp* expression to directly measure *lplA2* promoter activity under a variety of environmental conditions. Since the promoter region of *lplA2* is unknown and a small putative gene exists 100 bp upstream of the first gene in the *lplA2* operon (*LLM*), I generated a short (100 bp) and long (300 bp) pOS1-P_{lplA2}-*gfp* construct and transformed both into Δ*lipA* Δ*lplA1* and Δ*lipM* Δ*lplA1* strains in order to assess *lplA2* gene expression (Fig 16). To verify the presence of the desired promoter element in these plasmids, I PCR amplified both the 100 bp and 300 bp target regions and ran them on a 1.2% agarose gel. The outcome of these PCRs determined that the Δ*lipA* Δ*lplA1* and Δ*lipM* Δ*lplA1* strains each harbored the appropriate *gfp* reporter plasmid corresponding to the short (~100bp) and long (~300bp) putative *lplA2* promoter region when compared to the WT genomic DNA control (Fig 16). These data indicate that I have successfully transformed the short and long pOS1-P_{lplA2}-*gfp* promoter fusions into Δ*lipA* Δ*lplA1* and Δ*lipM* Δ*lplA1* strains. Due to time constraints and per recommendation by my committee, I decided to focus my attention on assessing the functional differences between LplA1 and LplA2 using biochemical approaches instead of *lplA2* expression under different stress conditions.

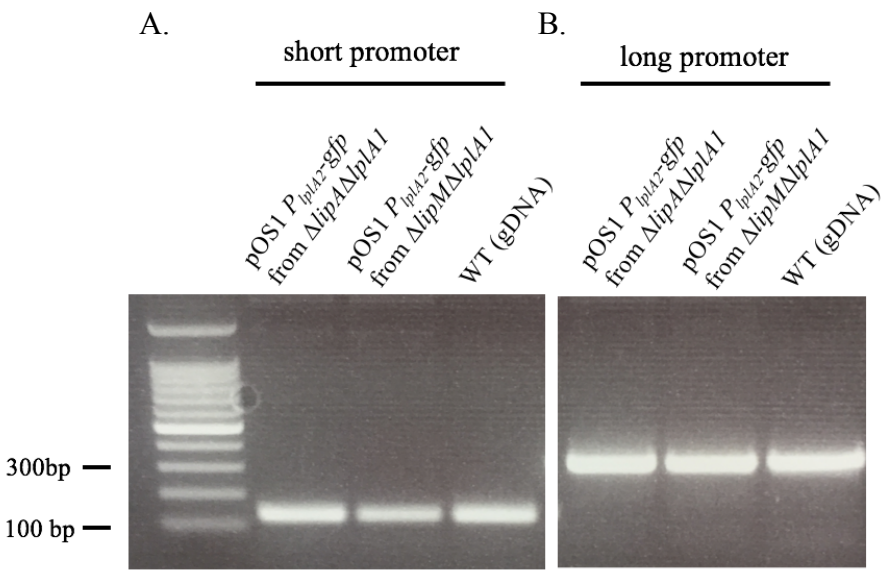


Figure 16. Verification of short and long pOS1-P_{lplA2}-*gfp* promoter fusions in Δ*lipA* Δ*lplA1* and Δ*lipM* Δ*lplA1* mutants via PCR
PCR samples were resolved on a 0.8% agarose gel to A. Verify the introduction of the short pOS1-P_{lplA2}-*gfp* plasmid into Δ*lipA* Δ*lplA1* and Δ*lipM* Δ*lplA1* strains and B. Verify the introduction of the long pOS1-P_{lplA2}-*gfp* into Δ*lipA* Δ*lplA1* and Δ*lipM* Δ*lplA1* strains.

Generation of 6x-Histidine Tagged Protein Expression Constructs of the Five Lipoic Acid Biosynthesis and Salvage Enzymes

In our previous studies, a genetic approach was used to identify the genes involved in lipoic acid biosynthesis and salvage in *S. aureus* (71). However, the precise activity of LplA1 and LplA2 and their substrate usage remains to be determined. I hypothesized that LplA1 and LplA2 are responsible for facilitating lipoylation of the metabolic complexes PDH, OGDH, BCODH, GcvH, and GcvH-L, although the two enzymes may have differing affinities and/or activities. In order to assess the functions of LplA1 and LplA2 biochemically, I first needed to express and purify recombinant LplA1 and LplA2 and their potential substrates/interacting partners. In order to purify the recombinant proteins, I generated 6x-Histidine tagged protein expression plasmids and screened for induction of LipM, LipA, LipL, LplA1, and LplA2 in the presence of 1mM IPTG. When IPTG was added to the culture medium to induce gene

expression, I observed overexpression of LipM, LipA, and LipL when whole cell lysates were resolved on an SDS-PAGE gel stained with Coomassie dye compared to uninduced controls (Fig 17). However, no apparent overexpression was seen for LplA1 and LplA2 (Fig 18), indicating that either they do not express the 6x-Histidine tagged protein or that expression is not high enough to detect on a Coomassie stained gel. In order to assess whether these 6x-Histidine tagged constructs are expressing LplA1 and LplA2, I performed immunoblots using an anti-6x-Histidine antibody to visualize the tagged proteins (Fig 18). These immunoblots indicated that LplA1 and LplA2 were being induced, albeit at lower levels than LipA, LipM, and LipL. Together these data indicate that N-terminal 6x-Histidine tagged LipM, LipA, LipL, LplA1, and LplA2 can successfully be overexpressed in *E. coli*.

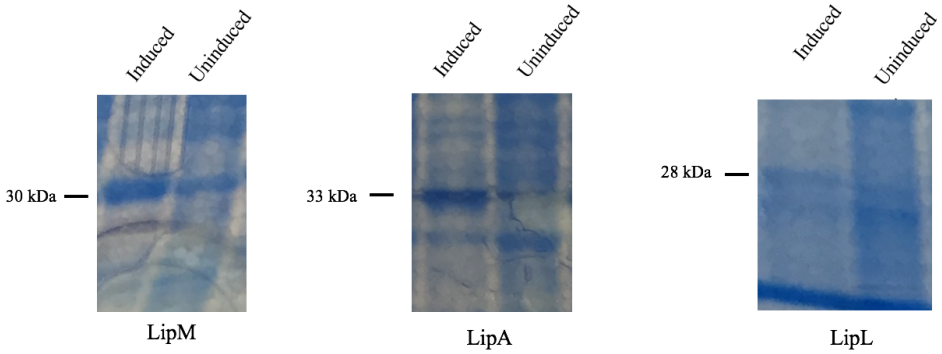


Figure 17. 6x-Hisitidine tagged LipM, LipA, and LipL of the *de novo* biosynthesis pathway can be successfully overexpressed with the addition of 1mM IPTG

Bacterial whole cell lysates of LysY I^Q *E. coli* strains harboring 6x-His-LipM, 6x-His-LipA, or 6x-His-LipL, induced with or without 1 mM IPTG, were resolved on an SDS PAGE gel and stained with Coomassie blue to assess the ability for each strain to overexpress the 6x-Histidine tagged protein.

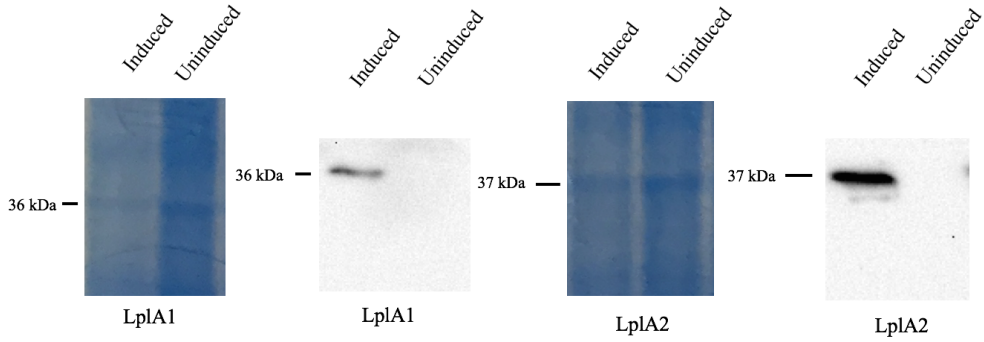


Figure 18. Trial Induction and Immunoblot of 6x-Hisitdine tagged LplA1 and LplA2
Bacterial whole cell lysates of LysY I^Q *E. coli* strains harboring 6x-His-LplA1 or 6x-His-LplA2, induced with 1mM IPTG, were resolved on an SDS PAGE gel and stained with Coomassie blue or transferred and used in immunoblots probed with mouse anti-6x-Histidine antibody.

Purification of 6x-Histidine Tagged Lipoic Acid Biosynthesis and Salvage Enzymes from *E. coli* and Apo E2-PDH, Apo E2-OGDH, Apo E2-BCODH, Apo GcvH, and Apo GcvH-L from Δ*lipA E. coli*

In order to evaluate the role of LplA1 and LplA2 in the lipoic acid salvage pathway and further investigate the sequential steps of lipoylation, I purified the five 6x-Histidine tagged proteins of the *de novo* biosynthesis and salvage pathway and the corresponding substrates E2-PDH, E2-OGDH, E2-BCODH, GcvH, and GcvH-L. To verify purity of the proteins, I ran an SDS PAGE gel of the five enzymes of the *de novo* biosynthesis and salvage pathway and the purified apo E2-subuntis of PDH, OGDH, BCODH, GcvH, and GcvH-L (~1 μg loaded) (Fig 19A and 19B). I expected to see a 36 kDa band for LplA1, a 37 kDa band for LplA2, a 30 kDa band for LipM, a 28 kDa band for LipL, and a 33 kDa band for LipA. In addition, I expected to see a 72 kDa band for PDH, a 65 kDa band for OGDH, a 51 kDa band for BCODH, a 23 kDA band for GcvH, and a 20 kDa band for GcvH-L. All purified proteins had the correct anticipated size (Fig 19). These data indicate that I have successfully purified all of the lipoic acid

biosynthesis and salvage enzymes, as well as apo E2-PDH, apo E2-OGDH, apo E2-BCODH, apo GcvH, and apo GcvH-L proteins.

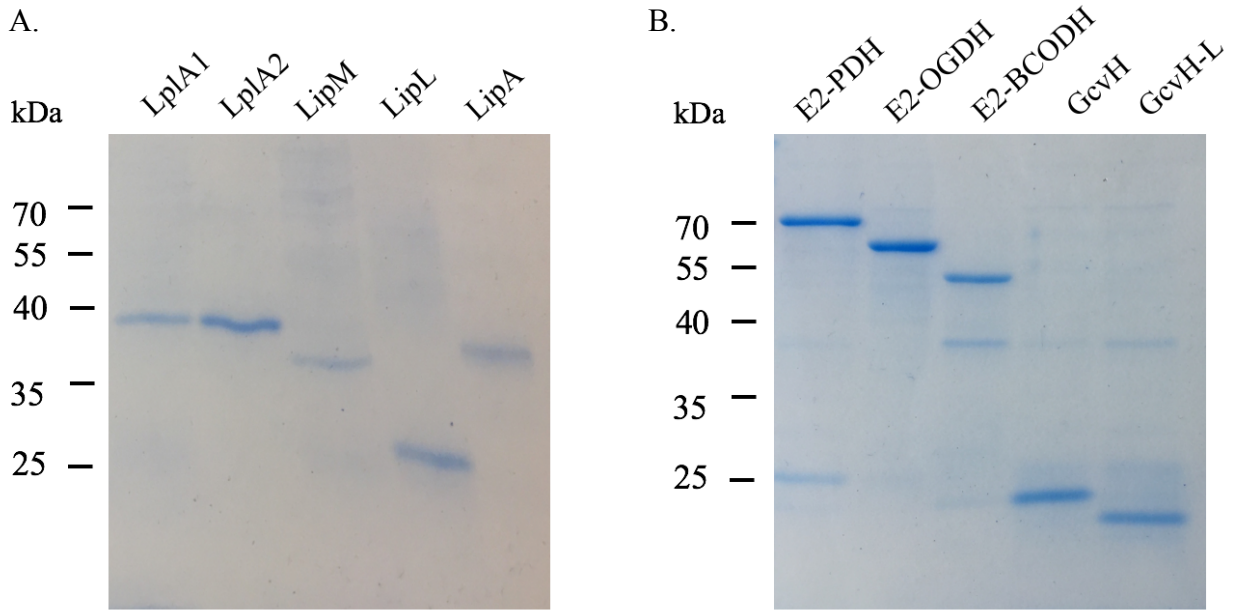


Figure 19. Purified proteins of the *de novo* biosynthesis and salvage pathway and purified lipoyl domains of the known lipoylated enzyme complexes
A. Coomassie blue staining of purified LplA1, LplA2, LipM, LipL, and LipA on a 12% SDS PAGE gel, ~1µg loaded B. Coomassie blue staining of purified apo E2-PDH, apo E2-OGDH, apo E2-BCODH, apo GcvH, and apo GcvH-L on a 12 % SDS PAGE gel, ~1µg loaded.

Assessing the Activity and Substrate Usage of LplA1 and LplA2 In Vitro

Only LplA1 Lipoylates Apo GcvH.

Our previous genetic evidence suggests that LplA1 is able to lipoylate GcvH using lipoic acid in vitro, however no such activity has been detected for LplA2 (71). Therefore, I tested whether or not LplA2 is capable of directly lipoylating H subunits by conducting lipoylation assays with GcvH in the presence of lipoic acid, lipoamide, the tripeptide $DK^{L}A$, and octanoic acid followed by resolving lipoylation reactions on an SDS-PAGE gel and staining with Coomassie dye or transferring to PVDF membranes and immunoblotting with anti-lipoic acid antibody. When the reaction was supplemented with free lipoic acid, I detected a lipoyl band and

shift in band size on the Coomassie-stained gel for the reaction containing LplA1, but not for LplA2 (Fig 20A). When incubated with lipoamide, I detected a lipoyl band but no shift in band size on the Coomassie-stained gel for LplA1, whereas no lipoylation was detected with LplA2 (Fig 20B). The lipoyl band detected upon incubation with LplA1 was more intense when the reaction was incubated with lipoic acid rather than lipoamide. I did not detect a lipoyl band for either LplA1 or LplA2 when the reaction was incubated with $DK^{L}A$ (Fig 20C). When I assessed the ability of LplA1 and LplA2 to octanoylate GcvH, I also detected a shift in band size on the Coomassie-stained gel for LplA1, but not LplA2 (Fig 20D). Currently the lab does not have an anti-octanoic acid antibody that is functional in immunoblotting procedures; as a result octanoylation was only evaluated via a shift in band size. Overall, these data indicate that LplA1, but not LplA2, directly lipoylates/octanoylates apo-GcvH using lipoic acid, lipoamide, and octanoic acid. However, due to the increased intensity of the lipoyl band and shift in band size in the presence of lipoic acid, it appears that lipoic acid is the preferred substrate used by LplA1 to lipoylate apo GcvH.

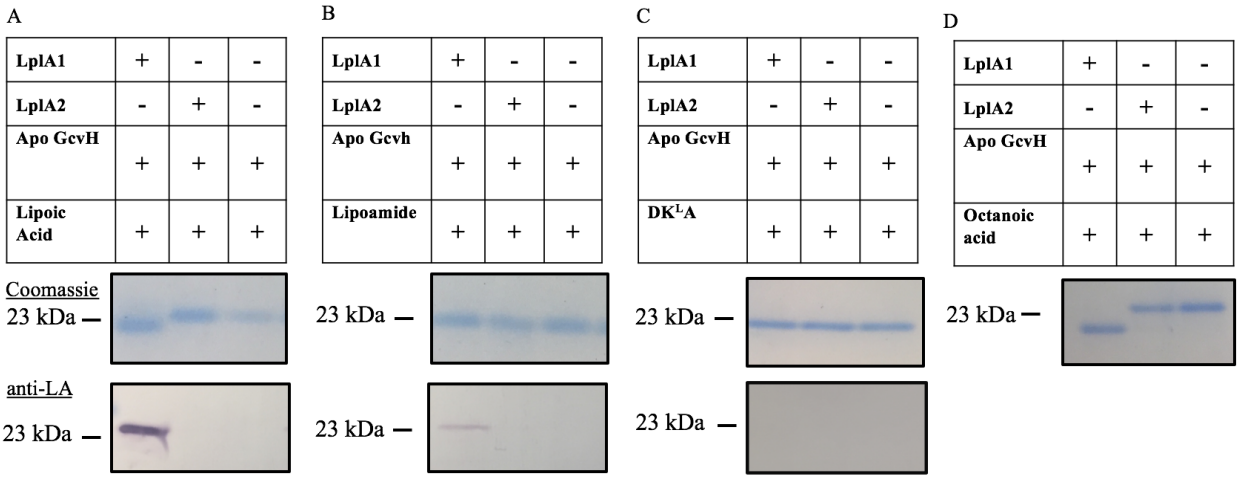


Figure 20. LplA1, but not LplA2, directly uses lipoic acid, lipoamide, and octanoic acid to lipoylate/octanoylate apo GcvH

Biochemical assessment of LplA1 and LplA2 lipoylation/octanoylation of apo GcvH using A. lipoic acid (2.4 μM) B. lipoamide (2.4 μM) C. DK^LA (2.4 μM) and D. octanoic acid (2.4 μM). Lipoylation was visualized by resolving reactions on a 12% SDS PAGE gel stained with Coomassie blue (top panel) and conducting an immunoblot (bottom panel) probed with a mouse anti-lipoic acid antibody (anti-LA). Octanoylation was visualized on a 12% SDS PAGE gel stained with Coomassie blue (top panel).

Both LplA1 and LplA2 Lipoylate Apo GcvH-L.

Based on the genome database, it is known that *S. aureus* encodes two GcvH proteins, GcvH and GcvH-L, and two lipoic acid ligases. In addition, it is known that both LplA2 and GcvH-L are encoded in the same operon. Since my data indicate that only LplA1 is responsible for lipoylating GcvH, I reasoned that LplA2 is responsible for lipoylating GcvH-L. To test this assertion, I determined whether LplA1 and LplA2 are able to lipoylate GcvH-L using lipoic acid, lipoamide, the tripeptide DK^LA, or octanoic acid. When the reaction was incubated with free lipoic acid, I detected lipoyl bands and observed a shift in GcvH-L band size on the Coomassie stained gel for reactions containing either LplA1 or LplA2 (Fig 21A). The lipoyl band from the reaction containing LplA1 appeared more intense compared to LplA2 (Fig 21A). In addition, I detected lipoyl bands for both LplA1 and LplA2 when the reaction was supplemented with lipoamide (Fig 21B). I did not detect lipoyl bands for either LplA1 or LplA2 when the reaction

was supplemented with $DK^{L}A$ (Fig 21C). Lastly, when incubated with octanoic acid, I observed a shift in GcvH-L band size on the Coomassie stained gel for the reactions containing both LplA1 and LplA2 compared to the negative control (Fig 21D). These data indicate that both LplA1 and LplA2 can use lipoic acid and lipoamide to lipoylate apo GcvH-L and octanoic acid as a source for octanoylation of apo GcvH-L.

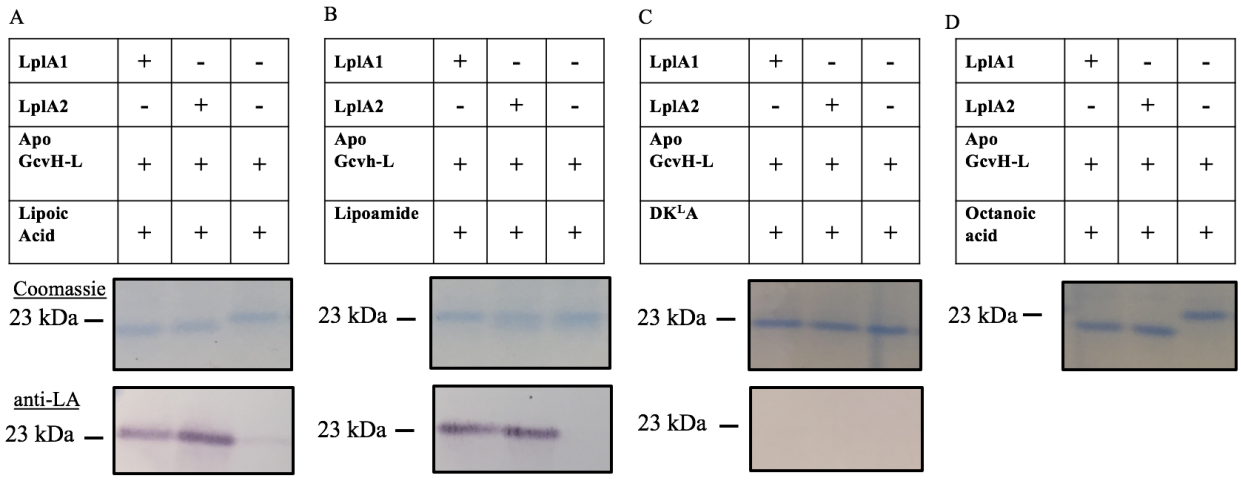


Figure 21. Both LplA1 and LplA2 directly lipoylate and octanoylate apo GcvH-L using lipoic acid, lipoamide and octanoic acid

Biochemical assessment of LplA1 and LplA2 lipoylation/octanoylation of apo GcvH-L using A. lipoic acid (2.4 μM) B. lipoamide (2.4 μM) C. $DK^{L}A$ (2.4 μM) and D. octanoic acid (2.4 μM). Lipoylation was visualized by resolving reactions on a 12% SDS PAGE gel stained with Coomassie blue (top panel) and conducting an immunoblot (bottom panel) probed with a mouse anti-lipoic acid antibody (anti-LA). Octanoylation was visualized on a 12% SDS PAGE gel stained with Coomassie blue (top panel).

Both LplA1 and LplA2 Lipoylate Apo E2-OGDH.

Prior genetic evidence suggested that LipL is primarily responsible for lipoylating apo E2-OGDH, however, when a Δ*lipL* mutant was grown in RPMI+BCFA, low levels of lipoylation on OGDH was seen (71). It was determined that these low levels of lipoylation were due to LplA1. To determine if LplA1 lipoylates E2-OGDH, I conducted lipoylation assays using apo E2-OGDH in the presence of lipoic acid, lipoamide, $DK^{L}A$, and octanoic acid. In the presence of free lipoic acid, I detected a lipoyl band for the reactions containing both LplA1 and LplA2 (Fig

22A). The intensity of the lipoyl band from the reaction containing LplA2 appeared more pronounced than the reaction containing LplA1 (Fig 22A). When the reaction was repeated using lipoamide as a substrate, I only detected a lipoyl band for the reaction containing LplA1 (Fig 22B). No lipoyl bands were detected for reactions containing LplA1 or LplA2 in the presence of DK^LA (Fig 22C). In addition, although I tested the ability of LplA1 and LplA2 to octanoylate apo E2-OGDH, my results were inconclusive because I do not have a positive control to verify that octanoylation occurred (Fig 22D). Together, these data indicate that both LplA1 and LplA2 directly lipoylate apo E2-OGDH using lipoic acid as a substrate, and LplA2 does so with apparent increased efficiency. In contrast, only LplA1 directly lipoylates apo E2-OGDH using lipoamide as a substrate.

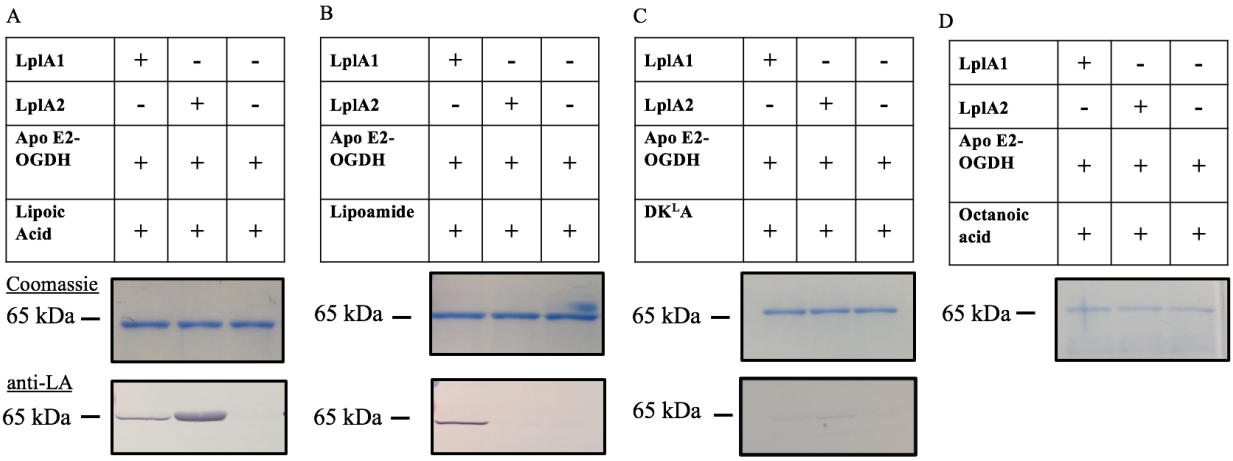


Figure 22. Both LplA1 and LplA2 lipoylate apo E2-OGDH using lipoic acid, however only LplA1 lipoylates apo E2-OGDH using lipoamide

Biochemical assessment of LplA1 and LplA2 lipoylation/octanoylation of apo E2-OGDH using A. lipoic acid (2.4 μM) B. lipoamide (2.4 μM) C. DK^LA (2.4 μM) and D. octanoic acid (2.4 μM). Lipoylation was visualized by resolving reactions on a 12% SDS PAGE gel stained with Coomassie blue (top panel) and conducting an immunoblot (bottom panel) probed with a mouse anti-lipoic acid antibody (anti-LA). Octanoylation was visualized on a 12% SDS PAGE gel stained with coomassie blue (top panel).

Only LplA2 Lipoylates Apo E2-PDH.

Based on my observation of different lipoylation activities for LplA1 and LplA2 with GcvH, GcvH-L and E2-OGDH, I reasoned that E2-PDH might also exhibit unique lipoylation characteristics that depend on lipoyl sources or ligases. To test the ability of LplA1 and LplA2 to lipoylate apo E2-PDH I conducted lipoylation assays in the presence of lipoic acid, lipoamide, and the tripeptide $DK^{L}A$ followed by resolving lipoylation reactions on an SDS-PAGE gel and staining with Coomassie dye or transferring to PVDF membranes and immunoblotting with anti-lipoic acid antibody. When incubated with free lipoic acid, I detected a lipoyl band for the reaction containing LplA2, but not LplA1 (Fig 23A). In contrast, no lipoyl protein bands were detected for reactions containing LplA1 or LplA2 in the presence of lipoamide or $DK^{L}A$ (Fig 23B and 23C). These data indicate that LplA2, but not LplA1 is able to lipoylate apo E2-PDH directly using lipoic acid as a substrate. Neither LplA1 nor LplA2 are able to incorporate lipoic acid using lipoamide or $DK^{L}A$ as a substrate.

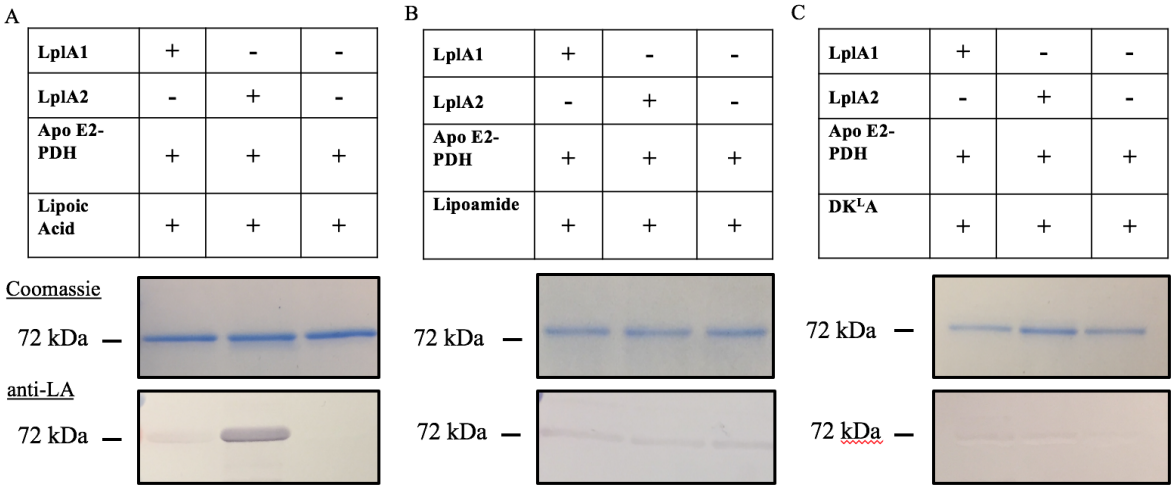


Figure 23. Only LplA2 directly lipoylates PDH using lipoic acid

Biochemical assessment of LplA1 and LplA2 lipoylation of apo E2-PDH using A. lipoic acid (2.4 μM) B. lipoamide (2.4 μM) C. $DK^{L}A$ (2.4 μM) and D. octanoic acid (2.4 μM). Lipoylation was visualized by resolving reactions on a 12% SDS PAGE gel stained with Coomassie blue (top panel) and conducting an immunoblot (bottom panel) probed with a mouse anti-lipoic acid antibody (anti-LA).

Only LplA2 Lipoylates Apo E2-BCODH.

Lastly, in order to test the ability of LplA1 and LplA2 to lipoylate apo E2-BCODH, I conducted the same lipoylation assays using lipoic acid, lipoamide, the tripeptide DK^LA, and octanoic acid as a substrate. In the presence of lipoic acid, I detected a lipoyl band for the reaction containing LplA2 (Fig 24A). However, no lipoyl bands were detected for reactions containing LplA1 or LplA2 in the presence of lipoamide and DK^LA (Fig 24B-C). Reactions were repeated using octanoic acid as a substrate, however without a positive control I could not determine whether or not octanoylation of apo E2-BCODH occurred (Fig 24D). Consistent with my results for apo E2-PDH, these data suggest that LplA2 can directly lipoylate apo E2-BCODH using lipoic acid as a substrate whereas, neither LplA1 nor LplA2 can lipoylate apo E2-BCODH using lipoamide or DK^LA as a source of lipoic acid.

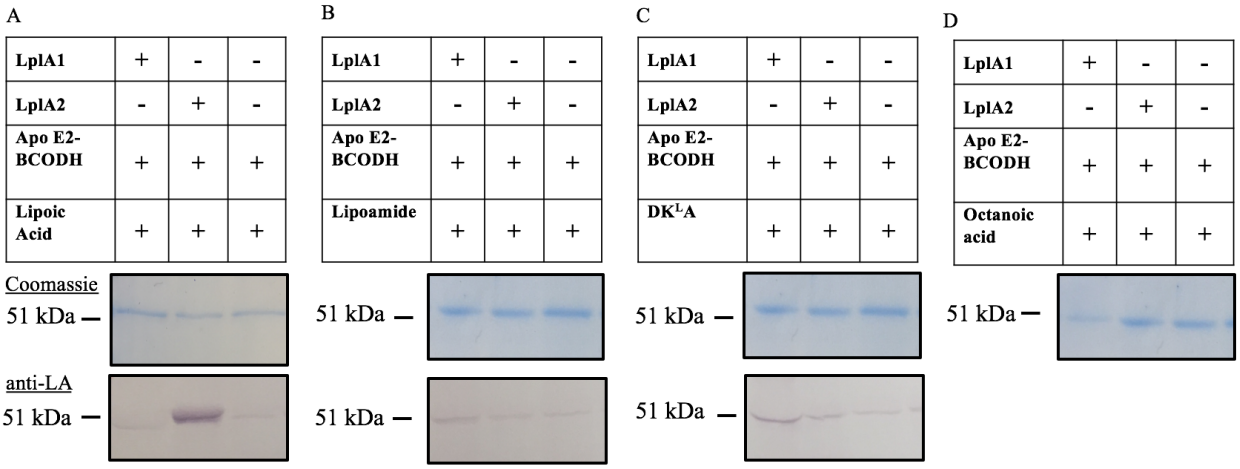


Figure 24. Only LplA2 lipoylates apo E2-BCODH using lipoic acid

Biochemical assessment of LplA1 and LplA2 lipoylation/octanoylation of apo E2-BCODH using A. lipoic acid (2.4 μM) B. lipoamide (2.4 μM) C. DK^LA (2.4 μM) and D. octanoic acid (2.4 μM). Lipoylation was visualized by resolving reactions on a 12% SDS PAGE gel stained with Coomassie blue (top panel) and conducting an immunoblot (bottom panel) probed with a mouse anti-lipoic acid antibody (anti-LA). Octanoylation was visualized on a 12% SDS PAGE gel stained with Coomassie blue (top panel).

Taken together, these data suggest that LplA1 appears to possess a more limited range of lipoylation targets that includes both H subunits (GcvH and GcvH-L) and one E2 subunit (E2-OGDH). In contrast LplA2 appears to have a broader capacity to target and lipoylate targets including all E2 subunits (E2-PDH, E2-OGDH, and E2-BCODH) as well as the secondary H subunit GcvH-L. Furthermore, each enzyme predominantly uses free lipoic acid as a substrate with limited ability to incorporate lipoamide and octanoic acid onto select targets. These data demonstrate that each ligase has its own preferred source of substrate and targets for lipoylation and that they can act independently from one another.

CHAPTER FOUR

DISCUSSION

Introduction

Pathogens such as *S. aureus* have developed pathways important for the acquisition of metabolites and nutrients. It has been shown that a number of these pathways are crucial for bacterial survival during host infection (15; 74). One such pathway is the lipoic acid biosynthesis and salvage pathway of *S. aureus*, which is responsible for the synthesis and scavenging of the vital cofactor lipoic acid. Unlike other pathogenic *Firmicutes*, the salvage pathway of *S. aureus* is unique as it is composed of two lipoic acid ligases, LplA1 and LplA2, important for scavenging lipoic acid from the environment (71). Prior work in the Alonzo lab determined that only LplA1 scavenges lipoic acid in vitro, whereas both ligases are crucial for host infection in vivo, leaving the exact role of LplA2 undetermined (71). My aim in this thesis was to further explore the functions of the lipoic acid ligases in *S. aureus* and assess their ability to use lipoic acid as well as alternative sources of lipoic acid. My data suggest that only LplA1, and not LplA2, can scavenge lipoic acid and peptide bound lipoyl domains from different sources under conditions used in this thesis, even when LplA2 is expressed from a constitutive promoter. However, my biochemical data indicate that both LplA1 and LplA2 are functional and can directly lipoylate *S. aureus* lipoyl enzyme complexes. In addition, my data suggest that both ligases in *S. aureus* have preferred targets for lipoylation and can act independently from one another. Overall, these data highlight the importance of the divergent functions of LplA1 and

LplA2 and may explain why *S. aureus* thrives so well when faced with low levels of free lipoic acid during host infection.

LplA1, but not LplA2, Can Use Free Lipoic Acid and Lipoylated Peptides to Stimulate Growth In Vitro

To test the possible divergent functions of LplA1 and LplA2, I first assessed the ability of either Δ*lipA* Δ*lplA1* and Δ*lipA* Δ*lplA2* mutants to use free lipoic acid and other lipoyl sources from proteins and small peptides. Prior data have shown that LplA2 is functional and stimulates bacterial growth when RPMI medium is supplemented with 20% FBS (71). However, addition of free lipoic acid to this medium does not increase bacterial growth, suggesting that LplA2-dependent growth requires FBS and is not enhanced by free lipoic acid (71). These data initially led me to hypothesize that LplA2 stimulates bacterial growth by facilitating the use of alternative sources of lipoic acid – perhaps lipoyl peptides in the serum. However, in the absence of LplA1, a Δ*lipA* Δ*lplA1* mutant and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2* strain, where *lplA2* is expressed under a constitutive promoter, were not able to grow in the presence of lipoic acid, lipoamide, octanoic acid, digested and undigested PDH, or digested and undigested OGDH. Contrary to my proposed hypothesis, these data suggest that LplA2 cannot recognize lipoic acid in the context of a peptide, even when it is being overexpressed. This is unusual considering our determination that FBS stimulates growth in an LplA2-dependent manner, which we presumed to be due to the use of lipoyl peptides or other derivatives. Interestingly, a Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2* strain, with constitutively expressed LplA2, does not grow when RPMI medium is supplemented with 20% FBS. It may be possible that *lplA2* is not being translated in a Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2* strain grown under these conditions. One way to address this issue would be to a run the bacterial

lysate of the Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2* strain on an SDS PAGE gel and conduct an immunoblot probed with an anti-LplA2 antibody to assess whether this strain produces LplA2. In addition, these data also suggest that additional factors not found under these conditions may be important for the LplA2 ligase to be functional.

From the genome sequence, I know that *lplA2* is encoded in an operon with four additional genes (*LLM*, *gcvH-L*, *macro*, and *sirTM*) (Fig 25). It is conceivable that expression of these genes is important for the function of LplA2 in vitro. In addition, research has shown that LplA2 is responsible for lipoylating GcvH-L under oxidative stress conditions in both *S. aureus* and *Streptococcus pyogenes* (72). Based on this information and the fact that I was not able to detect growth for the Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2* mutant in vitro but saw growth in vivo, I speculate that other components in vivo, such as oxidative stress, may be crucial for the expression of the additional genes in the LplA2 operon and required for LplA2 to function and stimulate bacterial growth. Evidence for this comes from Zorzoli *et al*., where mice were infected with WT, Δ*lipA* Δ*lplA1* Δ*lplA2*, Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1*, and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2* to determine if the ligases had overlapping functions in vivo (71). As expected, the triple mutant was nearly avirulent in mice since it does not have a functional lipoic acid *de novo* biosynthesis or salvage pathway, however both complement strains, Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1* and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2,* had similar bacterial burden compared to the WT implying that LplA2 is expressed and functional under these conditions (71). This was a very interesting finding since it supports the idea that additional factors found in vivo may be responsible for LplA2 functionality. It is tempting to speculate that the host environment leads to the upregulation of the *lplA2* operon to promote activity of LplA2. To further explore this notion,

I would repeat the growth curves described in this thesis under oxidative stress conditions to assess whether or not the Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2* strain is able to grow. In addition, I would repeat in vivo experiments with strains containing deletions of the other genes in the *lplA2* operon (*LLM*, *gcvH-L*, *macro*, and *sirTM*) to assess whether or not these genes are responsible for promoting LplA2 function in vivo. In sum, the work in my thesis implies that LplA2 has a distinct role in lipoic acid salvage compared to LplA1 and that additional, yet to be identified, factors may be important for LplA2 function.

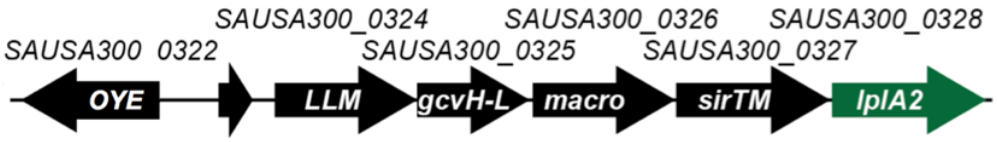


Figure 25. Gene arrangement of the LplA2 operon
The green arrow corresponds to the lipoic acid ligase, LplA2, which is part of the lipoic acid salvage pathway important for scavenging lipoic acid from the environment. The black arrows correspond to the additional genes, *LLM, gcvH-L, macro,* and *sirTM*, that are a part of the LplA2 operon.

Further analysis of the growth curves presented in this thesis showed that in the absence of LplA2, Δ*lipA* Δ*lplA2* and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1* mutants grew when supplied with lipoic acid, lipoamide, and digested PDH. However, surprisingly, the strains were not able to grow when the medium was supplemented with undigested PDH, possibly indicating that *S. aureus* is not able to break down the large PDH enzyme complex and import it for its own use. In addition to supplementing with PDH, the growth medium was also supplemented with OGDH, since both PDH and OGDH are the most abundantly found enzyme complexes in the body. However, in the presence of digested and undigested OGDH, only the WT strain was able to grow, indicating that LplA1 can only recognize peptide bound lipoic acid in the context of PDH and not OGDH. Since very minimal growth of Δ*lipA* Δ*lplA2,* Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1*,

Δ*lipA* Δ*lplA2,* and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1* was observed in RPMI supplemented with OGDH compared to WT, I speculate that this growth may be due to some other components of the OGDH porcine heart extract.

Since I had hypothesized that LplA1 and LplA2 can use peptide bound lipoic acid to stimulate growth, it was surprising to see that only LplA1 was able to use peptide bound lipoic acid specifically in the context of PDH, but not OGDH. To further explore the substrate specificity of LplA1 for PDH and not OGDH, I analyzed the E2-PDH and E2-OGDH amino acid sequences. I know that only the E2 subunits of OGDH and PDH contain a lipoyl moiety and when PDH is digested with proteinase K the smallest predicted lipoyl moiety achieved is a $DK^{L}A$ tripeptide (54). Analysis of both E2-PDH and E2-OGDH amino acid sequences showed that the lipoylated lysine in PDH is flanked by an aspartate and alanine, whereas the lipoylated lysine in OGDH is flanked by an aspartate and threonine. These tripeptides are conserved throughout multiple species such as human, pig, rat, and mouse (Fig 13A and 13B). In addition, the PDH enzyme complex harbors two lipoyl moieties compared to OGDH, which only has one. To ensure that other components of the OGDH and PDH complex did not facilitate growth by a mechanism that does not depend on lipoic acid to stimulate growth, I repeated the growth curves with the synthetic nonlipoylated (DKA) and lipoylated tripeptide ($DK^{L}A$), which has sequence similarity with E2-PDH. Just as anticipated, Δ*lipA* Δ*lplA2* mutant and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1* mutant grew compared to Δ*lipA* Δ*lplA1*, Δ*lipA* Δ*lplA1* Δ*lplA2* and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2* strain which did not grow, indicating that the growth I saw when the medium was supplemented with digested PDH was due to the lipoyl domain and not other components of the digested PDH enzyme complex.

My data show that LplA1 uses peptide bound lipoic acid in the context of PDH and not OGDH. As mentioned previously, from the genome database I know that the lipoyl lysine of PDH and OGDH are flanked with different amino acids. Based off of this information, I hypothesized that different amino acids may make it more difficult for the ligases to recognize the lipoyl domain. However, contrary to my hypothesis, I observed growth for both Δ*lipA* Δ*lplA2* and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1* but not for Δ*lipA* Δ*lplA1* and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2* when the medium was supplemented with a $DK^{L}T$ tripeptide, which has sequence similarity with E2-OGDH. These data indicate that it appears to be irrelevant which amino acids flank the lipoyl lysine. Nonetheless, I speculate that the broader domain architecture of PDH may provide specificity and allow LplA1 to use it, but not ODGH, for growth. In the future, it will be important to repeat these growth curves with large peptide sequences of PDH and OGDH that encompass the lipoyl domain to assess whether the lipoyl lysine in a broader domain determines specificity.

LplA1 and LplA2 Can Directly Use Lipoic Acid and Lipoamide to Lipoylate the Apo E2 and H Subunits

Based on my findings and previous results from Zorzoli *et al,* 2016, I have revised our model of *S. aureus* lipoic acid biosynthesis and salvage (Fig 27). My data indicate that LplA1 can directly use lipoic acid, and in some cases lipoamide, to lipoylate the H subunits (GcvH, and GcvH-L) and one E2 protein subunit (E2-OGDH), whereas LplA2 can directly use lipoic acid to lipoylate all *S. aureus* lipoyl domain-containing E2 subunits (E2-PDH, E2-OGDH, and E2-BCODH) as well as the accessory H subunit (GcvH-L). LplA2 appears to lipoylate E2-OGDH at an increased efficiency compared to LplA1, suggesting that LplA2 may be the preferred ligase

for lipoylation of E2-OGDH. In the future, it may be interesting to explore the differences between GcvH and GcvH-L which may provide greater insight as to why LplA1 can lipoylate both H subunits whereas LplA2 only appears to lipoylate one H subunit. Together these data indicate that both ligases can use different sources of lipoic acid for lipoylation, but lipoic acid seems to be preferred.

It is a well-known fact that lipoic acid is attached to a conserved lysine via an amide bond. For bacteria to use lipoamide or peptide bound lipoic acid, they first have to hydrolyze the amide bond to generate free lipoic acid, which can only be achieved with an enzyme that has lipoamidase activity. A prior review has suggested that the lipoic acid ligases may have lipoamidase activity (39). As a result, I anticipated either LplA1, LplA2, or both ligases would harbor lipoamidase activities, leading to similar lipoylation patterns when lipoic acid or lipoamide are used as substrates, since lipoamidase activity results in release of free lipoic acid. However, my data showed that all five known enzyme complexes were lipoylated using lipoic acid, whereas only GcvH, GcvH-L, and OGDH were lipoylated using lipoamide, indicating that lipoylation with lipoamide and lipoic acid does not result in similar patterns of lipoylation. In addition, I detected a shift in band size on an SDS PAGE gel for reactions supplemented with lipoic acid, whereas no shift was detected for reactions supplemented with lipoamide. Based on these observations, I speculated that the *S. aureus* ligases do not have lipoamidase activity and wondered whether the compositional differences between lipoic acid and lipoamide may explain the different patterns of lipoylation. Composition analysis of lipoic acid and lipoamide showed that lipoic acid consists of a dithiolane ring and a carboxyl group at its terminal end which is important for attachment to the lysine via an amide bond. Lipoamide on the other hand, does not

have a carboxyl group at its terminal end but instead has an amine group. These structural differences make it impossible for lipoamide to form an amide bond with the charged lysine, as only a carboxyl group and amine group can form an amide bond (Fig 26). As a result, I speculated that lipoamide may form an amide bond with amino acids nearby that have a carboxyl group. The only way an amino group can reasonably form an amide bond is with another hydroxyl group, which can be found on amino acids such as threonine and serine. Intriguingly, GcvH and GcvH-L both have a threonine flanking the lipoyl-lysine. Since threonine has a carboxyl group, lipoylation of the threonine rather than the lysine may occur during the biochemical reactions with lipoamide. It may be reasonable to suspect that both ligases, LplA1 and LplA2, have the ability to lipoylate other amino acids with carboxyl groups. Further analysis of the E2-subunits of PDH and BKDH, which do not get lipoylated with lipoamide, showed that neither had amino acids containing a carboxyl group flanking the lysine. However, investigation of the E2-subunit of OGDH also did not have an amino acid with a carboxyl terminus flanking the lysine. Nevertheless, I believe that it may be possible for lipoamide to form an amide bond with the carboxyl group of another adjacent amino acid when used as a substrate.

In addition to having different patterns of lipoylation with lipoic acid and lipoamide, I observed a lack of shift in band size on an SDS PAGE gel in reactions conducted with lipoamide compared to lipoic acid. Proteins on an SDS PAGE gel are separated based on their size, conformation, and charge. SDS is specifically important for coating the protein to provide a uniform negative charge. However, it has been suggested that the shift seen after lipoylation with lipoic acid is due to the increase in net negative charge after acylation of the lysine (75). If my earlier speculations about lipoylation of threonine with lipoamide are correct, it may also explain

why there is a lack of shift in band size in those reactions. Unlike lysine, which has a positive charge, threonine does not have a charge. Due to a lack of charge, lipoylation of a threonine would not alter its overall net charge, thus, these reactions might not run differently on an SDS PAGE gel compared to the non-lipoylated control.

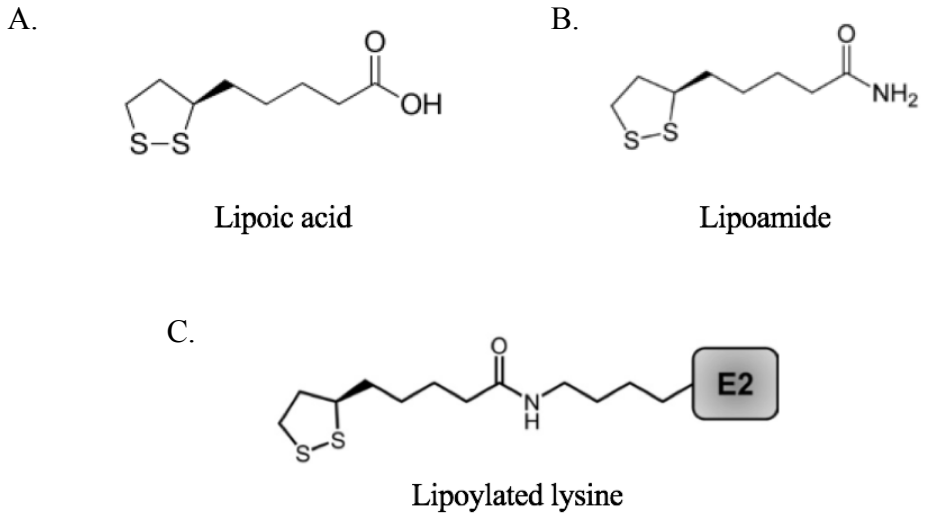


Figure 26. Structural differences between lipoic acid and lipoamide
A. Lipoic acid is an organosulfur compound containing a dithiolane ring B. Lipoamide is a derivative of lipoic acid with an amine terminal end and naturally found attached to a lysine via an amide bond. C. Lipoamide bound to a conserved lysine via an amide bond and attached to the E2 subunit of lipoylated enzyme complexes.

Both LplA1 and LplA2 Use Octanoic Acid to Octanoylate GcvH and GcvH-L

Prior genetic evidence suggested that LplA1 in *S. aureus* facilitates salvage of lipoic acid and octanoic acid to lipoylate/octanoylate apo GcvH in vitro (71). From these data, I hypothesized that LplA2 may also facilitate such activity and octanoylate GcvH-L, since both of them are encoded in the same operon. In order to investigate this activity and assess whether both LplA1 and LplA2 can lipoylate GcvH and GcvH-L, I supplemented the reactions with octanoic acid. My data indicated that both ligases use octanoic acid as a substrate to octanoylate apo GcvH-L, however only LplA1 appeared to be able to octanoylate apo GcvH. Additional

assessment of the ability of LplA1 and LplA2 to octanoylate E2-PDH, E2-OGDH, and E2-BCODH resulted in the inability to detect a shift in band size, indicating that either the ligases are not able to transfer octanoic acid onto E2-PDH, E2-OGDH, and E2-BCODH or that a shift in band size is very minor and not able to be detected by resolution on an SDS-PAGE gel. Nevertheless, it is still a possibility that the ligases octanoylate E2-PDH, E2-OGDH, and E2-BCODH. One way to address this question would be to conduct octanoylation assays with radioactive octanoic acid and detect octanoylation using autoradioagraphy. Another possible approach would be to run the octanoylation assays on a native gel and look for a shift in band size compared to the control indicating octanoylation.

S. aureus **May Have Potential Lipoamidase Activity**

Previous data indicated that LplA1 can use the lipoylated tripeptide (DK^LA) to stimulate growth in vitro. However, when I assessed the ability of LplA1 to directly use DK^LA as a source of lipoic acid in a biochemical assay, no lipoylation was detected. These data indicate that the two ligases themselves do not have lipoamidase activity, but rather *S. aureus* may encode an additional factor such as a lipoamidase in vitro that is vital for LplA1 to use peptide bound lipoic acid. In *L. monocytogenes,* which also encodes two lipoic acid ligases, studies have shown that a metal dependent lipoamidase is required for the ligases to use lipoyl tripeptides as a source of lipoic acid (55). These data suggest that *S. aureus* may also encode a yet to be identified lipoamidase that facilitates use of lipoylated tripeptides as a source of lipoic acid. In order to determine whether or not lipoamidase activity exists in *S. aureus*, a possible approach would be to conduct lipoylation assays with LplA1 and DK^LA supplemented with a crude extract from a Δ*lipA* Δ*lipM* Δ*lipL* Δ*lplA1* Δ*lplA2* strain to ensure that other components of lipoic acid

biosynthesis and salvage do not interfere. If my speculations of possible lipoamidase activity are correct, I would expect to see lipoylation of OGDH, GcvH, and GcvH-L. In addition, since a Δ*lipA* Δ*lplA2* strain grew in medium supplemented with $DK^{L}A$, it may also be possible that other proteins of the *de novo* biosynthesis pathway are important for optimal LplA1 function. To further explore this idea, a possible approach could be to repeat the biochemical assays with LplA1 and $DK^{L}A$ supplemented with LipM or LipL to assess whether they play a role in the ability of LplA1 to use peptide bound lipoic acid. Overall, these additional experiments may help explain the ability of LplA1 to use lipoylated tripeptides during growth in broth culture but not in biochemical assays.

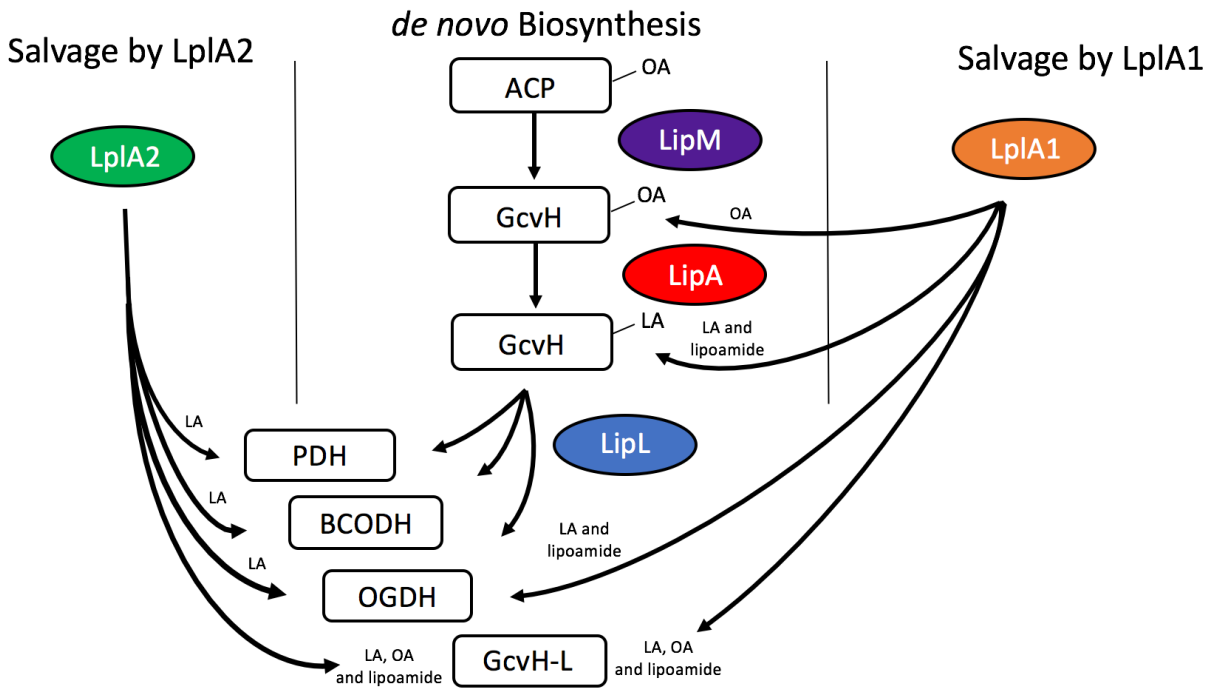


Figure 27. Lipoic acid biosynthesis and salvage pathway in *S. aureus*
S. aureus can acquire lipoic acid through the *de novo* biosynthesis pathway where octanoic acid is attached to the acyl carrier protein and then transferred to the H subunit of GcvH with the help of the octanoyl transferase (LipM). Next, the octanoyl moiety is converted to lipoic acid via the lipoic acid synthetase (LipA). Lastly, LipL, another transferase, transfers the lipoyl moiety to different E2-subunits. During the lipoic acid salvage pathway, LplA1, transfers free lipoic acid (LA), lipoamide, and octanoic acid (OA) onto GcvH and GcvH-L, and free lipoic acid (LA) and lipoamide onto OGDH. LplA2 on the other hand, transfers free lipoic acid (LA) onto PDH, OGDH, BCODH and free lipoic acid (LA), octanoic acid (OA) and lipoamide onto GcvH-L.

Concluding Remarks

In this thesis, I provide direct biochemical evidence demonstrating functional lipoic acid ligase activity for both LplA1 and LplA2 of *S. aureus*. Each ligase functions independently, but has the capacity to act on both overlapping and distinct targets that comprise a range of H and E2 subunits of key metabolic enzyme complexes. Furthermore, both ligases have the potential to universally scavenge lipoic acid and, in some cases, octanoic acid and lipoamide, thereby expanding their functional plasticity. The ability of LplA2 to lipoylate nearly all potential targets (all E2 proteins and GcvH-L) implies a greater metabolic reach compared to LplA1, which has a more limited repertoire of targets (H proteins and E2-OGDH). Furthermore, the varied substrates of these ligases (lipoic acid, lipoamide, and octanoic acid) may help explain the ease with which *S. aureus* is able to infect a wide range of tissues, which harbor varied levels of free lipoic acid or lipoyl derivatives. This is further supported by my in vitro demonstration of ligase-dependent utilization of lipoyl peptides by *S. aureus* in broth culture and prior data that supports the use of lipoyl peptides by *S. aureus* during infection (71). Altogether these findings highlight the remarkable adaptability of *S. aureus* as it relates to nutrient acquisition and provides novel insights into mechanisms by which the bacterium maintains metabolic homeostasis when challenged with severe nutritional restrictions. My findings have the potential to be instrumental in the development of novel therapeutics designed to block *S. aureus* acquisition of lipoic acid, thereby perturbing bacterial replication in vivo and facilitating disease resolution.

APPENDIX A

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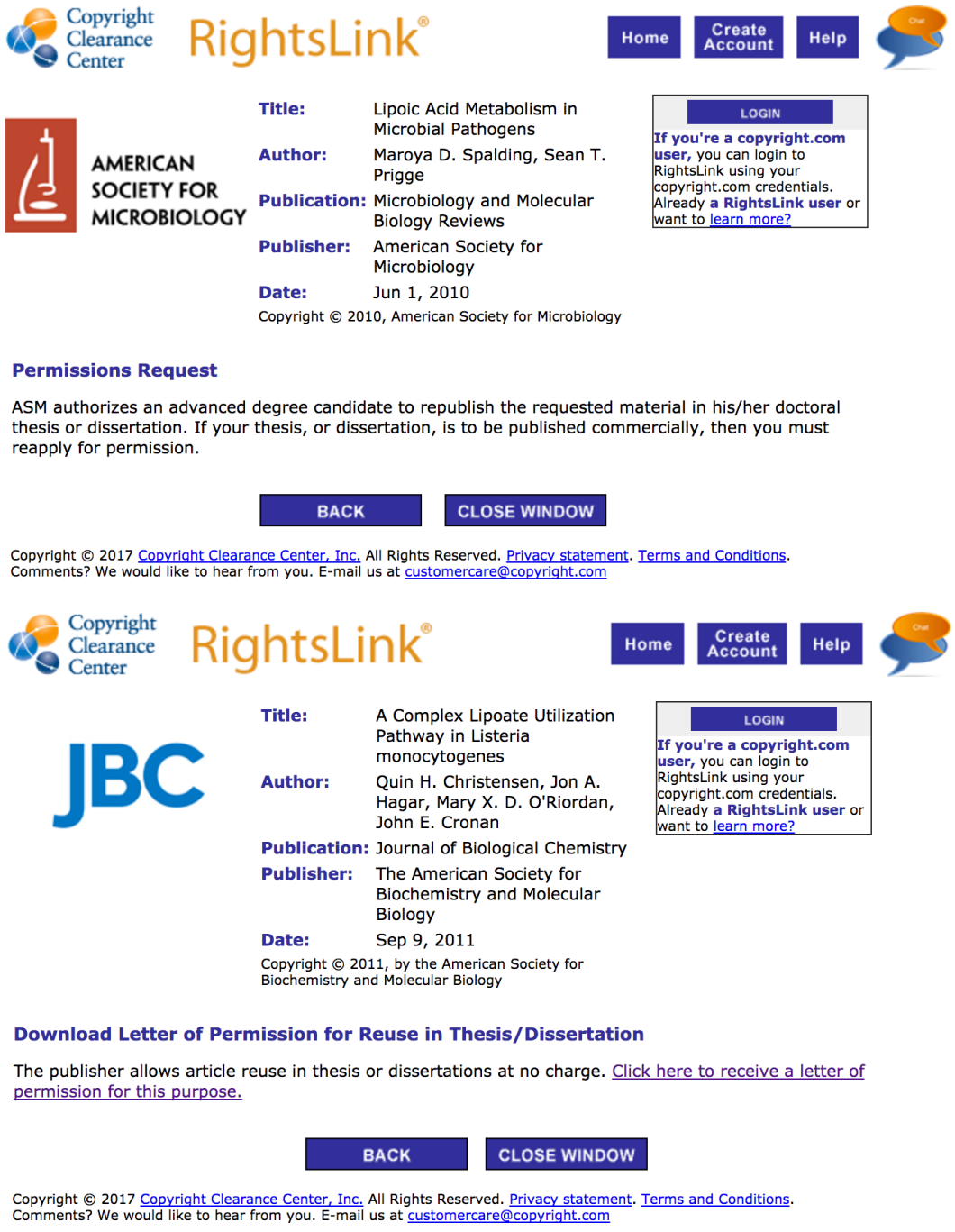
Figure 28. Authorization to republish



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VITA

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