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Lipoate Metabolism in Staphylococcus Aureus Pathogenesis

Maria Azul Zorzoli
Loyola University Chicago

LOYOLA UNIVERSITY CHICAGO

LIPOATE METABOLISM IN *STAPHYLOCOCCUS AUREUS* PATHOGENESIS

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MARIA AZUL ZORZOLI

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A Luciana, por darme la mano en la jungla

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LIST OF ABBREVIATIONS

ACKA	Acetate Kinase
ACP	Acyl carrier protein
AoDH	Acetoin Dehydrogenase
AMP	Ampicillin
BCODH	Branched Chain 2- Oxoacid Dehydrogenase
BCFA	Branched Chain Fatty Acid
CFU	Colony Forming Units
CHIPS	Chemotaxis inhibitory protein of staphylococci
CY	Casamino acids Yeast
ERM	Erythromycin
GCS	Glycine cleavage system
GcvH	H subunit of the Glycine cleavage system
ISD	Iron-regulated surface determinant
KAN	Kanamycin
LA	Lipoic Acid
MRSA	Methicillin Resistant *Staphylococcus aureus*
OA	Octanoic Acid
OGDH	2-Oxoglutarate Dehydrogenase
PCR	Polymerase Chain reaction

PDH	Pyruvate Dehydrogenase
POR	Pyruvate::flavodoxin oxydoreductase
PTA	Phosphotransacetylase
PVL	Panton-Valentine leucocidin
RPMI	Roswell Park Memorial Institute
SCC*mec*	Staphylococcal cassette chromosome *mec*
SCIN	Staphylococcal complement inhibitor
SOC	Super Optimal broth with Catabolite repression
SOEing	Splicing by Overlap Extension
TCA	Tricarboxylic acid
T3SS	Type 3 secretion system
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
WT	Wild type

ABSTRACT

Lipoate is an essential cofactor of several proteins involved in cellular energy homeostasis and catabolism. Lipoate metabolism has been linked to pathogenesis in some microbial species, but its role in *Staphylococcus aureus* infections had not been explored. In this thesis, we tested the hypothesis that lipoate acquisition mechanisms promote *S. aureus* infectivity. We used a bacterial genetics approach to elucidate the biological function of the *S. aureus* genes involved in lipoate metabolism. Our findings allowed us to propose a model for lipoic acid *de novo* biosynthesis and salvage pathways in *S. aureus*. Moreover, we detail hitherto undescribed genetic arrangements of lipoate *de novo* biosynthesis and salvage genes in the *S. aureus* genome, which suggest a potential role for lipoate acquisition mechanisms in metabolic regulation and oxidative stress defense. Also, we have identified critical requirements for gene products involved in lipoate metabolism in murine sepsis. Our data indicate that *S. aureus* is capable of using bacterial and host-derived lipoate during infection in a tissue-specific manner, thereby promoting survival in diverse nutrient-restricted environments. Overall, our findings suggest that the *S. aureus* lipoate *de novo biosynthesis* and salvage pathways offer potential for the development of novel therapeutics that target key metabolic programs in *S. aureus*.

CHAPTER ONE

LITERATURE REVIEW

Introduction

Staphylococcus aureus is a commensal bacterium that can be found colonizing the skin in humans. About a fifth of the human population are permanently colonized with *S. aureus* and almost of 60% of humans are occasional carriers (1-3). *S. aureus* is also a prominent pathogen capable of invading several organs and tissues causing mild to severe, or even fatal infections (2-4). Special concern has been raised in society over the increasing number of patients infected with hypervirulent or antimicrobial-resistant *S. aureus* strains, acquired by both immunocompromised patients at health-care facilities and otherwise healthy individuals from community environments (5-8).

S. aureus has developed sophisticated strategies to survive in different mammalian tissues, both as a commensal and as a pathogen. Upon infection, it evades the host immune responses by secreting numerous virulence factors, such as pore-forming toxins and proteases (3, 4). Moreover, this species readily adapts to nutrient restriction to survive within the host; iron acquisition mechanisms are a clear example of the remarkable ability of *S. aureus* to hunt for nutrients (9, 10).

Lipoate is an essential cofactor in central metabolism in bacteria since it enables the function of several proteins that lead to the production of cellular energy and vital metabolites, such as amino acids and fatty acids (11, 12). Interestingly, lipoate acquisition pathways have been linked to pathogenesis in several bacterial and malarial species (13-18). For example, *Listeria monocytogenes*, which is a Gram-positive pathogen that belongs to the same taxonomic phylum as *S. aureus,* requires lipoate salvage to infect the host, demonstrating that the mechanism for incorporating this cofactor could promote pathogenesis in a wide range of bacteria (16, 19).

A deep understanding of the elements that promote *S. aureus* fitness and prevalence during infection is a matter of paramount importance for the design of new therapeutics against this pathogen. Lipoate metabolism in *S. aureus* is poorly understood and the role of lipoate acquisition during infection is currently unknown (16). Therefore, investigating *S. aureus* lipoate metabolism and its role in pathogenesis could reveal novel targets for developing antimicrobial agents against this major pathogen.

Staphylococcus aureus

S. aureus is a major human pathogen

Skin and soft tissue infections such as impetigo, folliculitis and cellulitis are amongst the most common diseases caused by *S. aureus* (5, 7, 8, 20). This pathogen can also cause respiratory diseases such as pneumonia and can induce sepsis upon spread into the bloodstream (3, 21). Furthermore, *S. aureus*

is the leading cause of infectious endocarditis in the United States and is often associated with osteomyelitis in young children (22-25). Mortality and morbidity due to staphylococcal infections constitute a significant economic burden for society (5, 26, 27).

A variety of factors can increase the risk of infection in patients. These include the use of foreign bodies such as catheters or prostheses, the presence of underlying health conditions, as well as current or recent hospitalization. In addition, treatment of *S. aureus*-derived diseases constitutes a current challenge in public health due to the increasing antimicrobial resistant strains found in the clinic (5, 8, 22, 27, 28).

According to the World Health Organization's First Global Report on Antibiotic Resistance (28), people infected with MRSA (methicillin-resistant *S. aureus*) are estimated to be 64% more likely to die than those infected with non-resistant strains. A clear understanding of *S. aureus* pathogenesis could have a significant socio-economic impact on the prevention and treatment of both community-acquired and hospital-acquired infections (5, 7, 8, 29).

Numerous virulence factors contribute to *S. aureus* pathogenicity

The ability to infect and survive in a broad spectrum of host tissues makes *S. aureus* a major pathogen. In order to cause disease, the bacterium has to colonize the host while simultaneously evading and/or suppressing host immune responses (6, 30). To counter these defense mechanisms and succeed at infecting the host, *S. aureus* produces numerous virulence factors (1, 3, 6, 31).

Among the arsenal of *S. aureus* virulence factors are several key surface proteins (6, 32). These cell wall-anchored (CWA) proteins are attached to peptidoglycan and mediate processes such as attachment to host cells, disruption of immune responses or stimulation of biofilm formation (6, 33). For example, the protein Clumping factor B is capable of binding fibrinogen and molecules expressed by squamous cells, thus promoting attachment to the skin surface (3). Another group of *S. aureus* CWA proteins, the iron-regulated surface determinant (Isd) proteins, facilitate iron binding and uptake, and support survival in iron-limiting environments including the skin and mucosa (31, 34). Expression of several CWA proteins depends on growth conditions and *S. aureus* nutritional status, highlighting their role in promoting adaptation to fluctuations in environmental conditions (1, 3, 32). Another major CWA *S. aureus* virulence factor is the deeply studied Protein A, which sequesters antibodies by binding IgG molecules by the Fc region (6, 31, 35). This prevents proper recognition by Fc receptors on phagocytic leukocytes interfering with phagocytosis and activation of the complement cascade.

As highlighted by the function of the CWA Protein A, *S. aureus* is capable of producing a wide variety of both cell wall linked and secreted factors that protect against major host immune defenses including, but not limited to, phagocytosis and complement activation. During infection with *S. aureus*, one of the most important mechanisms used to combat the pathogen is opsonophagocytosis and subsequent induction of the respiratory burst (30, 31,

36). This process is initiated by migration of neutrophils to the site of infection, an event that is mediated by a gradient of diffusible peptides derived from the pathogen (i.e. N-formyl peptides) and the host, (i.e. complement degradation products). Upon reaching the site of infection, neutrophils pass through the blood vessel endothelium and migrate into the tissue. *S. aureus* interferes with these processes by secreting inhibitory molecules that can block neutrophil adhesion and migration (30, 31, 36). For example, *S. aureus* produces the staphylococcal superantigen-like proteins -5 and 11, which compete with neutrophil cell receptors and decrease neutrophil binding and rolling through the endothelium (3, 37). Additionally, *S. aureus* is capable of inhibiting neutrophil migration to infected tissues by secreting small proteins such as CHIPS, chemotaxis inhibitory protein of staphylococci, and SCIN, Staphylococcal complement inhibitor, which binds receptors on the membrane of neutrophils, preventing the process of chemotaxis and allowing the bacterium to evade phagocytosis (38-40).

Although inhibiting phagocyte recruitment and complement activation are key virulence strategies, *S. aureus* also employs other methods to evade the respiratory burst initiated after phagocytosis. For example, *S. aureus* can detoxify reactive oxygen species by producing enzymes such as catalase, superoxide dismutase and a variety of other proteins with antioxidant properties (1, 3, 6, 35).

Secreted toxins also play a major role in the pathogenesis of *S. aureus*. Most noteworthy are the myriad cytotoxic agents that can damage host cell membranes. The membrane damaging α-toxin, also known as Hla or alpha-

hemolysin, is one of the most studied virulence factors in *S. aureus* (40, 41). The toxin binds to specific receptors on susceptible cells to induce lysis, releasing cytosolic content that trigger a potent inflammatory reaction. Another group of secreted proteins, named leukocidins, specifically target immune cells by forming pores after oligomerization on the cell surface. The leucocidins include HlgAB, HlgCB, LukAB/HG, LukMF, Panton-Valentine leucocidin (PVL), and LukED. This wealth of bicomponent pore-forming proteins leads to a broad spectrum of hemolytic, leukotoxic and dermonecrotic activities (23, 31, 42). Finally, phenol-soluble modulins are a group of small amphipathic cytolytic peptides produced by *S. aureus* that can target white and red blood cells and promote inflammation in the host (2, 3, 31, 42).

S. aureus enhances its pathogenicity through its ability to recover essential nutrients from the site of infection. The scavenged compounds are then used for all sorts of biochemical processes, from DNA synthesis to the production of energy. Moreover, alterations in *S. aureus* nutrient-acquisition mechanisms have been shown to affect its virulence (4, 10). The acquisition of transition metals is a great example that demonstrates how *S. aureus* competes with the host for essential nutrients (43). Mammalian cells have evolved mechanisms that restrict iron availability, such as the intracellular location of iron and its sequestration within iron-binding proteins, such as transferin, lactoferrin or hemoproteins (34, 44). These strategies limit the proliferation of pathogens in a defense process known as “nutritional immunity” (45). To circumvent this

nutritional restriction, *S. aureus* has evolved mechanisms to release and incorporate iron from host deposits, mainly from heme, which is the primary iron reservoir in mammals. Proteins from the heme uptake systems extract sequestered iron and the iron-chelators staphyloferrin A and B and aurochelin facilitate the passage of this essential nutrient to the bacterial cytosol. These iron-dependent siderophores are upregulated in iron-deprived conditions (34, 44). Interestingly, heme uptake systems, such as the Isd system, have been shown to be essential for *S. aureus* pathogenesis by promoting trace metal acquisition that leads to bacterial proliferation and persistence in the host (3, 10, 34, 43, 46).

A note on antimicrobial resistance

While the production of virulence factors is critical to *S. aureus* pathogenesis, additional acquisition of a broad range of antibiotic resistance genes has further augmented *S. aureus* pathogenicity and adverse outcomes associated with failed treatment. Infections with antibiotic resistant bacterial decrease the available tools to treat diseases and increase the patient's risk of suffering from more severe complications. The prevalence and widespread dissemination of antibiotic resistance genes has become a serious problem and a major public health crisis. Meanwhile, very few novel therapeutic strategies are being developed.

The extensive use of antibiotics both in the community, the clinic and in the food industry has contributed to the selection of antimicrobial resistance determinants in bacteria and promoted the emergence of pan-resistant species

(47). Despite the role of the selective pressure derived from antibiotic utilization, antimicrobial resistance in bacteria is a phenomenon rooted in complex genetic mechanisms, including chromosomal alterations and trade of genetic information among species (47, 48). (49).

For *S. aureus*, resistance to antimicrobials resides in its high adaptability and mutability, which facilitate gene variations and the rapid incorporation of extrachromosomal DNA (1, 5, 26). As a consequence of its multi-drug resistant phenotype and its incidence worldwide, methicillin resistant *S. aureus* (MRSA) has been widely studied (8, 20). Methicillin resistance in *S. aureus* occurred after acquisition of a mobile genetic element known as the staphylococcal cassette chromosome *mec* (SCC*mec*); SCC*mec* contains the *mecA* gene, which confers resistance to penicillin drugs and β-lactam antibiotics, and it may carry additional genes involved in resistance to other classes of antimicrobials and toxins, such as the Panton-Valentine leucocidin (8, 50).

The rise of antibiotic resistance has not been restricted to methicillin since a few *S. aureus* strains have begun to show reduced susceptibility to antimicrobials commonly used to fight MRSA and infections caused by other pathogens. Researchers hypothesize that intense use of the antibiotic vancomycin as the therapeutic approach infections constituted a strong selective pressure that fostered the acquisition of the *vanA operon* from vancomycin-resistant enterococci (8, 47, 51). Additionally, resistance to non-β-lactam antibiotics such as clindamycin has been detected in MRSA isolates from the

community. This antibiotic resistance has been associated with the presence of the pUSA03 plasmid containing the resistance genes *ermA* and *ermC* (5, 8)

The growing number of infections caused by multi-drug resistant and highly virulent *S. aureus* strains isolated from community settings is a major public health concern. Thus, further research on potential antimicrobial targets and novel therapeutic approaches is required to reverse the increasing trend on multi-drug resistance in *S. aureus*.

Final considerations

S. aureus is a fascinating and exceptionally successful pathogen. The combined action of immunoevasion, immunosuppression, and nutrient scavenging activities, in addition to its rapid acquisition of antibiotic resistance, allow it to thrive in disadvantageous circumstances. In light of the information provided in the literature, we can surmise that the outcome of a staphylococcal infection is the result of a fine balance between the host immune responses and the ability of the pathogen to subvert those defensive actions. On one side of the spectrum, *S. aureus* infections can remain asymptomatic or localized due to effective host defenses; on the other side lays the risk of bacterial dissemination and disease due to bacterial counter-measures.

An increasing number of MRSA strains have begun to display complete or partial resistance to previously effective drugs, reducing the spectra of available effective antimicrobial drugs against this pathogen (5, 8, 50). The increased frequency of infections caused by hyper virulent strains that show resistance to

multiple antibiotics emphasizes the urgency of comprehensive studies on the causes, severity and treatment of *S. aureus* infections.

Lipoate metabolism

Lipoate is required for metabolism and redox homeostasis

Lipoate is the deprotonated form of lipoic acid (Figure 1) (16). It is highly conserved among species of different domains of life and acts as an essential organosulfur cofactor in key multienzyme complexes that are involved in oxidative and one-carbon metabolism (11). Lipoate itself and lipoylated complexes are well conserved in nature, yet an important versatility exists among organisms regarding their requirements for lipoate and its acquisition (11, 16).

Five multienzyme complexes depend on lipoate to function and these have been extensively described in the literature: pyruvate dehydrogenase complex (PDH); 2-Oxoglutarate dehydrogenase (OGDH); branched-chain 2-Oxoacid dehydrogenase (BCODH); the glycine cleavage system (GCS) and acetoin dehydrogenase complex (AoDH). Of note, all except one are encoded in S. aureus genome; the AoDH is not synthesized or required for its metabolic functions (11, 16).

The three 2- oxoacid dehydrogenases are composed of multiple copies of three enzymatic subunits, E1, E2 and E3. The core of the complex contains the E2 subunit bearing the lipoate molecule covalently attached to a well-conserved lysine through an amide bond (Figure 1B) (16). Pyruvate dehydrogenase (PDH) converts pyruvate to acetyl coenzyme A; 2-Oxoglutarate dehydrogenase (OGDH)

is involved in the transformation of α-ketoglutarate into succinyl-CoA and the branched-chain 2-Oxoacid dehydrogenase (BCODH) participates in the initial steps of branched chain fatty acid synthesis (11, 16). The glycine cleavage system (GCS) catalyzes the reversible decarboxylation of glycine and shows differences in both sequence and structure compared to the 2- oxoacid dehydrogenases. The GCS also contains a lipoate molecule attached to a conserved lysine, but it is bound to the H subunit of this system (GcvH) (6). An additional gene product related to the GCS has been found within the SirtM operon of S. aureus, which encodes an ADP-ribosyltransferase and a non-canonical form of the Glycine cleavage complex H (gcvH-L) that appears to be upregulated under conditions of redox stress (52, 53). Finally, the acetoin dehydrogenase complex (AoDH), which is not present in S. aureus, also has three catalytic subunits and is functionally related to PDH, but it does not catalyze the oxidative decarboxylation of branched-chain 2-Oxoacids (16).

Despite their structural and functional differences, PDH, OGDH, BCODH and GCS share an E3 subunit, which is responsible for recycling the disulfide bond for a new catalytic cycle (11, 16). In these multienzyme complexes, the lipoate molecule is responsible for binding substrates through its thioester bond, where it tunnels those substrates between the active sites of the different subunits (11). In addition to its role in catalysis, the disulfide bond formed by the thiol groups is thought to be involved in redox reactions, conferring an important role for lipoate as an antioxidant (11, 12, 16).

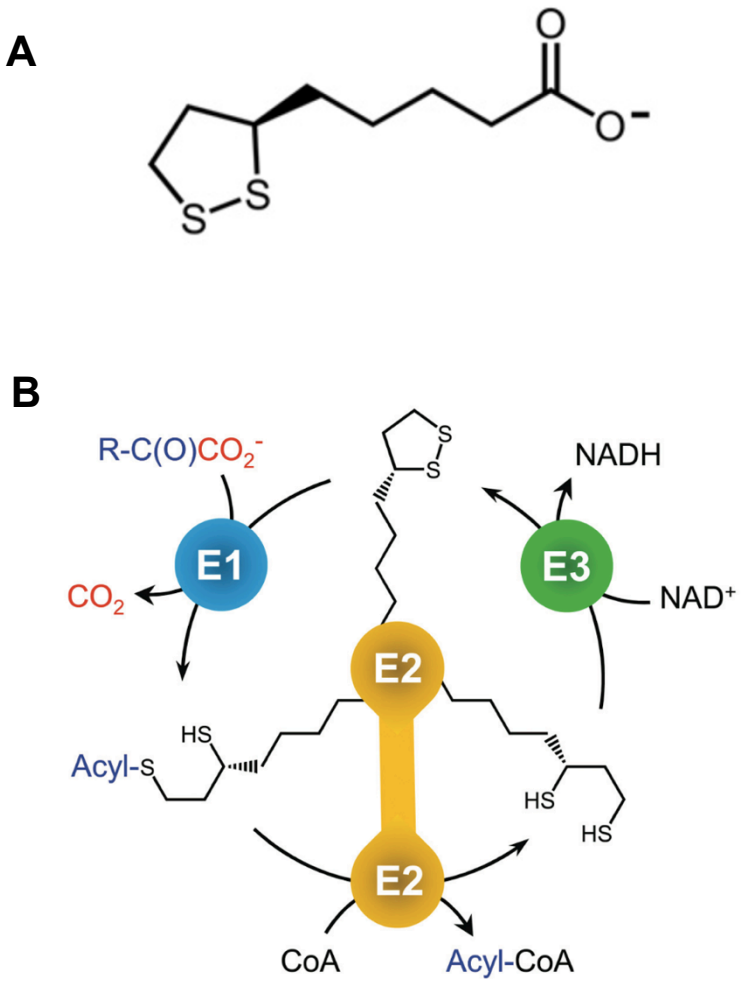

Figure 1: Structure and function of the cofactor lipoate

A. The biologically active form of lipoic acid, lipoate. B. Reactions catalyzed by 2-Oxoacid dehydrogenase complexes. First, the E1 subunit decarboxylates the 2- oxoacid substrate, subsequently, the lipoyl cofactor on the E2 subunit transfers the acyl group to coenzyme A (CoA). At the end of the cycle, lipoate is regenerated after reduction of NAD^+. Reprinted with permission from Spalding and Prigge (Figure 16 - Appendix I)

Lipoate can be scavenged or generated endogenously

Lipoylation is the posttranslational modification of proteins with lipoate, and it can occur by two known mechanisms: via lipoate biosynthesis or through lipoate scavenging from environmental sources (11). For those organisms that can generate lipoate, *de novo* biosynthesis is catalyzed by the action of a lipoate synthetase LipA, which uses octanoate, a molecule derived from fatty acid metabolism, as a precursor (16, 54, 55). Lipoate scavenging, instead, involves the acquisition of this cofactor from the environment and the direct ligation onto proteins. This process is referred to as the salvage pathway and is catalyzed by lipoate ligases (11, 15, 56).

Lipoate *de novo* biosynthesis occurs in two sequential steps, the transfer of octanoate from an acyl carrier protein (ACP) to a protein subunit (generally a member of one of the metabolic complexes described above), followed by the insertion of two sulfur atoms into the octanoate backbone (11). The initial ligation of octanoate to ACP depends on the enzymatic activity of the type II fatty acid synthetase; the direct transfer of octanoate from ACP to a conserved lysine of the target protein is catalyzed by an octanoyl transferase, which has been well characterized in numerous species (11, 54, 57). Finally, LipA catalyzes the irreversible conversion of the octanoyl group to a lipoate molecule by generating a disulfide bond (11, 57).

Lipoate biosynthesis can be bypassed by the action of lipoate ligases, which catalyze the insertion of free lipoate through generation of an amide bond

directly to the E2/H subunit of the enzyme complexes (11). Since free lipoate is rarely found to the environment due to its exclusive linkage to the protein subunits of PDH, OGDH, BCODH, AoDH complexes, as well as GCS, free lipoate has to first be made available by enzymes capable of breaking the lipoyl-protein amide bond (11, 16). Free lipoate *in vivo* is believed to come mostly from the gastrointestinal tract, where lipoate is released from lipoyl-proteins by the action of an intestinal amidase. For bacterial pathogens, lipoate most likely comes from damaged tissue, though it is not known whether or not all or some bacterial species encode an analogous amidase or similar enzyme that releases lipoate from source lipoyl-peptides (16). Of note, some ligases are also involved in the salvage of octanoate, which means that they can incorporate octanoate into target proteins, bypassing the action of the octanoyl transferase; yet, the use of octanoate as an exogenous source necessitates that the bacterium have a functional lipoate synthetase to generate lipoic acid (11, 16, 54).

Escherichia coli, *Bacillus subtilis*, and *Listeria monocytogenes* are three bacteria whose lipoate biosynthesis and salvage pathways are well characterized. In *E. coli*, *de novo* lipoate biosynthesis occurs after the sequential action of an octanoyl transferase, LipB, and a lipoate synthetase, LipA, as described above. The lipoate ligase, LplA, is capable of using both free lipoate and octanoate to bypass this *de novo* biosynthesis pathway (58).

Figure 2 depicts *B. subtilis* pathways for protein lipoylation. Unlike *E. coli*, the *B. subtilis* lipoate biosynthesis and salvage pathway requires four enzymes

and is now considered the model pathway of bacterial lipoylation in Gram-positive bacteria (11, 54, 59). Despite having a low degree of amino acid sequence homology, *B. subtilis* LipM catalyzes the same reaction as *E. coli* LipB; both are octanoyl transferases that remove an octanoate group exclusively from an octanoyl-ACP and neither enzyme has ligase activity. In *B. subtilis* the octanoyl group is transferred from the ACP to the H subunit of the GCS. This observation is consistent with the hypothesis that GcvH is the first protein to be lipoylated through the *de novo* biosynthesis in other microorganisms, such as *Plasmodium facilparum* and *Toxoplasma gondii* (14, 59, 60). In addition to LipA and LipM, the *B. subtilis* genome encodes a lipoate ligase, LplJ, and the amidotransferase, LipL, capable of transferring both lipoate and octanoate (11, 59).

The food-borne human pathogen *L. monocytogenes* is a lipoate auxotroph; it does not have a LipM or LipA homologs, thus, it has no means to generate lipoate endogenously (16, 61). Nevertheless, this Gram-positive bacterium has retained an amidotransferase enzyme, LipL and has two lipoate ligases, LplA1 and LplA2 (16, 61). Remarkably, whole cell extracts of *L. monocytogenes* have shown to have amidase activity, which facilitates the release of lipoate from mammalian tissues followed by incorporation into E2 and H subunits via the ligase LplA2 (15, 61).

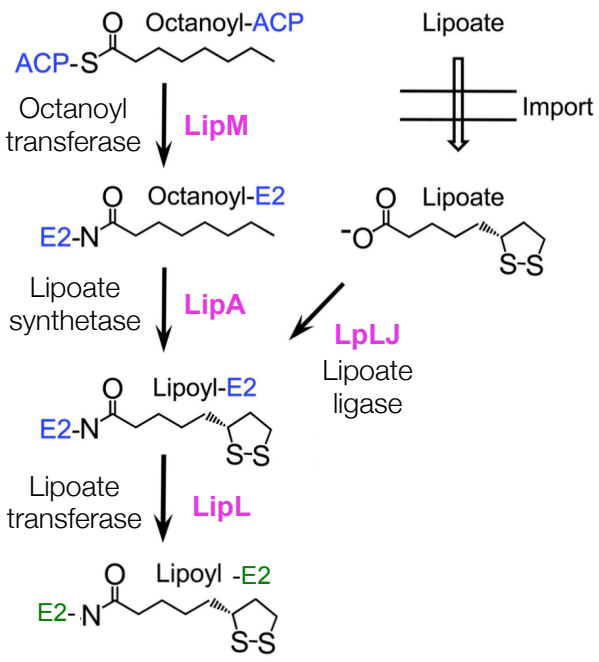

Figure 2: *B. subtilis* lipoylation mechanisms

This is the model lipoate biosynthesis pathway for *B. subtilis* (modified from Spalding *et al*. Reprinted with permission from Spalding and Prigge (Figure 16 - Appendix I). Lipoate *de novo* biosynthesis initiates with the transfer of an octanoyl group from an acyl carrier protein (ACP) to a metabolic complex protein subunit (Octanoyl-E2) by the octanoyl transferase LipM. Subsequently, the lipoate synthetase LipA catalyzes the irreversible conversion of octanoate to lipoate (Lipoyl-E2). LipL is involved in transferring lipoate groups among different subunits of the lipoylated enzyme complexes (Lipoyl-P2). Through the lipoate salvage pathway free lipoate is directly bound to the protein subunit through the action of the lipoate ligase, LplJ (16, 54, 59).

Mechanisms of lipoate acquisition vary among microbial species

Phylogeny and functional analysis of the enzymes involved in lipoate acquisition have revealed an interesting genetic diversity among species, even

within the same phylum. In 2010, M.D. Spalding and S.T. Prigge surveyed the literature on lipoic acid metabolism in bacterial, fungal, and protozoan microorganisms and described the remarkable degree of diversity that exists among microbial species (16). For example, the review indicates that some species lack the enzymatic machinery to produce lipoate endogenously, while others cannot scavenge lipoate or do not require lipoate to support survival at all. Furthermore, not all species express the five known lipoylated enzyme complexes, highlighting the diversity of lipoate acquisition and its metabolic enzyme complexes in nature (11, 16, 61).

A comparison between *Pseudomonas aeruginosa* and *Helicobacter pylori* highlights the diversity of lipoylation strategies found in Gram-negative bacteria. *H. pylori* does not require lipoate enzymes to sustain growth. The bacterium maintains an active TCA cycle by replacing the requirement of lipoic acid-containing enzymes, KDH and PDH, with the alternate 2-Oxoglutarate oxidoreductase (KOR) and the pyruvate:flavodoxin oxidoreductase (POR) (16). In contrast, *P. aeruginosa* uses both the salvage and the biosynthesis pathway to incorporate lipoate to five lipoylated enzyme complexes required for metabolic efficiency (16).

Mycobacterium tuberculosis, *Mycobacterium leprae* and *Corynebacterium diphtheriae* are three species of Actinobacteria that appear to depend exclusively on the endogenous synthesis of lipoate, as they do not encode lipoate ligases. These species have a lipoate metabolic pathway that resembles that of Gram-

negative bacteria, although they lack of a KDH complex, whose function is replaced by a 2-Oxoglutarate decarboxylase (16).

Gram-positive bacteria belonging to the phyla *Firmicutes* display additional diversity in their lipoate utilization strategies. Some *Clostridia* species are lipoate auxotrophs and encode a single lipoate ligase, while the major pathogenic species within the order *Bacillales* can encode multiple lipoate ligases, a lipoate synthetase, or both (16). For example, *L. monocytogenes* is a lipoate auxotroph that encodes two lipoate ligases, LplA1 and LplA2 (61). Interestingly, these lipoate ligases use different lipoylated substrates to replicate and have discrete biological roles *in vivo* and *in vitro* (15, 19). *B. subtilis* encodes one lipoate ligase, LplJ and has a complete set of enzymes dedicated to *de novo* biosynthesis pathway (54, 59). The lipoic acid biosynthesis and salvage pathways of *S. aureus* are currently unknown, but this species seems to encode a lipoate synthetase and several lipoate ligases (16). Additionally, *L. monocytogenes, B. subtilis* and *S. aureus* have different sets of lipoylated enzyme complexes; *B. subtilis* codes for PDH, OGDH, BCODH, GCS and AoDH; *S. aureus* lacks AoDH but encodes two GcvH proteins and *L. monocytogenes* lacks both KDH and AoDH (16).

Protein lipoylation in yeasts is restricted to the mitochondria. These microorganisms encode only three lipoylated enzyme complexes, PDH, KDH and GCS and three enzymes involved in lipoate metabolism, Lip2, Lip3 and Lip5. *Saccharomyces cerevisiae* and *Candida albicans* have the functional enzymatic

machineries to generate lipoate endogenously (Lip2 and Lip3 are *E. coli* LipB and LipA orthologs, respectively), but cannot scavenge lipoate despite encoding a lipoate synthetase (Lip3). Interestingly, Lip3 is an amidotransferase involved in transporting lipoyl groups from GCS to PDH and KDH. Thus, Lip2 and Lip5 are required to lipoylate GCS and Lip3 confers the ability to lipoylate the additional enzyme complexes found in the mitochondria (16).

In the pathogenic protozoans *Plasmodium falciparum* and *Toxoplasma gondii*, lipoate is attached to the E2 subunits of PDH, OGDH, BCODH and the H subunit of GCS, resembling the enzyme complexes found in Gram-positive bacteria (13, 14, 18). Interestingly, the lipoylated complexes have different subcellular location inside these parasites; PDH is located in the apicoplast and GCS and BCODH are present in the mitochondrion (16, 60). Furthermore, protein lipoylation differs in each organelle, with lipoate biosynthesis occurring in the apicoplast and lipoate salvage occurring in the mitochondrion (17, 62). In addition, malarial parasites encode LipA and LipB orthologs and two lipoate ligases (14). As in *L. monocytogenes, P. falciparum* lipoate ligases have substrate specificity; lipL1 preferentially lipoylates OGDH and PflipL2 catalyzes the lipoylation of PDH (16).

In summary, lipoate is a conserved molecule that permits the function of well-conserved enzyme complexes. Importantly, microbial species display a remarkable diversity regarding lipoylation strategies, highlighting the role of lipoate metabolism in adaptation to a variety of environmental niches.

To date, the *S. aureus* lipoate biosynthesis and salvage pathways have not been elucidated. Sequence alignment analyses suggest that this pathogen encodes a lipoate synthetase ortholog and several lipoate ligases, but no octanoyl transferase seems to be present in its genome. However, attempts to predict functions based exclusively on amino acid homology in other species have led to mistaken annotations and thus, the enzymatic activity of these putative lipoate ligases deserves further investigation (6, 15).

Lipoate biosynthesis and salvage in microbial pathogenesis

Intuitively, the fundamental role of lipoate in catalysis and redox reactions might have important implications for microbial pathogens, which often face nutritional restrictions and reactive oxygen intermediates during host infection. When considering microbial pathogenesis, the mechanisms of lipoate acquisition have been found to promote survival and adaptation in different pathogens, from bacteria to parasites and fungal species (11, 16). For example, it has been shown that PDH mediates expression of T3SS in *P. aeruginosa* by stabilizing the bacterial metabolic status through the production of acetyl-CoA (63). Additionally, it has been established that the disruption of a lipoylated complex in *M. tuberculosis* leads to increased susceptibility to macrophage killing and reduces its pathogenicity (64). Finally, evidence suggests that lipoate scavenging in the malarial parasite *P. falciparum* is required to infect the host (13, 16-18, 62).

The distinct roles that *L. monocytogenes* lipoate ligases, LplA1 and LplA2, have *in vivo* and *in vitro* constitute a remarkable example of adaptation to nutrient

deprivation during infection (61). One of its lipoate ligases, LplA1 has evolved to function during infection, sequestering lipoate from the host. Interestingly, this enzyme is dispensable in culture media (15, 61). Moreover, LplA1 and LplA2 are capable of using alternative sources of lipoate, an effect that is facilitated by a yet to be identified amidase present in whole cell lysates, which allows this pathogen to take better advantage of restricted nutrients that are not immediately bioavailable within the host (15, 59).

L. monocytogenes lipoate metabolism has also been topic of interest in the context of protein secretion since the E2 PDH and GcvH subunits have been found associated with the external bacterial surface. It is thought that these proteins are secreted by an accessory secretion system in *L. monocytogenes*, SecA2, which is required for virulence (16, 65). Although is currently unknown if these E2 and H subunits are being actively secreted to support pathogenesis, this raises the possibility that lipoylated proteins have a secondary function, dissimilar from its original metabolic role, in what is referred to as a moonlighting capacity (16, 65).

Having alternative lipoate acquisition mechanisms that enable lipoate scavenging from resource-deprived contexts constitutes an important asset for bacterial pathogens, beyond that of conferring metabolic adaptability. As stated above, lipoate can confer antioxidant protection by quenching free radicals that can ultimately kill bacterial cells. It does so by forming a redox couple with its reduced form, the molecule dihydrolipoate. The dihydrolipoate-lipoate pair is

capable of neutralizing superoxide radicals, the hydroxyl radical and hydrogen peroxide, as well as interacting with other antioxidants present in the cell membrane and the cytosol (6, 17, 18).

Concluding remarks

As described above, *S. aureus* is a versatile bacterium that produces myriad virulence factors in order to support colonization, tissue damage and dissemination in the host. Moreover, *S. aureus* has an astonishing capacity to adapt to different nutritional conditions as shown by its ability to scavenge essential nutrients, such as iron (43, 44). Unraveling the existing mechanisms behind *S. aureus* infection strategies is of paramount importance to develop new therapeutic strategies with the potential to reduce the morbidity and mortality associated with clinical disease. This thesis aims to study the role of one essential nutrient, lipoate, to determine its contribution to *S. aureus* fitness and pathogenesis.

The intersection of lipoate metabolism and virulence was first delineated by the discoveries that linked lipoate acquisition to bacterial and malarial pathogenesis, as shown for *L. monocytogenes*, *T. gondii* and *P. falciparum* (14, 15, 19, 62). The association of lipoate acquisition with microbial infection might be based on the fundamental role that this cofactor has in aerobic metabolism and redox homeostasis or may extend to additional moonlighting functions as suggested in *L. monocytogenes*. At this point, our knowledge of the underlying

processes that support this connection is minimal, in part due to the vast diversity of lipoate acquisition mechanisms that exist among species.

It is somewhat surprising that lipoate metabolism and its role in pathogenesis have not yet been explored in *S. aureus,* despite being one of the most important human pathogens. Homology-based studies revealed that *S. aureus* has a putative lipoate synthetase and several putative salvage ligases, indicating that this pathogen is capable of both lipoate biosynthesis and scavenging (16). The potential capacity to synthesize lipoate in addition to the unusual number of putative lipoate ligases predicted in the *S. aureus* genome open the question of whether this pathogen is capable of using different protein lipoylation approaches during infection.

Important human pathogens have been shown to use alternative sources of lipoate within the host (15, 61, 65) or compartmentalize the lipoylation mechanisms in their cells, relying on lipoate biosynthesis to lipoylate proteins in one organelle and supporting lipoylation through scavenging in another (14, 17, 18). Thus, it is conceivable that *S. aureus* has evolved alternative strategies to incorporate this essential nutrient in different environments; For example, it may use lipoate to adapt as a commensal bacterium and survive as an invasive pathogen or to colonize a specific tissue and survive inside phagocytes.

An especially intriguing connection drawn by *Rack et al.* is the potential relationship between protein lipoylation and oxidative stress responses, as well as the potential role of lipoate in the regulation of essential metabolic processes

in *S. aureus* (53). Furthermore, the existing evidence supporting the role of free lipoic acid as a redox modulator could have implications in pathogenesis, as this cofactor by itself could be used by *S. aureus* as a biological tool to cope with oxidative stress caused by the host defenses.

The answer to many of these questions, especially the role of lipoate in *S. aureus* pathogenesis, is hampered by our lack of knowledge on the mechanisms that this pathogen uses to lipoylate proteins. The scope of this work is to characterize the lipoate biosynthesis and salvage pathway in *S. aureus* and to establish whether the acquisition of this cofactor has a role in its pathogenesis.

CHAPTER TWO

MATERIALS AND METHODS

Bacterial strains and culture conditions

All bacterial strains used in this study are listed in Table 1. *S. aureus* pulse field gel electrophoresis type USA300 isolate LAC cured of its plasmids was used as the parental (wild type) strain for all genetic manipulations. *S. aureus* NCTC8325 derivative RN4220, *S. aureus* RN9011, *E. coli* DH5α and DC10B were used as host strains for propagation of recombinant plasmids and plasmid integration.

All *E. coli* strains were grown in Lysogeny Broth (LB) (Amresco) supplemented with antibiotics as indicated below. For replication of *S. aureus* strains, Tryptic Soy Broth (TSB) (Criterion) was used as a frich medium, and Roswell Park Memorial Institute 1640 medium (RPMI) (Corning) supplemented with 1% casamino acids (Amresco) was used as a defined medium lacking lipoate and octanoate. Unless otherwise specified, cultures were incubated at 37°C in a shaking incubator at 200 rpm with tubes held at a 45° angle. When required, LB and TSB media were solidified using 1.5% Agar (Amresco).

Where necessary, media were supplemented with the following antibiotics or chemicals at the following final concentrations: ampicillin (AMP) at 100 μg/ml,

Table 1: List of strains used in this study

Strain	Genotype	Designation
USA300 LAC	*S. aureus* USA300 Strain LAC. Plasmid cured.	LAC (WT)
DH5α	*E. coli* strain for recombinant pIMAY and pJC plasmids	
RN4220	Restriction deficient *S. aureus* for plasmid passage	RN4220
RN9011	RN4220 + pRN7203 expressing SaPI integrase	RN9011
FA-S831	AH-LAC with an in-frame deletion of *lipA*	Δ*lipA*
FA-S842	AH-LAC with an in-frame deletion of *lipM*	Δ*lipM*
FA-S841	AH-LAC with an in-frame deletion of *lplA1*	Δ*lplA1*
FA-S837	AH-LAC with an in-frame deletion of *lplA2*	Δ*lplA2*
FA-S1176	AH-LAC with an in-frame deletion of *lipL*	Δ*lipL*
FA-S1182	AH-LAC with in-frame deletions of *lipA* and *lipM*	Δ*lipA*Δ*lipM*
FA-S1249	AH-LAC with in-frame deletions of *lipA* and *lplA1*	Δ*lipA*Δ*lplA1*
FA-S1180	AH-LAC with in-frame deletions of *lipA* and *lplA2*	Δ*lipA*Δ*lplA2*
FA-S977	AH-LAC with in-frame deletion of *lipA* and Δ*lipL:kan*	Δ*lipA*Δ*lipL*
FA-S912	AH-LAC with in-frame deletions of *lplA1* and *lplA2*	Δ*lplA1*Δ*lplA2*
FA-S1251	AH-LAC with in-frame deletions of *lipM* and *lplA1*	Δ*lipM*Δ*lplA1*
FA-S957	AH-LAC with in-frame deletion of *lipM* and transposon insertion in *lplA2* transduced from NE266 (*lipM:erm*)	Δ*lipM*Δ*lplA2* (Δ*lipM-lplA2::erm*)
FA-S994	AH-LAC with in-frame deletion of *lipM* and Δ*lipL:kan*	Δ*lipM*Δ*lipL*
FA-S1210	AH-LAC with in-frame deletions of *lipL* and *lplA1*	Δ*lipL*Δ*lplA1*
FA-S998	AH-LAC with in-frame deletions of *lplA2* and Δ*lipL:kan*	Δ*lipL*Δ*lplA2*
FA-S1178	AH-LAC with in-frame deletions of *lipA, lplA1,* and *lplA2*	Δ*lipA*Δ*lplA1*Δ*lplA2*
FA-S992	AH-LAC with in-frame deletion of *lipA*, Δ*lipL:kan*, and transposon insertion in *lipM* transduced from NE1334	Δ*lipL*Δ*lipA*Δ*lipM* (Δ*lip-L*Δ*lipA-lipM::erm*)
FA-S1038	AH-LAC with in-frame deletion of *gcvH*	Δ*gcvH*
FA-S1041	AH-LAC with in-frame deletion of *E2-PDH*	Δ*E2-PDH*
FA-S1042	AH-LAC with in-frame deletion of *E2-OGDH*	Δ*E2-OGDH*
FA-S877	FA-S831 complemented with pJC1112-*lipA*	Δ*lipA+lipA*
FA-S1119	FA-S842 complemented with pJC1111-*lipM*	Δ*lipM+lipM*
FA-S1190	FA-S1176 complemented with pJC1111-*lipL*	Δ*lipL+lipL*
FA-S1258	FA-S1176 complemented with pJC1111-*pta*	Δ*lipL+pta*
FA-S1257	FA-S1176 complemented with pJC1111-*pta-lipL*	Δ*lipL+pta-lipL*
FA-S1206	FA-S912 complemented with pJC1111-*lplA1*	Δ*lplA1*Δ*lplA2+lplA1*
FA-S1208	FA-S912 complemented with pJC1111-*lplA2*	Δ*lplA1*Δ*lplA2+lplA2*
FA-S1259	FA-S1249 complemented with pJC1111-*lplA1*	Δ*lipA*Δ*lplA1+lplA1*
FA-S1205	FA-S1180 complemented with pJC1111-*lplA2*	Δ*lipA*Δ*lplA2+lplA2*
FA-S1260	FA-S1251 complemented with pJC1111-*lplA1*	Δ*lipM*Δ*lplA1+lplA1*
FA-S1222	FA-S1182 complemented with pJC1111-*lipM*	Δ*lipA*Δ*lipM+lipM*
FA-S1200	FA-S1178 complemented with pJC1111-*lplA1*	Δ*lipA*Δ*lplA1*Δ*lplA2+lplA1*
FA-S1212	FA-S1178 complemented with pJC1111-*lplA2*	Δ*lipA*Δ*lplA1*Δ*lplA2+lplA2*

The mutant strains listed in this table were generated with the help of former and current members of the lab under supervision of Dr. Francis Alonzo: Azul Zorzoli (~40% of the mutant strains), Ryan Novak, James Grayczyk and Irina Laczkovich.

erythromycin (ERM) at 3 μg/ml, kanamycin (KAN) at 50 μg/ml, neomycin (NEO) at 50 μg/ml, chloramphenicol (CM) at 10 μg/ml (Amresco), anhydrous tetracycline (ANTET) (Acròs Organics) at 1 μg/ml, $CdCl_2$ (Alfa Aesar) 0.3mM, Na

Citrate (Sigma) 10mM. Media used for experiments in which the requirement for lipoate or octanoate had to be bypassed were supplemented with the following short branched-chain carboxylic acids (Sigma): 10.8 mM isobutyric acid, 9.2 mM 2-methylbutyrate and 9 mM isovalerate, and 10 mM sodium acetate (Sigma). This medium has been previously used to bypass the metabolic requirement for lipoylated enzyme complexes involved in TCA cycle and branched chain amino acid catabolism (54) and will be referred as +BCFA throughout this document.

Molecular genetic techniques

S. aureus chromosomal DNA was isolated using Wizard Genomic DNA purification kit following the manufactures protocol (Promega). A 2 mg/ml stock solution of lysostaphin (Ambi Products, NY) was stored at -80°C in 20 mM sodium acetate, pH 4.5. For genomic DNA isolation, 2.5ul of the stock solution was added to a 1.2 mL culture of *S. aureus* that had been pelleted by centrifugation and resuspended in 200 ul of TSM buffer (50mM Tris, 0.5M D-Sucrose,10 mM $MgCl_2$ pH 7.5), followed by incubation for 15 minutes at 37°C to digest the cell wall. After lysostaphin treatment, the bacteria were centrifuged at maximum speed for 3 minutes in a microcentrifuge and bacterial DNA extracted following the remainder of the manufacturer's protocol. QIAGEN Mini / Midi plasmid isolation kits were used to extract recombinant plasmids. DNA gel extraction was performed using QIAGEN QIAquick kit. Polymerase chain reaction (PCR) was performed in Flexid Mastercycler (eppendorf) using Phusion High-Fidelity DNA Polymerase (New England Biolabs), oligonucleotides from

Eurofins and dNTPs from Quanta Biosciences. DNA ligation was performed in eppendorf ThermoMixer C using T4 DNA ligase (New England Biolabs). PCR purification was done using a QIAquick kit from QIAGEN. Genscript performed sequencing. All restriction endonucleases were purchased from New England Biolabs. 0.8% agarose gels (Amresco) was used for DNA electrophoresis.

E. coli competent cells preparation

A 3 mL culture of *E. coli* was grown overnight at 37°C in a shaking incubator. The following day, the strain was subcultured at a 1:55 dilution (2 mL into 110 mL) of LB in a 250 mL flask. Bacteria were incubated at 37°C with shaking for ~2.5 hr until reaching mid-logarithmic phase (OD600 0.3 -0.4). All remaining steps were conducted using pre-chilled solutions, flasks, and tubes. Four 50 mL tubes containing 25 mL of the log-phase culture were kept on ice for a period of 10 min, after which they were centrifuged at 4,000 rpm for 10 min at 4°C. Bacterial cells were harvested after decanting the supernatant and washed twice in 10 mL of filter-sterilized Transformation Buffer 1 (TBF-1) (30 mM KOAc, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% Glycerol, adjusted at pH 5.8 with 0.2 M Acetic Acid). Cells were incubated on ice for 10 minutes between washes. After a final centrifugation step at 4,700 rpm for 5 min the bacterial pellet was suspended in 1 mL of filter-sterilized Transformation Buffer 2 (TBF-2) (10 mM MOPS, 10 mM $RbCl_2$, 75 mM $CaCl_2$, 15% Glycerol (Amresco), adjusted at pH 6.5 with KOH). Finally, 100 µL of the competent cells were immediately aliquoted into 1.5 mL microcentrifuge tubes and stored at -80°C.

E. coli heat transformation

In order to transform chemically competent *E. coli*, 2 µL of the ligation product or purified plasmid was incubated with 50 µL competent cells on ice for 20 min. Afterwards, cells were incubated at 42°C for 45 seconds, placed on ice for 2 min and then suspended in 250 µL of SOC medium. (tryptone (Amresco), 0.5 % yeast extract (Amresco), 0.05 % NaCl (Amresco) in distilled water, supplemented with 10% 250mM KCl (Amresco) and adjusted to a pH 7.0 using 5M NaOH (Amresco). After autoclaving and cooling, 20% filter-sterilized 1M glucose solution was added. Incubation was performed with shaking at 30°C for 1 hr, after which 100 µL of the culture was spread onto LB selection plates containing the required antibiotic supplements.

Preparation of *S. aureus* electrocompetent cells

S. aureus competent cells were prepared by inoculating 300 µL of an overnight culture into 30 mL of fresh TSB. Cells were incubated with shaking at 37°C for 3 hr until reaching mid-logarithmic stage followed by centrifugation at 8,000 rpm for 10 min at 4°C. Unless indicated, cells were kept on ice throughout the entire process and all solutions and tubes used were pre-chilled. The bacterial pellet was washed 2 times in 30 mL ice-cold 10% glycerol. Cells were then resuspended in 15 mL 10% glycerol followed by additional centrifugation at 8,000 RPM for 10 min to pellet the bacteria. Lastly, cells were resuspended in 3 mL 10% glycerol and 200-500 µL aliquots were distributed into microcentrifuge tubes and stored at -80°C. Strains that harbored antibiotic resistance cassettes

were grown in media supplemented with antibiotics. Strains that showed impaired growth due to lipoate biosynthesis defects were grown in BCFA media.

S. aureus transformation via electroporation

Transformation was performed by incubating a mixture of 50 µL of thawed *S. aureus* competent cells with 10 µL of purified plasmid (~1 µg) at room temperature for 30 min. Cells were transferred to a 2 mm electroporation cuvette (VWR) and pulsed at 1,800 V, 10 µF, and 600 Ω in a GenePulser Xcell BIORAD electroporator. Cells were recovered using TSB or TSB+BCFA as needed, transferred to a microcentrifuge tube and incubated for 1 hr and 30 min. Afterwards, 100 µL were spread onto TSA plates supplemented with the appropriate antibiotics and incubated at 30°C for 24-48 hr.

Construction of mutagenesis vectors

Two fragments corresponding to ~500 nucleotides upstream (us) of the start codon (ATG) and ~500 nucleotides downstream (ds) from the stop codon (TAA) of the target gene of interest were amplified using oligonucleotide pairs shown in Table 2. Specifically, each us fragment was PCR amplified from wild type *S. aureus* genomic DNA using oligonucleotide #1 and oligonucleotide #2. ds fragments were generated using oligonucleotide #3 and oligonucleotide #4 (Table 2). Both us and ds amplicons were purified and used as templates in a SOEing PCR reaction to obtain the final amplicon required for mutagenesis. Each amplicon of ~1000 nucleotides was subcloned into pIMAY using KpnI and SacI restriction endonucleases.

Table 2: List of oligonucleotides used in this study

Name	Ref	Sequence	Final purpose
0829SOE1-Kpn	1	CCC-GGTACC(KpnI)-GCACAATGTGCCATCATCAA	*lipA* deletion
0829SOE2-Kas	2	CCTTATTAATGGTTAAATATTAACT-GGCGCC(KasI)-CGCCATAACAACACATACCC	*lipA* deletion
0829SOE3-Kas	3	GGGTATGTGTTGTTATGGCG-GGCGCC(KasI)-AGTTAATATTTAACCATTAATAAGG	*lipA* deletion
0829SOE4-Sac	4	CCC-GAGCTC(SacI)-ATTAATGTTCAGTATCTTGAATG	*lipA* deletion
0930SOE1-Kpn	1	CCC-GGTACC(KpnI)-GCATTATACCTGTATAAATAC	*lplA1* deletion
0930SOE2-Kas	2	ATAGTCCTTTAATCGTTTATGA-GGCGCC(KasI)-TTTCATTACAATCTCTCCCTT	*lplA1* deletion
0930SOE3-Kas	3	AAGGGAGAGATTGTAATGAAA-GGCGCC(KasI)-TCATAAACGATTAAAGGACTAT	*lplA1* deletion
0930SOE4-Sac	4	CCC-GAGCTC(SacI)-ATAAGCAAAACCTCGCTTTAT	*lplA1* deletion
0328SOE1-Kpn	1	CCC-GGTACC(Kpn1)-TATTTCATATACAAGGGGAGTATA	*lplA2* deletion
0328SOE2-Kas	2	CATACATAAAATAACAATATTAACT-GGCGCC(KasI)-GTACATCTTCTGTCTCTCCA	*lplA2* deletion
0328SOE3-Kas	3	TGGAGAGACAGAAGATGTAC-GGCGCC(KasI)-AGTTAATATTGTTATTTTATGTATG	*lplA2* deletion
0328SOE4-Sac	4	CCC-GAGCTC(SacI)-CTTGTGATAAATCCGCTTCG	*lplA2* deletion
1494SOE1-Kpn	1	CCC-GGTACC(KpnI)-TTATAGCCGCCTTTTAACATA	*lipM* deletion
1494SOE2-Kas	2	CATTTTTTATTCTAAAAACTACTT-GGCGCC(KasI)-AGTCATATTCAAGAACTCCTA	*lipM* deletion
1494SOE3-Kas	3	TAGGAGTTCTTGAATATGACT-GGCGCC(KasI)-AAGTAGTTTTTAGAATAAAAAATG	*lipM* deletion
1494SOE4-Sac	4	CCC-GAGCTC(SacI)-ATATTCAAAGTGCTCACACTT	*lipM* deletion
0571SOE1-Kpn	1	CCC-GGTACC(KpnI)-AAGTGCAAAATCAGCATTAAG	*lipL* deletion
0571SOE2-Kas	2	GGCTGTTAAATATTTAACTATTG-GGCGCC(KasI)-TTGCATAGGTTCTAATCCAG	*lipL* deletion
0571SOE3-Kas	3	CTGGATTAGAACCTATGCAA-GGCGCC(KasI)-CAATAGTTAAATATTTAACAGCC	*lipL* deletion
0571SOE4-Sac	4	CCC-GAGCTC(SacI)-GTTGCATACGCATTCTCGT	*lipL* deletion
0791SOE1-Kpn	1	CCC-GGTACC(KpnI)-AGGTTGCAGTCGTATGATTA	*gcvH* deletion
0791SOE2-Kas	2	ATTAAGGAGTTACACGGTGA-GGCGCC(KasI)-GAGAATCCCCTCCTAATTAA	*gcvH* deletion
0791SOE3-Kas	3	TTAATTAGGAGGGGATTCTC-GGCGCC(KasI)-TCACCGTGTAACTCCTTAAT	*gcvH* deletion
0791SOE4-Sac	4	CCC-GAGCTC(SacI)-CAGACATTCCATACATTTGATC	*gcvH* deletion
0995SOE1-Kpn		CCC-GGTACC(KpnI)-TGAAGAAGTACCTGAAGAAG	*e2-pdh* deletion
0995SOE2-Kas	2	GAAATCTCCAACTACCATGTT-GGCGCC(KasI)-GTTTTTGCCCTCCTAAGATT	*e2-pdh* deletion
0995SOE3-Kas	1	AATCTTAGGAGGGCAAAAAC-GGCGCC(KasI)-AACATGGTAGTTGGAGATTTC	*e2-pdh* deletion
0995SOE4-Sac	4	CCC-GAGCTC(SacI)-TACTTCTTGTAAGTTTAAAGCA	*e2-pdh* deletion
1305SOE1-Kpn	1	CCC-GGTACC(KpnI)-TGCACAAGCGGCTAGTTTA	*e2-ogdh* deletion
1305SOE2-Kas	2	TAAACTATTTTGTGTTGTGGA-GGCGCC(KasI)-GACTTATTTCCCCCTAGTTA	*e2-ogdh* deletion
1305SOE3-Kas	3	TAACTAGGGGGAAATAAGTC-GGCGCC(KasI)-TCCACAACACAAAATAGTTTA	*e2-ogdh* deletion
1305SOE4-Sac	4	CCC-GAGCTC(SacI)-AATCATAAATTATAGAATATCGG	*e2-ogdh* deletion
1464SOE1-Kpn	1	CCC-GGTACC(KpnI)-GCTAAAGGGCTTTTATTATCA	*e2-bcodh* deletion
1464SOE2-Kas	2	ATAGATGCATCTATGTTATCA-GGCGCC(KasI)-ACTTTCCCTCCCTAGAATT	*e2-bcodh* deletion
1464SOE3-Kas	3	AATTCTAGGGAGGGAAAGT-GGCGCC(KasI)-TGATAACATAGATGCATCTAT	*e2-bcodh* deletion
1464SOE4-Sac	4	CCC-GAGCTC(SacI)-GCTGGTCTTGCGATACCA	*e2-bcodh* deletion
KanF-Kas		TCCC-GGCGCC(KasI)-CTCGACGATAAACCCAGCGAAC	Kan^R cassette insertion
KanR-Kas		TCCC-GGCGCC(KasI)-CTTTTTAGACATCTAAATCTAGGTAC	Kan^R cassette insertion
0829CompF-Bam		GGC-GGATCC(BamHI)-GTAAGATTATTGGATTTTCATTT	*lipA* complement
0829CompR-EcoRI		GGC-GAATTC(EcoRI)-CTATCTATGACAATGAAAGG	*lipA* complement
UniCompSOE1-Pst	C1	ATAT-CTGCAG(PstI)-ATCCCATTATGCTTTGGCA	P_{HELP} amplification
0328CompSOE2	C2	AATCGGTTCTATTAAGTACATGGGTTTCACTCTCCTTCTA	P_{HELP} amplification
0328CompSOE3	C3	TAGAAGGAGAGTGAAACCCATGTACTTAATAGAACCGATT	*lplA2* complement
0328CompSOE4-Sac	C4	ATAT-GAGCTC(SacI)-TAGAAACAAAAACCATATATATA	*lplA2* complement
0930CompSOE2	C2	ATTATTATTACTAATGAATTTCATGGGTTTCACTCTCCTTCTA	P_{HELP} amplification
0930CompSOE3	C3	TAGAAGGAGAGTGAAACCCATGAAATTCATTAGTAATAATAAT	*lplA1* complement
0930CompSOE4-Sac	C4	ATAT-GAGCTC(SacI)-ACTATTCTTAACATCTCATAC	*lplA1* complement
1494CompSOE2	C2	AAAATTCCAAGTTTCAGTCATGGGTTTCACTCTCCTTCTA	P_{HELP} amplification
1494CompSOE3	C3	TAGAAGGAGAGTGAAACCCATGACTGAAACTTGGAATTTT	*lipM* complement
1494CompSOE4-Sac	C4	ATAT-GAGCTC(SacI)-TGTAGGTAAAATAGTCTACG	*lipM* complement
0571CompSOE2	C2	CGAATGCGAAAGATTGCATGGGTTTCACTCTCCTTCTA	P_{HELP} amplification
0571CompSOE3	C3	TAGAAGGAGAGTGAAACCCATGCAATCTTTCGCATTCG	*lipL* complement
0571CompSOE4-Sac	C4	ATAT-GAGCTC(SacI)-AAAAATAACAGCCCCAAACG	*lipL* complement
ptaSOE1-Pst	C1	ATAT-CTGCAG(PstI)-ATCCCATTATGCTTTGGCA	P_{HELP} amplification
ptaSOE2	C2	ATACATTTAATAAATCAGCCATGGGTTTCACTCTCCTTCTA	*pta* complement
ptaSOE3	C3	TAGAAGGAGAGTGAAACCCATGGCTGATTTATTAAATGTAT	*pta* complement
ptsSOE4	C4	ATAT-GAGCTC(SacI)-TTATTGTAAGGCTTGCGCT	*pta* complement
pta-0571SOE1-Pst	C1	ATAT-CTGCAG(PstI)-ATCCCATTATGCTTTGGCA	P_{HELP} amplification
pta-0571SOE2	C2	ATACATTTAATAAATCAGCCATGGGTTTCACTCTCCTTCTA	*pta lipL* compl.
pta-0571SOE3	C3	TAGAAGGAGAGTGAAACCCATGGCTGATTTATTAAATGTAT	P_{HELP} amplification
pta0571SOE4-Sac	C4	ATAT-GAGCTC(SacI)-AAAAATAACAGCCCCAAACG	*pta lipL* compl.

Generation of in-frame deletion mutants

Briefly, recombinant pIMAY containing 500 bp regions of homology us and ds of the target gene were transformed into *S. aureus* LAC via electroporation and propagated at 28-30°C in the presence of chloramphenicol. Plasmid recombination into the chromosome was induced by shifting to 37°C, a non-permissive temperature for pIMAY replication, in the presence of chloramphenicol. Plasmid integrants were then incubated at 30°C without antibiotics, to induce plasmid replication and excision from the chromosome. Bacteria were subsequently plated on medium containing anhydrous tetracycline (AnTet), which induces the expression of a *secY* antisense RNA transcript under control of the Pxyl/tetO promoter and serves as an effective counter-selection against any *S. aureus* still harboring pIMAY. Following counter-selection, chloramphenicol sensitive bacteria were screened for the presence of the desired mutation by PCR.

Construction of marked deletion mutants

To overcome difficulties encountered when generating mutants with growth deficiencies (*lipL, e2-pdh, e2-ogdh,* and *gcvH)*, we constructed mutagenesis plasmids containing an antibiotic resistance marker, *aphA (kan^R).* The kan resistance gene was PCR amplified from plasmid pBTK with oligonucleotides TCCC-GGCGCC-CTCGACGATAAACCCAGCGAAC and TCCC-GGCGCC-CTTTTTAGACATCTAAATCTAGGTAC and sub-cloned into a unique KasI site engineered between the us and ds regions of

homologypreviously cloned into pIMAY. Once constructed, the pIMAY gene replacement constructs were transformed into wild type *S. aureus* as described above. Allelic replacement was carried out as already described and gene replacement mutants were selected on Kan/Neo plates. This procedure was used to generate the following mutant strains: Δ*lipL*::*kan,* Δ*e2-pdh*::*kan,* Δ*e2-ogdh*::*kan,* and Δ*gcvH*::*kan*.

Construction of *lipL* markerless deletion mutant

The first attempts to construct clean deletions of *lipL* failed to yield mutants, likely due to the role of LipL in supporting the function of essential metabolic pathways in *S. aureus*. To generate this growth-defective mutant strain, we constructed the above-mentioned gene replacement mutant carrying an antibiotic selection marker in place of *lipL*. This initial strain, Δ*lipL*::*kan*, was then used as parental strain to construct a Δ*lipL* clean deletion mutant as well as Δ*lipA*Δ*lipM*Δ*lipL.* The excision of the antibiotic resistance gene was achieved by using pIMAY containing *lipL* us and ds 500bp regions of homology, constructed as described above leading to the generation of a Δ*lipL* mutant strain, whose identity was validated by DNA sequencing.

Construction of Δ*lipM* Δ*lipL*

Δ*lipL::kan* was introduced into Δ*lipM* via generalized transduction using bacteriophage ϕ11. The transduction process is described later in this section, in page 45.

Construction of Δ*lipM* Δ*lplA2*

Δ*lipM* Δ*lplA2* was constructed by transducing Δ*lplA2::erm* mutation into Δ*lipM* using bacteriophage ϕ11. Δ*lplA2::erm* (NE266) was obtained from the University of Nebraska USA300 *S. aureus bursa aurealis* transposon mutant library.

Construction of ΔlipA Δlip MΔlipL

This mutant was generated from *S. aureus* LAC with an in-frame deletion of *lipA* that was transduced with both a transposon inserted into *lipM,* obtained from NE1334 (*lipM::erm*), and the *lipL* deletion mutant bearing a kanamycin resistance cassette (Δ*lipL::kan*). All transductions were conducted using bacteriophage ϕ11, generating Δ*lipA* Δ*lipM* Δ*lipL*.

Bacteriophage-mediated generalized transduction

S. aureus specific bacteriophage ϕ11 was used for all transductions. To package phage with donor DNA, a 3 mL overnight culture of the donor strain containing the marked gene of interest was incubated with shaking at 37 °C in TSB-LB media (mixed 1:1) supplemented with 5mM $CaCl_2$ and 5mM $MgSO_4$. The overnight culture was diluted 1:100 into 10 mL of TSB-LB media supplemented with 5 mM $Cacl_2$ and 5 mM $MgSO_4$, and was then grown at 37°C in the shaking incubator for 2.5-3 hours, until reaching a OD 600 nm of 0.3 to 0.9. After this step, 500 µL of bacterial culture were incubated at room temperature with 10-fold serial dilutions of ϕ11 phage lysate stock in TMG (10 mM Tris pH 7.5, 5 mM $MgCl_2$, 0.01% gelatin (v/v)), aiming to achieve an approximate multiplicity of

infection of 1:1. After 30 minutes, the tubes containing the bacterial suspension and the phage dilutions were mixed with 3 mL of CY Top agar (Casamino acids 5 g/L, Yeast Extract 5 g/L glucose 5 g/L NaCl 6g/L, 7.5 g/L agar, +/- BCFA as needed) cooled at 55°C and supplemented with 5 mM $CaCl_2$ and 5 mM $MgSO_4$ and immediately poured on TSA (+/-BCFA as needed) plates supplemented with the corresponding antibiotic. Once solidified, plates were incubated overnight at 30°C. Following overnight growth, the top agar of 2-3 plates was scraped from those with confluent plaques using a sterile scoopula and introduced into a single 50 ml tube. Top agar was then suspended in 2 mL of phage buffer (TMG) per plate worth of Top agar. After extensive vortexing, the tubes were centrifuged for 15 min at maximum speed and the supernatant was filter-sterilized twice using 0.45 µm and 0.22 µm filters. Phage stocks were stored at 4°C.

To transduce recipient strains, overnight cultures containing 20 mL of the recipient cells in TSB:LB supplemented with 5mM $CaCl_2$ were centrifuged at maximum speed for 15 min and resuspended in 3 mL TSB:LB media (1:1), also supplemented with 5 mM $CaCl_2$. Phage infection was carried out by incubating 500 µL serial dilutions (1; 1:10; 1:100) of the bacterial suspension with either 100 µL of ϕ11 phage (10^8-10^9 PFU) or 100 µL of TMG buffer (control uninfected) at room temperature for 30 min, inverting the tubes every 10 min. After incubation, 40 mM final sodium citrate were added to the tubes from a 1M stock solution and samples were incubated for an additional 30 min period, inverting tubes every 10 min. Samples were then centrifuged at maximum speed for 5 min and washed in

500 µL LB:TSB medium supplemented with 40mM final sodium citrate. After a second centrifugation step cells were resuspended in 200 µL TSB containing 40 mM final sodium citrate and 100 µL were spread onto TSA BCFA plates containing 10 mM sodium citrate supplemented with the necessary antibiotics. Plates were incubated at 30°C for 24-48 hr or until detection of bacterial colonies. Potential transductants were later validated via PCR.

Generation of complemented strains

S. aureus complementation strains were generated using plasmid pJC1111(66) for all strains, except for complementation with *lipA*, for which we used the vector pJC1112 (67). These vectors were propagated in *E. coli* DH5α and selected for ampicillin resistance. Both pJC1111 and pJC1112 stably integrate into the SaPI-1 site of the *S. aureus* chromosome after passage through strain *S. aureus* RN9011 containing pRN7203 expressing SaPI-1 integrase, leading to single-copy stable integration of the plasmid.

In order to generate complementation strains using pJC1111 we first generated constructs that drive expression of the *lipM*, *lipL*, *pta*, *pta-lipL*, *lplA1* or *lplA2* genes under the control of the constitutive P_{HELP} promoter obtained from pIMAY (68). All oligonucleotides designed for amplification of these complement constructs are listed in Table 2. Primers designated as C1 and C2 were used to amplify the P_{HELP} promoter using pIMAY as template DNA. Primers identified as C3 and C4 were used to amplify the open reading frame corresponding to the gene of interest for complementation including ~150 nucleotides ds. These two

DNA fragments were joined via SOEing PCR as described above. All amplicons were subsequently purified and cloned into pJC1111 after digestion with restriction enzymes Pst1 and Sac1 and subsequent ligation into pJC1111 generating pJC1111-*lipM,* pJC1111-*lipL,* pJC1111-*lplA1,* pJC1111-*lplA2,* pJC1111-*pta* and pJC1111-*pta-lipL.*

All complementation plasmids were propagated in *E. coli* DH5α, followed by isolation and subsequent electroporation into *S. aureus* RN9011 and plating on TSA+BCFA plates supplemented with chloramphenicol and $CdCl_2$. Integrants were selected based upon CM^r $CdCl_2^r$ and were used as donor strains to package and transduce the complementation allele into the desired mutant strain. Complemented strains were selected based on their $CdCl_2$ resistance and validated via PCR. This process resulted in the generation of strains Δ*lipM+lipM;* Δ*lipl+lipl;* Δ*lipL+pta;* Δ*lipL+pta-lipL;* Δ*lipA*Δ*lplA1+lplA1;* Δ*lipA*Δ*lplA2+lplA2;* Δ*lipM*Δ*lplA1+lplA1;* Δ*lipA*Δ*lipM+lipM;* Δ*lipA*Δ*lplA1*Δ*lplA1+lplA1, and* Δ*lipA*Δ*lplA1*Δ*lplA1+lplA2.*

Complementation of Δ*lipA* was accomplished by amplification of the *lipA* gene with its native promoter using primers 0829CompF-Bam and 0829CompR-EcoRI. The resultant amplicon was cloned into pJC1112 after digestion with enzymes BamHI and EcoRI. Plasmid propagation in *E. coli* DH5α was performed as described above and pJC1112-*lipA* was subsequently electroporated into *S. aureus* RN9011. The chromosomally integrated complementation vector was later packaged into bacteriophage ϕ11 and transduced into a Δ*lipA* background.

Positive transformants were selected based on their erythromycin resistance (3.5 µg/mL) and designated Δ*lipA+lipA.*

Requirement of lipoate or octanoate for bacterial growth

Bacterial growth curves were carried out in four types of media: RPMI, RPMI+BCFA, RPMI+Lipoate and RPMI+Octanoate. Overnight cultures were prepared in triplicate from three individual colonies by inoculating 200 µL of RPMI+BCFA in adjacent wells of a round-bottom 96-well polystyrene plate (Corning) followed by incubating overnight with shaking at 200 RPM at 37°C. The following morning, cells were pelleted at 3,700 rpm for 15 min and washed three times with 200 µL of RPMI to remove any remaining branched chain carboxylic acids. After washing cells, 2 µL of each triplicate sample was inoculated into flat-bottom polystyrene 96-well plates (Corning) containing 198 µL of RPMI, RPMI+BCFA, RPMI+25 nM of α-Lipoic acid (Sigma) or RPMI+250 µM Octanoic acid (Sigma). Bacterial growth was assessed over time by measuring optical density at 550 nm (OD550), using microplate reader ELx800 (Biotech) until reaching stationary phase (9-10 hours). Results were graphed and analyzed using Prism® (GraphPad Software, Inc., San Diego, CA). All growth curves were conducted at least three times in triplicate. The mean optical density at 550 nm and standard deviation are shown for all curves.

Whole cell lysate preparation

S. aureus wild type and mutant strains were grown overnight with shaking at 37°C in 15 mL conical tubes containing 5 mL of RPMI+BCFA media. A

subculture of 60 µL of these samples was inoculated into 15 mL conical tubes containing 6 mL of RPMI+BCFA, RPMI+BCFA+5 µM α-Lipoic acid, or RPMI+BCFA+170 µM Octanoic acid. Samples were incubated with shaking at 200 RPM for 9 hr and bacterial growth was determined by measuring optical density at 600nm with 1 mL of the culture using a Genesys 10S UV-Vis spectrophotometer (Thermo). The remaining culture volume was centrifuged at 4,200 rpm for 15 min, the supernatant was discarded, and the bacterial pellets were stored at -80°C until whole cell lysates were prepared. After thawing frozen pellets on ice, the bacteria were suspended in 250 µL of PBS and transferred to screw cap microcentrifuge lysing tubes (Fisher Scientific) containing 250 µL 0.1mm glass cell disruption beads (Scientific Industries, Inc.). Cells were lysed using a Fast Prep-24 5G (MP Biomedicals) in two sequential steps, at 5.0 speed for 20 seconds and at 4.5 speed for 20 seconds, each separated by a 5 min incubation period on ice. After cell disruption, samples were centrifuged at 13,000 rpm for 15 min. 130 µL of the supernatant were collected in microcentrifuge tubes containing 43 µL of 6X SDS loading buffer (1.11mL 0.9M Tris-HCl pH 6.8; 0.16g SDS; 2 mL 100% Glycerol; 0.2 mL 14.7M BME; 0.5 mL 0.5 M EDTA, 4 mg bromophenol blue in a 10 mL final volume) and samples were boiled for 10 min prior to storage at -20 C.

Protein lipoylation assessment

Proteins samples from OD-normalized whole cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in

12% polyacrylamide gels at 120 volts for approximately 4 hours. Coomassie staining was performed to evaluate protein patterns and equivalent loading of samples using GelCode Blue stain reagent (Thermo) with Precision Plus Protein Ladder™ (Thermo) used as a molecular weight marker. Briefly, resolved proteins were transferred from polyacrylamide gels to 0.2 µm PVDF membranes (Immobilon, Roche) at 200 V for 1 hr in a Quadra Mini-Vertical PAGE/Blotting System (CBS Scientific). After transfer, membranes were incubated overnight with PBST (0.1% Tween-20 in PBS) supplemented with 5% BSA at 4°C. Nonspecific binding of antibody to *S. aureus* antibody binding proteins, Protein A and Sbi, was blocked by incubating the membranes with 0.9 mg/mL human IgG (Sigma) for 1hr. A 1:3000 dilution of rabbit polyclonal anti-lipoic acid antibody (Calbiochem) in PBST was added to the membrane followed by incubation for 1 hour and three subsequent washed in ~20mL of PBST. Goat anti-Rabbit IgG (H+L) HRP conjugate (Thermo) was then added at a 1:200 dilution in PBST for an additional hour followed by three 15 minutes washes in ~20 mL of PBST. Western blot images were captured on a FluorChem System (Protein Simple) using SuperSignal West Pico Chemiluminiscent Substrate (Thermo).

Murine systemic infections

All animal experiments were performed in ABSL2 facilities with protocols approved by Loyola University of Chicago in accordance with the standards of the Institutional Animal Care and Use Committee. Each experiment was performed at least three times with cohorts of at least 4 animals. Overnight

cultures of each strain were inoculated from freshly isolated single colonies struck out from the freezer the day prior and grown with shaking at 37°C for ~16 hours. A 1:100 subculture into 15 mL of TSB+BCFA was performed followed by incubation with shaking at 200 rpm, 37°C for 3-4 hr (until reaching optical density at 600 nm near 1.0). Cultures were then centrifuged for 5 minutes at maximum speed in a tabletop centrifuge. Cell pellets were washed twice in 5 mL PBS and 2 mL of the final bacterial suspension was added to a 15 mL conical tube containing 8 mL of PBS. Bacterial suspensions were then normalized with PBS to reach to an O.D. of 0.32 - 0.33 (1 x 10^8 CFU/mL). Six to eight week old female Swiss Webster mice obtained from Envigo (formerly Harlan) were used in all experiments. Mice were deeply anesthetized with 2,2,2-tribromoethanol (Avertin; Sigma) via intraperitoneal injection, followed by inoculation with 100 µL PBS containing 1 x 10^7 CFU wild type *S. aureus* or mutant strains directly into the bloodstream via injection into the retro-orbital venous plexus. After infection, the remaining bacterial-PBS suspension was plated on TSA-BCFA plates to ensure viability of cells in PBS and accurate infection inoculum. All strains were fully viable in PBS and all animals received between (1.0 and 2.0 x 10^7 CFU). Infected mice were monitored daily and their kidneys and hearts were isolated immediately after euthanasia, at 96 hr post infection. Tissues were aseptically isolated, homogenized and spread onto TSA-BCFA agar plates and incubated overnight at 37°C in order to enumerate CFU.

Statistical Analysis

Data analysis was performed via nonparametric 1-way ANOVA using Prism Graphpad Software V.7 with evaluation of statistical significance done with Krustal-Wallis multiple comparison post-test. N values in mice experiments are indicated in the corresponding figures. Growth curves were conducted at least three times and each one was generated from three independent single colonies. For each experiment, mean and standard deviations of each data point were obtained after averaging the values in optical densities. Immunoblots were conducted a minimum of four times from freshly prepared samples of whole cell lysates normalized according to their optical density values after 9 hours of incubation at 37°C.

CHAPTER THREE

EXPERIMENTAL RESULTS

Introduction

Staphylococcus aureus is both a skin commensal bacterium and one of the most relevant human bacterial pathogens, capable of causing minor infections and life-threatening diseases. In the last few years, there has been an increase in the onset of *S. aureus* community-acquired infections, some of which are caused by hypervirulent strains with an unusual resistance to antimicrobials. The reason that *S. aureus* is such a versatile and efficient pathogen stems from its production of an artillery of virulence factors, along with its ability to hunt for essential metabolites in nutritionally restricted environments (1, 7).

In a transposon mutagenesis screen designed to identify novel genes with potential immunomodulatory or virulence functions, Dr. Alonzo identified a transposon insertion in the gene *SAUSA300-0829*, encoding a putative lipoate synthetase (*lipA)*. Supernatant derived from this transposon mutant elicited a hyper inflammatory response *in vivo* and *in vitro* (unpublished data), suggesting a potential link between lipoate biosynthesis in *S. aureus* and pathogenesis. Based on the information available from other bacterial species that harness lipoate biosynthesis for pathogenesis, and considering the major role for lipoate

in metabolism in bacterial survival, we hypothesize that the mechanisms of lipoate acquisition in *S. aureus* contribute to fitness and pathogenesis by promoting survival in infectious sites with limited access to lipoate.

Until now, the mechanisms of lipoate biosynthesis and salvage, as well as the role of lipoate in *S. aureus* infection biology, remain unknown. The work in this thesis aimed to address these topics.

The model pathway for lipoate acquisition

The mechanisms of lipoylation in bacteria were first described in *Escherichia coli*, considered the model organism on which studies of lipoate *de novo* biosynthesis and salvage are based. However, more recent work using *Listeria monocytogenes* and *Bacillus subtilis* revealed significant differences in the lipoate biosynthesis and salvage pathways of these microorganisms, suggesting divergence from those of *E. coli* (16). Due to the moderate amino acid sequence similarities of *S. aureus* lipoate biosynthesis and salvage genes compared to that of *E. coli*, our work instead derives from comparisons to *B. subtilis* lipoate ligases and biosynthesis genes. A simplified pathway for the *B. subtilis* lipoate biosynthesis and salvage pathways is depicted in Figure 2.

In *B. subtilis*, the endogenous synthesis of lipoate occurs in two sequential steps catalyzed first by an octanoyl transferase (LipM) and then by a lipoate synthetase (LipA). The first step consists of transferring an octanoate group from an octanoylated acyl carrier protein (ACP) to a target E2 or H protein subunit. The second step entails the synthesis of lipoate via the insertion of two sulfur atoms into the octanoate backbone by LipA. Additionally, *B. subtilis* requires a

third enzyme for lipoylation of the target proteins known as LipL, a lipoyl transferase. By using its lipoate salvage pathway, *B. subtilis* can bypass both octanoyl transferase and the lipoate synthetase activities using LplJ, a lipoate ligase capable of scavenging free lipoate and octanoate from environmental sources.

Based on the comparison of amino acid identity of *S. aureus* USA300 isolate FPR3757 lipoate biosynthesis/salvage pathway, this pathogen appears to be more complex than most other Gram-positive pathogens. Current gene annotations suggest that *S. aureus* encodes four putative lipoate ligases and one lipoate synthetase. According to this annotation, *S. aureus* would not encode an octanoyl transferase or a lipoyl transferase. Nevertheless, our bioinformatics analyses revealed similarities to *B. subtilis* enzymes involved in *de novo* lipoate biosynthesis and salvage and to *L. monocytogenes* lipoate ligases (**.**

Table 3 and Fig. 16 Appendix II). For instance, in *S. aureus* a number of gene products annotated as lipoate ligases exhibit significant amino acid sequence identity to *B. subtilis*: SAUSA-300-0571 54% similarity to LipL and SAUSA300-1494 has 62% identity to LipM, indicating that some of these gene products might be lipoyl/octanoyl transferases. Additionally, in contrast to *B. subtilis,* which only has one lipoate ligase (LplJ)*, S. aureus* appears to have two gene products, 0328 and 0930, with predicted ligase functionality. The amino acid sequence alignment shows that SAUSA-300-0328 has 39% similarity, while SAUSA-300-0930 has 57% amino acid sequence identity to *B. subtilis* LplJ.

The presence of several putative lipoate ligases and a lipoate synthetase in the genome of *S. aureus* implies that this pathogen uses both the *de novo* biosynthesis and salvage pathways to acquire lipoate. Interestingly, the existence of two putative ligases in *S. aureus* genome is a unique characteristic that distinguishes this species from other Gram-positive pathogens. The presence of more than one lipoate ligase is a feature most commonly associated with organisms that lack a *de novo* lipoate biosynthesis pathway, such as *L. monocytogenes* (16, 19). When we compared 0930 and 0328 with the sequences coding for *L. monocytogenes* lipoate ligases, we observed that *S. aureus* putative lipoate ligases genes had a moderate (37-41%) amino acid sequence similarity to *L. monocytogenes* LplA1 and LplA2 (Table 3 and Fig. 16 Appendix II). This information may imply that *S. aureus* encodes two lipoate ligases in addition to potentially having the enzymes required for lipoate *de novo* biosynthesis.

Our bioinformatics analyses revealed a number of distinct genes with potential novel roles in lipoate metabolism within the genome of S. aureus that deserved further investigation; Figure 3 depicts their genomic localization and operon organization. For clarity purposes, we have decided to adopt relevant B. subtilis and L. monocytogenes nomenclatures. Thus, S. aureus genes potentially involved in lipoate de novo biosynthesis (0829, 1494 and 0571) are named according to B. subtilis classification (lipA, lipM and lipL, respectively) and the genes potentially involved in lipoate salvage (*0930* and *0328*) are named following *L. monocytogenes* gene nomenclature (*lplA1* and *lplA2*, respectively)**.**

Table 3: ***S. aureus*** **putative genes involved in lipoate metabolism**

S. aureus annotation	Gene Product	% Identity to *B. subtilis* / Predicted function
Lipoate synthetase	0829 (LipA)	LipA - Lipoate synthetase (79%)
Lipoate Ligase	1494 (LipM)	LipM - Octanoyl transferase (62%)
	0571 (LipL)	LipL - Lipoyl transferase (54%)
	0930 (LplA1)	LplJ - Lipoate ligase (57%)
	0328 (LplA2)	LplJ - Lipoate ligase (39%)

In addition to their similarity to *B. subtilis* LplJ, the putative lipoate ligases 0930 and 0328 share between 37%-41% amino acid identity with *L. monocytogenes* lipoate ligases LplA1 and LplA2. Due to the fact that *S. aureus* potentially encodes two lipoate ligases and not one, we have decided to adopt the nomenclature established for *L. monocytogenes* for the genes involved in lipoate salvage. Thus, genes 0930 and 0328 will be referred as LplA1 and LplA2.

The fact that the two ligases in *L. monocytogenes* have different affinities for lipoylated peptides *in vivo* (15) suggests potential unique functions for the *S. aureus* lipoate ligases in fitness and pathogenesis.

Of note, two genes associated with lipoate metabolism in *S. aureus* were found in operon arrangements. Our bioinformatics analysis of S. aureus gene arrangements showed that *lipL* gene is clustered in a putative operon with the *pta* gene, presumably responsible to encode the phosphotransacetylase in *S. aureus*. Pta is linked to the acetyl CoA kinase (ACKA); together they catalyze the reversible interconversion of acetyl CoA to acetate and ATP during overflow

metabolism. The *S. aureus 0328* (*lplA2*) putative lipoate ligase gene is the second gene with a unique genetic arrangement in the chromosome. It is located in an extended operon that encodes the sirtuin SirtM and its macrodomain, along with GcvH-L (53). Interestingly, LplA2 modifies GcvH-L in a lipoylation reaction that precedes the reversible modification of GcvH-L via SirtM (53).

In a few bacterial and malarial pathogens *lplA* orthologs can be found near the genes that encode for E2-PDH and other lipoylated complexes (16). This association could, in principle, facilitate the association between a lipoate enzyme to its target proteins. Whether these unique genetic arrangements are involved in additional functions or regulation of metabolic processes remains to be tested.

In this study, I planned to determine the biological role of the components of the lipoate *de novo* biosynthesis and salvage pathway in *S. aureus* in order to fully establish the mechanisms used to acquire this essential cofactor. Specifically, I aimed to test the hypothesis that not all *S. aureus* genes annotated as lipoate ligases are actually involved in lipoate salvage, but rather have functions related to either lipoate salvage or *de novo* biosynthesis. Also, I proposed to study the requirement for this pathway in *S. aureus* aerobic growth. Lastly, I aimed to determine whether lipoate *de novo* biosynthesis and salvage pathways facilitate *S. aureus* metabolic adaptability during infection.

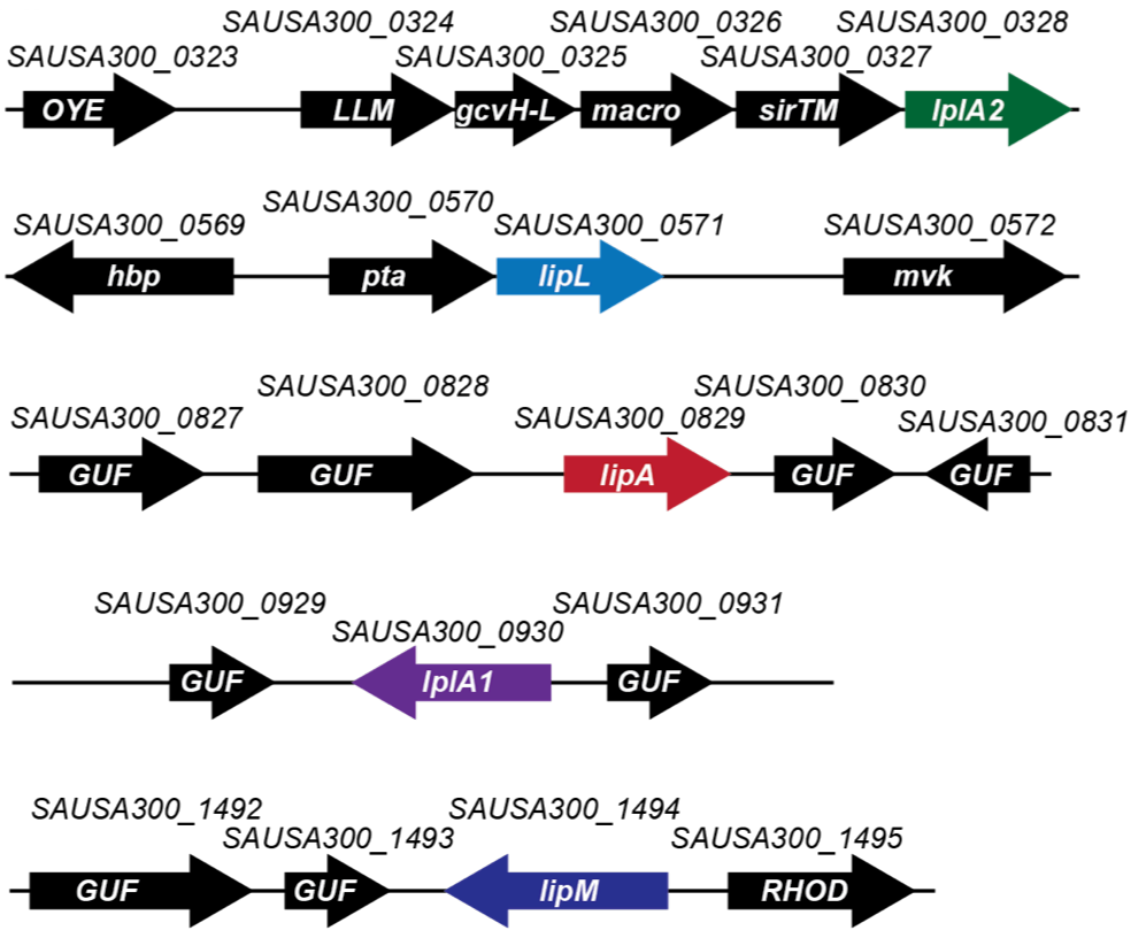

Figure 3: Genome organization of *S. aureus* lipoate predicted genes

Boxed arrows indicate open reading frames. LplaA2 is encoded in the SirTM operon (53). The gene encoding the putative lipoyl transferase (amidotransferase) LipL is located in an operon with the gene coding for the enzyme phosphotransacetylase. The following flanking genes do not seem to be relevant to lipoate metabolism or regulation: hbp: heme binding protein; mvk- mevalonate kinase - rhod: rhodanase domain protein - guf: gene of unknown function.

For clarity purposes and based in the information provided above, we propose the following hypothetical pathway for the acquisition of lipoate via *de novo* biosynthesis and salvage.

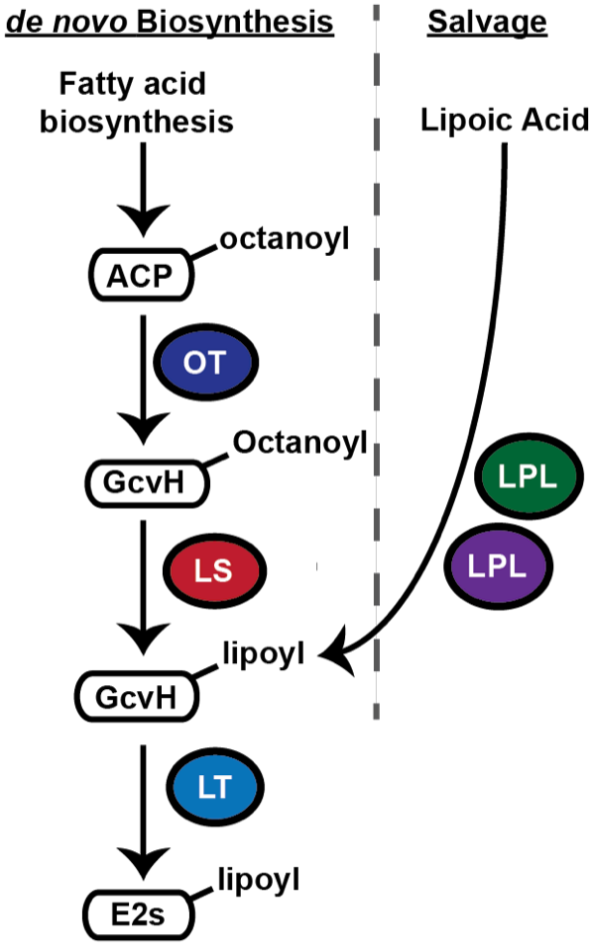

Figure 4: Hypothetical lipoate *de novo* biosynthesis and salvage pathway

This is a simplified model for lipoate *de novo* biosynthesis and salvage in *S. aureus*. Lipoate biosynthesis is catalyzed by a lipoate synthetase (LS) prior octanoylation of GcvH by an octanoyl transferase (OT). Lipoyl groups are subsequently transferred to E2 subunits of target proteins by a lipoyl transferase (LT). In the salvage pathway, lipoate is incorporated from the environment through the action of lipoate ligases (LPL)

Construction of deletion mutants to study *S. aureus* lipoate pathway

We constructed nonpolar in-frame deletion mutants of all five genes predicted to be involved in lipoate acquisition, as well as their corresponding complementation strains to evaluate the requirement for each gene in *de novo* lipoate biosynthesis. Based on our bioinformatics analyses, we anticipated the single deletion mutants of the putative lipoate ligases, Δ*lplA1* and Δ*lplA2*, would not be sufficient to elucidate the biological role of these enzymes, since these mutant strains would still be capable of generating lipoate *de novo* due to the presence of *lipA* in each strain. Therefore, in order to ascertain the function of the lipoate ligases, we also needed to construct double and triple in-frame deletion mutants of both ligase genes in the background of a *lipA* deletion mutant. Lastly, to fully dissect the steps in *S. aureus* protein lipoylation, we also generated double deletion mutants of the putative lipoate ligases in both Δ*lipM* and Δ*lipL* genetic backgrounds. As required, we generated complementation strains for all biosynthesis and salvage mutants displaying phenotypic variances from wild type.

Table 1 lists the mutant strains generated to fully dissect *S. aureus de novo* biosynthesis and salvage pathways. Unless indicated, all strains were generated by myself or with the assistance of others in the lab. The table also includes our WT strain USA300 LAC, *E. coli* DH5α, *S. aureus* RN4220 and *S. aureus* RN9011, all of which were required to propagate plasmids and for the construction of mutants or their complements. Table 2 lists the oligonucleotides

used for mutant and complemented strains. For the generation of mutant strains with growth deficiencies (*lipL, e2-pdh, e2-ogdh,* and *gcvH),* we conducted mutagenesis using plasmids containing a kanamycin resistance marker.

Identification of the lipoylated protein subunits in *S. aureus*

To elucidate the lipoate *de novo* biosynthesis and salvage pathway in *S. aureus,* we assessed in-frame deletion mutants of the putative genes involved in lipoate metabolism for their ability to replicate and lipoylate metabolic E2 and H subunits. As mentioned previously, the literature suggests that *S. aureus* possesses four lipoate-containing multienzyme complexes, PDH, OGDH, BCODH and GCS, each bearing a lipoate molecule attached to a core E2 or H subunit (16). These lipoylated subunits can be detected as four distinct bands in immunoblots of *S. aureus* whole cell lysates using anti-lipoic acid antibody. GcvH-L, the protein described by Rack *et al.* (53)

To determine the identity of the four lipoylated proteins detected in *S. aureus* whole cell lysates, we evaluated the lipoylation profiles of ΔE2-pdh, ΔE2-ogdh and ΔgcvH (H subunit of the glycine cleavage complex) mutants (Figure 5). constructed by James Grayczyk, a current member of our lab. Unfortunately, generation of a Δ*E2-bcodh* mutant was unsuccessful, which suggests a major role of the branched-chain 2-Oxoacid dehydrogenase complex in maintaining *S. aureus* viability. If *E2-pdh*, *E2-ogdh*, and *gcvH* encode proteins that get lipoylated in *S. aureus*, then we should see an absence of lipoylation corresponding to the product encoded by that gene, regardless of whether or not lipoate is supplemented exogenously.

Wild type *S. aureus* lipoylates four protein species at 72, 65, 51, and 23 kilodaltons (Figure 5). After deletion of the E2 subunit of PDH, the ~72 kDa band is no longer detectable. When we tested Δ*E2-ogdh* in the presence or absence of lipoate supplementation, we were unable to detect the ~65 kDa band. The ~23 kDa lipoylated protein observed in the wild type lipoylation profile was no longer detectable after deletion of the H subunit of the GCS complex, in the presence or absence of lipoate supplementation. In the absence of exogenous lipoate, the Δ*gcvH* mutant has no detectable lipoylated proteins. After supplementation with lipoate, protein lipoylation was restored to a Δ*gcvH* mutant, with the exception of the ~23 kDa protein band. These results indicate that the ~72 kDa lipoylated protein corresponds to E2-PDH, the ~65 kDa molecular weight species to E2-OGDH, and the ~23 kDa protein to the H subunit of the GCS. By process of elimination, E2-BCODH corresponds to the 51 kDa lipoylated protein band (Figure 5B). The defects in global protein lipoylation observed for the Δ*gcvH* mutant in the absence of lipoate suggests that *de novo* biosynthesis of lipoate and subsequent protein transfer in *S. aureus* occurs sequentially, with GcvH required for the lipoylation of other protein subunits when lipoate is not supplemented exogenously.

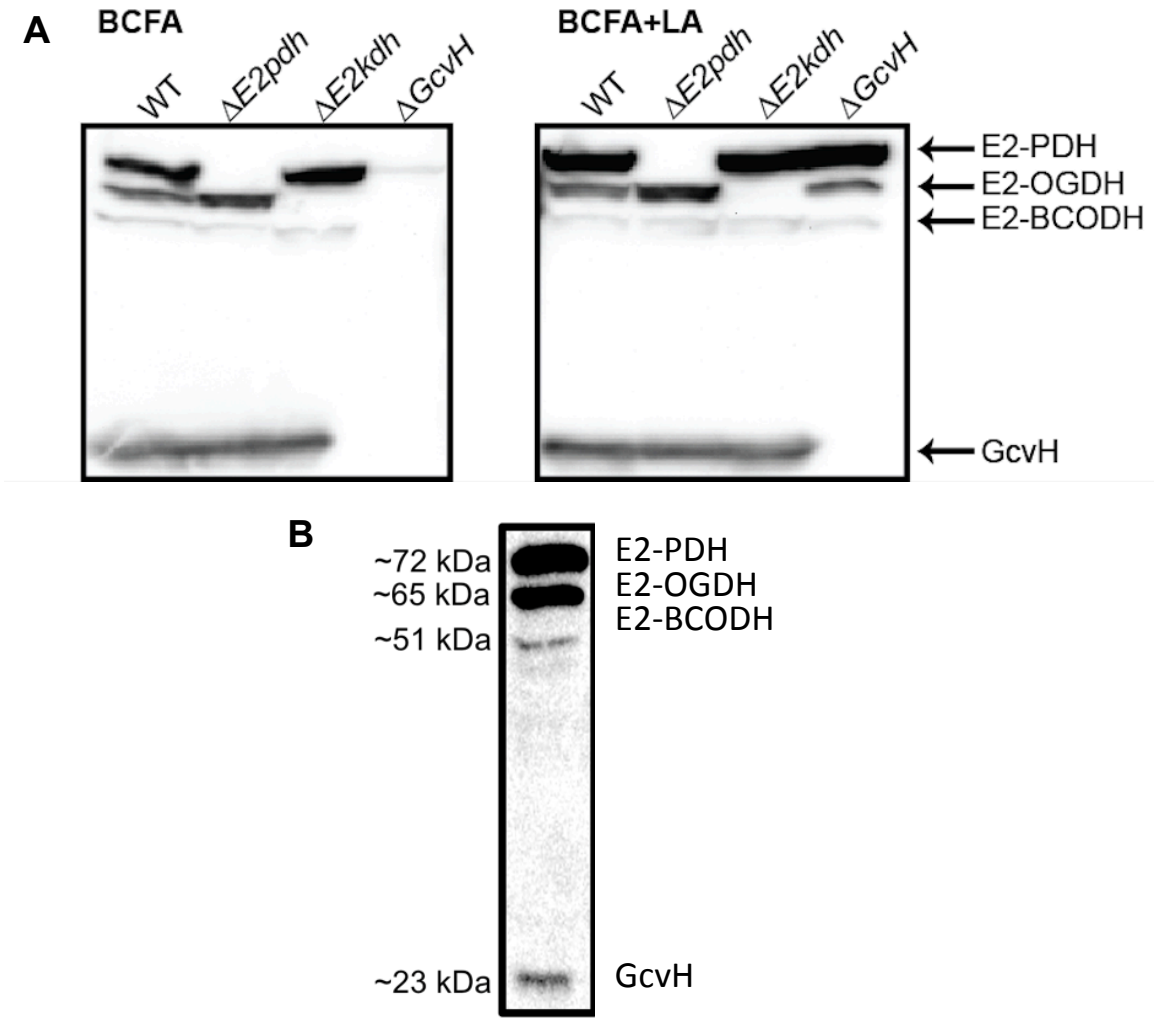

Figure 5: Identification of lipoylated proteins in wild type *S. aureus*

Total cell lysates were obtained from wild type *S. aureus* or the indicated mutant strains grown until early stationary phase (9 h). Protein lysates were resolved by SDS-PAGE and detected with anti-lipoic acid antibody. A. Immunoblot of wild type *S. aureus* and mutant strains in the absence (BCFA) or presence of lipoate supplementation (BCFA-LA). B. Identity of each lipoylated protein in *S. aureus.*

The role of *S. aureus* putative gene products involved in *de novo* biosynthesis of lipoate

Assessing the ability to replicate and lipoylate proteins in the presence or absence of lipoate/octanoate is a useful tool that allows us to identify gene products with roles in *de novo* biosynthesis versus lipoate salvage. Any lipoate biosynthesis mutant whose gene product plays a strict role in *de novo* biosynthesis will be rendered auxotrophic for lipoate. To test the roles of *lipA*, *lipL*, *lipM*, *lplA1*, and *lplA2* in *de novo* biosynthesis and salvage we generated the following deletion mutant strains: Δ*lipA*, Δ*lipA* + *lipA,* Δ*lipM,* Δ*lipM* + *lipM*, Δ*lipL,* Δ*lipL+pta lipL* (referred in figure 6 as Δ*lipL+ lipL*), Δ*lplA1*, Δ*lplA2.*

The mutant strains were studied using two straightforward assays, (i) assessment of bacterial growth and (ii) protein lipoylation in the presence or absence of lipoate and octanoate. Interestingly, supplementation of 25 nM lipoate was sufficient to stimulate growth, but 5 μM lipoate was needed to detect lipoate in the immunoblot. In contrast, higher concentrations of lipoate (250 μM) were required to promote growth *in vitro*, while lipoylated proteins after octanoate supplementation could be detected using 175 μM of octanoate. The high concentrations of octanoate required for cell replication in our studies were consistent to what has been found in *E. coli*, for which it has been shown a decreased rate of octanoate uptake compared to lipoate uptake (69).

As expected, the lipoate auxotroph Δ*lipA* mutant was not able to grow in the absence of lipoate. (Figure 6A), nor was it able to replicate after octanoate supplementation (Figure 6C), but it could replicate when lipoate was

supplemented in the medium (Figure 6B), confirming a role in *de novo* biosynthesis of lipoate. Immunoblots revealed that Δ*lipA* is unable to lipoylate the target proteins when grown in medium that lacks free lipoate (Figure 6A), noted by the absence of the 4 bands corresponding to lipoylated E2 and H subunits found in wild type. In contrast, we detect wild-type lipoylation levels after lipoate supplementation, but not after octanoate supplementation (Figure 6B and C). These results, along with the successful complementation in Δ*lipA* + *lipA,* supports a role for LipA as a lipoate synthetase, which converts the octanoyl group to lipoate in GcvH.

A Δ*lipM* mutant was unable to replicate in RPMI (Figure 6A) without lipoate or octanoate supplementation. This indicates that the *lipM* gene product is involved in *de novo* biosynthesis despite its annotation as a salvage enzyme. Restoration of Δ*lipM* replication was achieved by supplementing either lipoate or octanoate (Figure 6B and C). Similar to Δ*lipA*, we detected no lipoylated proteins for Δ*lipM* in the absence of exogenous lipoate (Figure 6A). Lipoylation occurred in the presence of lipoate (Figure 6B), presumably because the salvage function performed by the lipoate ligases remained intact. In addition, lipoylation was also restored after octanoate supplementation, indicating that LipM is capable of ligating octanoate to the target proteins, which is then irreversible converted to lipoate by the action of LipA (Figure 6C). Complementation with *lipM* further supported the role of the *lipM* gene product as an octanoyl transferase. Thus, our results suggest that the *lipM* gene product is involved in promoting lipoate biosynthesis from an octanoate precursor in the step that precedes protein lipoylation, consistent with its role as a putative octanoyl transferase.

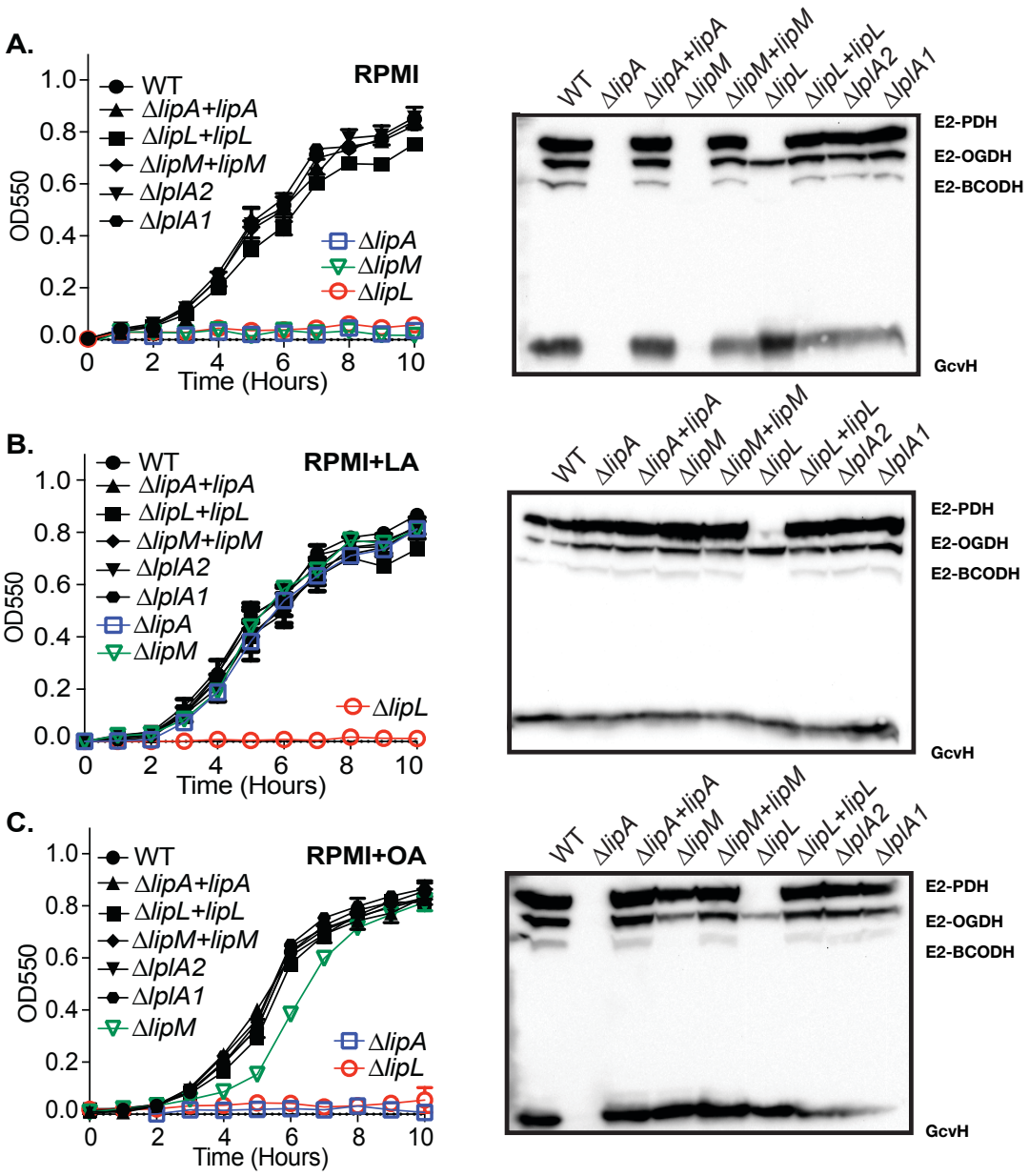

Figure 6: Identification of *de novo* biosynthesis enzymes

LipA and LipM are required for *de novo* biosynthesis of lipoate. Growth curves (left) and immunoblots using anti-lipoic acid antibody (right) of whole cell lysates of strains grown in RPMI+BCFA (A), RPMI+BCFA+Lipoate (B), RPMI+BCFA+Octanoate (C). All strains were able to replicate in RPMI+BCFA (Fig. 18 A- Appendix II). Coomassie Blue stained protein patterns are shown in Figure 20- Appendix II.

We were concerned about the low level of detection of the modified E2-BCODH subunit as well as a growth delay observed when assessing a *lipM* mutant in RPMI + Octanoate (Figure 6C). We hypothesized that these defects in lipoylation and replication were caused by a decreased efficiency to incorporate octanoate through the salvage pathway, as opposed to an inability to transfer an octanoyl group to BCODH. To test this hypothesis, we repeated immunoblots but loaded 1.5X the amount of sample, after which we confirmed the presence the E2-BCODH subunit in the immunoblots (Figure 19). To address the delay in replication, we titrated the concentration of octanoate in growth curves and detected normal cell replication after increasing its concentration to 310 μM (data not shown), suggesting that inefficient use of octanoate caused the lipoylation defect and reduced growth rate linked to BCODH function in *lipM* mutants.

The deletion mutant Δ*lipL* was unable to grow regardless of lipoate and octanoate supplementation (Figure 6B and C), which suggests that it is not directly involved in lipoate salvage or lipoate biosynthesis; rather, it appears to be essential for aerobic growth in some capacity. To rule out the possibility of a switch in bacterial metabolism we tested aerobic and anaerobic growth for Δ*lipA* and Δ*lipL* on tryptic soy agar (TSA) plates (data not shown). While Δ*lipA* was able to replicate in both aerobic and anaerobic conditions, Δ*lipL* was only able to grow in anaerobic conditions. This supports the hypothesis that *lipL* gene product is an essential component for *S. aureus* aerobic growth. In addition, Δ*lipL* displayed only partial lipoylation regardless of lipoate or octanoate supplementation (Figure 6A-C). Lipoylated proteins corresponding to E2- OGDH

and GcvH were detected before and after lipoate supplementation. In contrast, the E2-PDH and E2-BCODH lipoylated proteins were absent. These findings lead us to conclude that the *lipL* gene product is likely not involved in *de novo* biosynthesis or in lipoate salvage *per se,* yet its presence is required for survival *in vitro* and efficient lipoylation of at least two of the five E2 subunits. The fact that the E2-PDH and E2-BCODH bands are missing in the lipoylated protein profile for all conditions tested indicates that *lipL* encodes a protein responsible for transferring lipoate groups to those two proteins. We can presume that the lack of lipoylation in E2-PDH and E2-BCODH is probably causing the replication arrest observed in our growth curves. Lastly, we can infer from Figure 6 that protein lipoylation might be occurring in sequential steps, which would coincide with what has been described in the literature for *B. subtilis* (16, 54, 61).

Construction of the complementation strain for Δ*lipL* was more complicated than expected. We were unable to restore growth or protein lipoylation after introduction of pJC1111-*lipL*. After examining the genetic organization of *lipL,* we noticed that this gene is located in what appears to be a two-gene operon along with *pta* (SAUSA300-*0570*)*,* the gene coding for the phosphate acetyl transferase (Pta) (Figure 3). Pta is the enzyme responsible for the synthesis of acetyl phosphate from acetyl-CoA and phosphate and, along with the enzyme acetyl kinase (AckA), is required for *S. aureus* survival during overflow metabolism (70). Because of this genetic arrangement, we decided to test the hypothesis that the entire *pta-lipL* operon is required for full complementation of a Δ*lipL* mutant. Indeed, we were able to successfully

complement a Δ*lipL* mutant after transduction of the integrative plasmid pJC1111-*pta-lipL* (Figure 7). Further investigation of the interplay between Pta and LipL will help us elucidate potential connections between metabolic output and protein lipoylation or novel regulatory schemes associated with protein lipoylation that may dictate shifts in metabolic flux.

Lastly, the putative lipoate ligase deletion mutants, Δ*lplA1* and Δ*lplA2,* were able to replicate and to lipoylate proteins in all three conditions tested (Figure 6A-C), which suggests that these enzymes are not involved in *de novo* biosynthesis, and may play a role in lipoate/octanoate salvage. However, we cannot yet assign a lipoate ligase function to these two genes until we test them in a genetic background that cannot use *de novo* biosynthesis to generate lipoate.

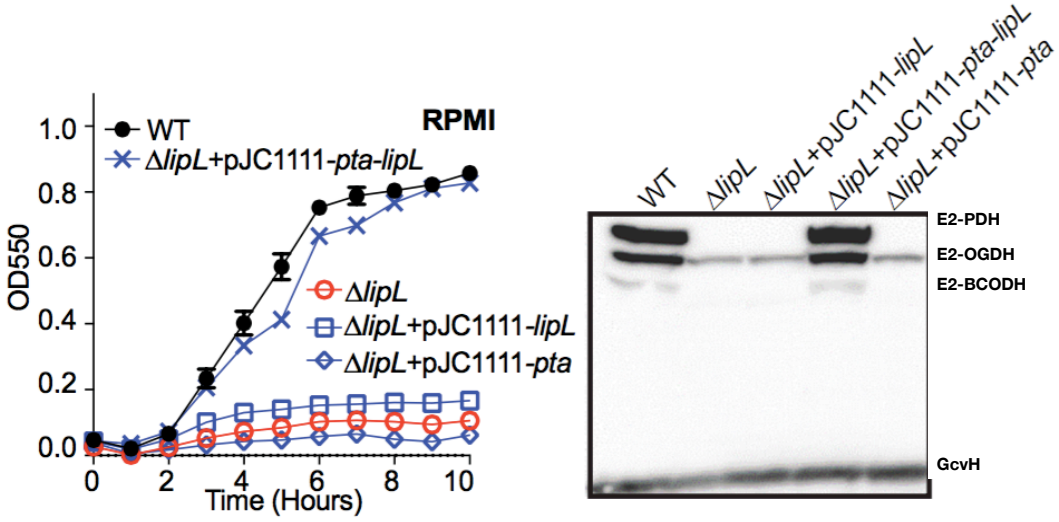

Figure 7: Complementation of Δ*lipL*

Δ*lipL* growth and lipoylation defects are restored after complementation with the *pta-lipL* **operon.** Growth curves (left) in RPMI and immunoblots with anti-lipoic acid antibody (right) from whole cell lysates of strains grown in RPMI+BCFA.

The requirement of lipoate *de novo* biosynthesis *in vivo*

Our results have revealed that *S. aureus* requires the lipoate salvage pathway to replicate *in vitro* when *de novo* biosynthesis is not functional. To test whether or not *de novo* biosynthesis of lipoate is required for survival of *S. aureus in vivo,* we assessed the ability of a Δ*lipA* mutant to colonize and infect mice using murine sepsis model. Six to eight week old female Swiss Webster mice were infected by retro-orbital venous plexus injection with ~1x10^7 colony forming units (CFU)/mL of WT, Δ*lipA* and Δ*lipA* + *lipA* and infection was allowed to proceed for 96 hours. We suspected that if *de novo* biosynthesis of lipoate were promoting *S. aureus* survival *in vivo*, then we should detect a decreased bacterial burden in infected organs. In contrast, if *de novo* of lipoate were dispensable, we should observe equivalent pathogenesis to that of wild type.

In the kidney, we detected no significant changes in bacterial burden between the wild type strain, Δ*lipA* or Δ*lipA*-*lipA* (Figure 8). However, in the heart, we observed a drastic decline in the ability of Δ*lipA* to colonize, with a 3-log reduction in bacterial CFU and a significant number of mice (N=8) having no recoverable bacteria. We also evaluated *S. aureus* seeding in liver and spleen of mice after systemic infection. Regrettably, no CFU for WT and other strains were detected in these tissues at 96 hours post infection.

From these results, we can conclude that *de novo* biosynthesis of lipoate is required to colonize the heart of mice in a systemic model of infection. Based on our previous results, we hypothesize that the efficient infection in the kidneys

relies upon the enzymatic activity of the lipoate ligases to maintain infectivity. Thus, when the ability to synthesize lipoate is blocked, *S. aureus* can satisfy the requirement for lipoate by scavenging this cofactor from the kidney environment. To fully assess the validity of this assumption, we will need to identify the lipoate ligases and determine their ability to incorporate lipoate *in vivo*.

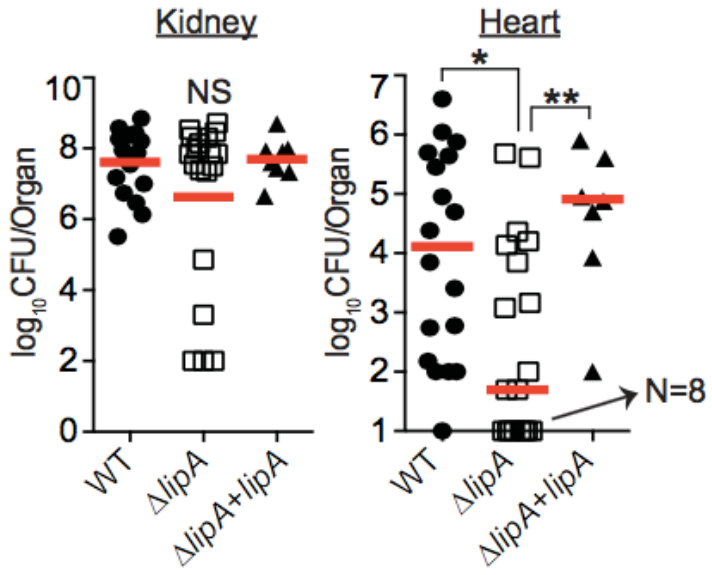

Figure 8: Requirement for lipoate *de novo* biosynthesis *in vivo*

De novo biosynthesis of lipoate is required for infection of the heart but not the kidney during sepsis. Bacterial burden displayed as $\log_{10}$CFU in kidneys and heart at 96 hours post bloodstream infection with 1.0 x 10^7 CFU of WT (N=8), Δ*lipA* (N=19) and Δ*lipA* + *lipA* (N=8). Median expressed as a measure of central tendency. In the heart, only one mouse had no recoverable bacteria, compared to 8 mice with undetectable CFU in the *lipA* deletion mutant. Statistics were calculated via nonparametric 1-way ANOVA and statistical significance in CFU values was evaluated using Krustal-Wallis multiple group comparisons. Evaluation of the results found in the heart between WT and Δ*lipA* showed a $P<0.05$ (*). Statistical significant differences where found in Δ*lipA* compared to Δ*lipA* + *lipA* had $P<0.001$ (**).

Characterization of the lipoate ligases constructed in a Δ*lipA* background

To evaluate the lipoate salvage function in isolation, we generated ligase mutants in a Δ*lipA* mutant background of *S. aureus.* This blocks the synthesis of lipoate *de novo* and permit assessment of ligase activity. The following mutants were constructed: Δ*lipA* Δ*lplA1,* Δ*lipA* Δ*lplA2* and their complemented strains Δ*lipA* Δ*lplA1+lplA1,* Δ*lipA* Δ*lplA2+lplA2.* Additionally, we generated Δ*lplA1* Δ*lplA2*, which lacks both putative lipoate ligase genes, but is still capable of engaging in *de novo* biosynthesis of lipoate. With these mutants, if the genes encoding putative lipoate ligases are involved in lipoate salvage, then they should no longer replicate and lipoylate when supplemented with lipoate.

As expected, the double ligase mutant Δ*lplA1* Δ*lplA2* replicates and lipoylates proteins in all three conditions tested (Figure 9). In contrast, Δ*lipA* Δ*lplA1* and Δ*lipA* Δ*lplA2* were unable to replicate and lipoylate proteins in RPMI lacking lipoate or octanoate (Figure 9A and C). When supplemented with lipoate (Figure 9B), a Δ*lipA* Δ*lplA2* replicates normally and exhibits WT lipoylation profiles. Thus, lipoate salvage *in vitro* appears to depend on LplA1. Strikingly, when we tested the Δ*lipA* Δ*lplA1* strain, we saw no restoration of growth and the ability to lipoylate proteins was lost, implying LplA1 is the primary lipoate ligase *in vitro*. Importantly, complementation of Δ*lipA* Δ*lplA1* with their corresponding genes fully restored wild type growth phenotypes and lipoylation profiles after lipoate supplementation (Figure 9B).

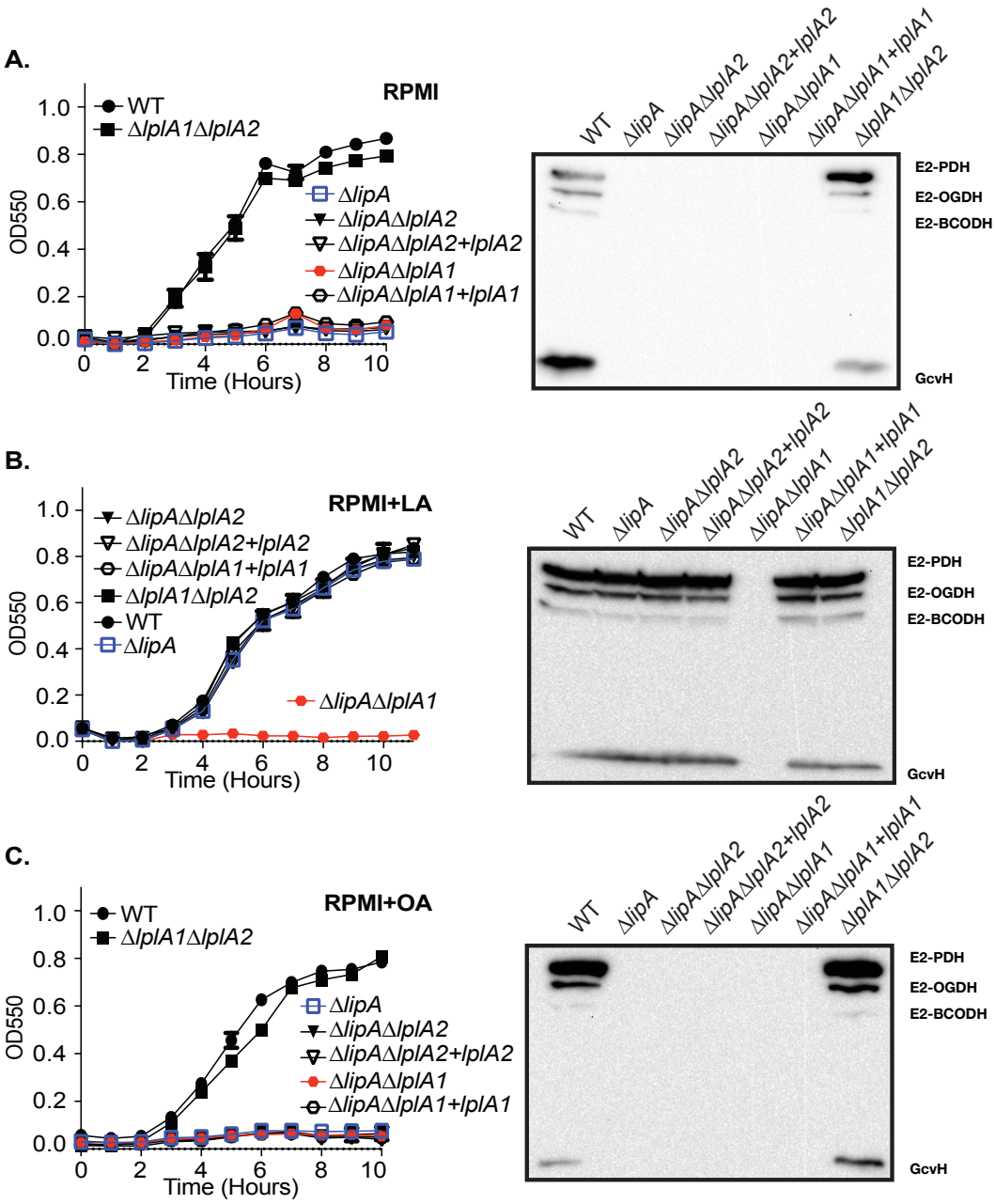

Figure 9: Assessment of lipoate ligases in the background of Δ*lipA*

LplA1 behaves as a lipoate ligase *in vitro*. Growth curves (left) and immunoblots detected with anti-lipoic acid antibody (right) of whole cell lysates of strains grown in RPMI+BCFA (A), RPMI+BCFA+Lipoate (B), RPMI+BCFA+Octanoate (C). All strains were able to replicate in RPMI+BCFA (Fig. 18 B- Appendix II). Coomassie Blue stained protein patterns are shown in Figure 20- Appendix II.

These results suggest that the two putative lipoate ligases, LplA1 and LplA2 do not behave similarly *in vitro*, with only LplA1 playing a role in lipoate salvage under these conditions. Nevertheless, it is possible that we are not detecting LplA2 activity with the methods used in this study. Alternatively, *lplA2* might not be expressed in broth culture. Studies evaluating LplA2 preferred lipoylated substrates and target proteins as well as *lplA2* expression would be necessary to fully understand the behavior of LplA2 *in vitro*.

Evaluation of the lipoate salvage pathway in a triple deletion mutant strain

Our results with ligase mutants constructed in a *lipA* deletion background showed that LplA2 was not engaging in lipoate ligation *in vitro*. This contrasted with the observations in *L. monocytogenes,* in which both of the lipoate ligases are capable of using lipoate *in vitro*, albeit with different affinities (61).

Additionally, the literature indicates that LplA2 is encoded in an operon that is upregulated under oxidative stress conditions (52, 53), so it is plausible that this gene is not sufficiently expressed under our experimental conditions. To test whether the constitutive expression of LplA2 renders protein lipoylation and allows replication *in vitro*, we generated the Δ*lipA* Δ*lplA1* Δ*lplA2* mutant strain and we individually complemented this strain with *lplA1* and *lplA2.* If LplA2 has the ability to ligate lipoate *in vitro*, then we should observe restoration of wild type growth and protein lipoylation phenotypes when supplemented with lipoate.

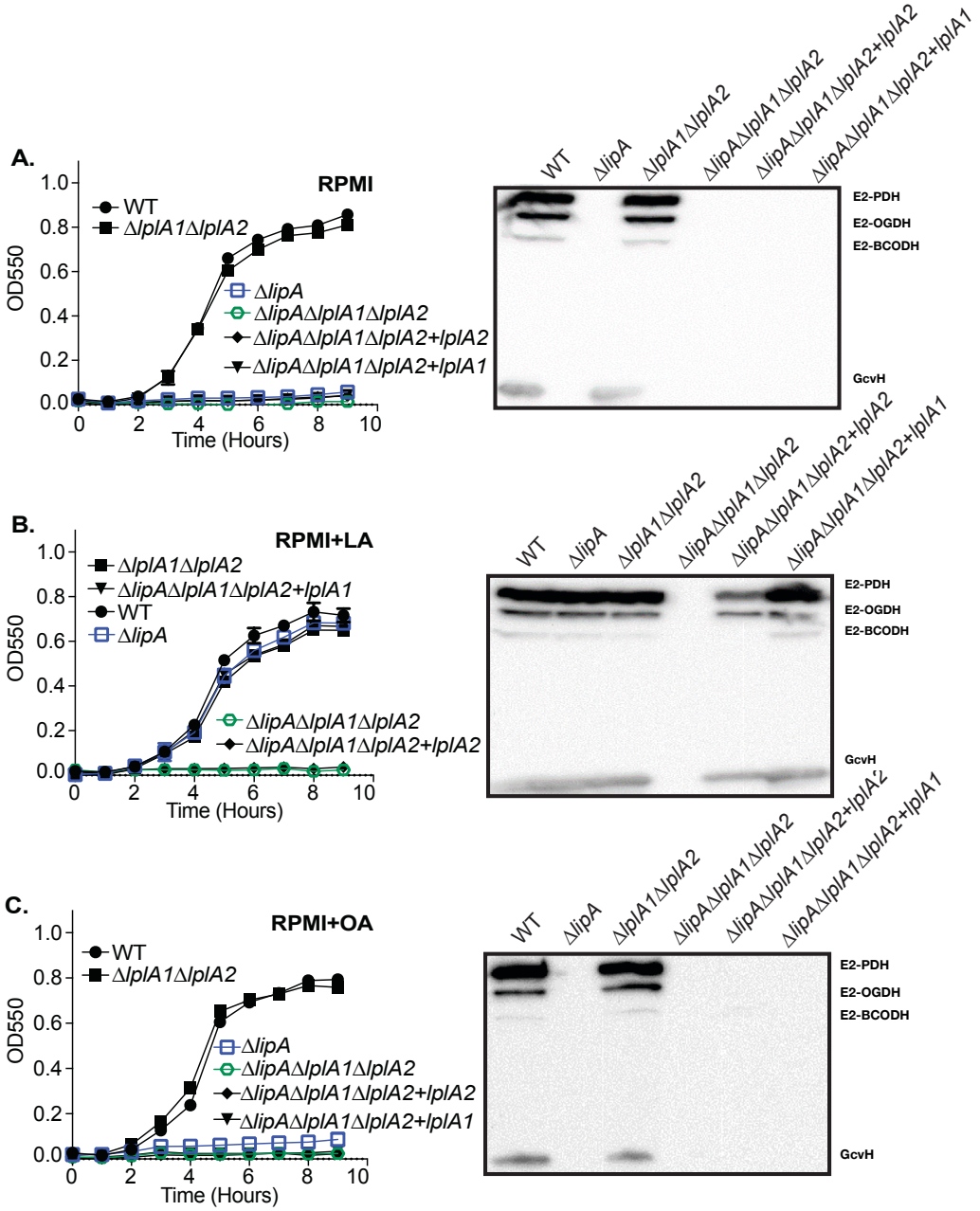

Figure 10: Assessment of lipoate ligases in triple deletion mutants

LplA2 facilitates lipoylation of E2 subunits and GcvH *in vitro* when constitutively expressed**.** Growth curves (left) and immunoblots detected with anti-lipoic acid antibody (right) of whole cell lysates of strains grown in RPMI+BCFA (A), RPMI+BCFA+Lipoate (B), RPMI+BCFA+Octanoate (C). All strains were able to replicate in RPMI+BCFA (Fig. 18 D- Appendix II). Coomassie Blue stained protein patterns are shown in Figure 21 where necesar- Appendix II.

As expected, Δ*lipA* Δ*lplA1* Δ*lplA2* was incapable of replication and protein lipoylation in the absence and presence of supplements (Figure 10). In contrast, Δ*lplA1* Δ*lplA2* replicates and lipoylates proteins at wild type S. aureus levels in all three conditions tested. The supplementation of lipoate in a triple mutant complemented with *lplA1* also restored protein lipoylation and growth to wild type levels. Interestingly, complementation with *lplA2* led to partial protein lipoylation and no restoration of growth. These results suggest that LplA2 is capable of ligating lipoate in vitro when constitutively expressed albeit at levels that are insufficient to restore growth.

The contribution of lipoate ligases to lipoate salvage in a *lipM* deletion background

We constructed strains in a Δ*lipM* mutant background to determine whether the lipoate ligases LplA1 and LplA2 play a role in salvage of lipoate or octanoate during the initial steps of lipoate *de novo* biosynthesis. A Δ*lipM* single deletion mutant behaved as previously described (Figure 6), while a Δ*lipA* Δ*lipM* double mutant was incapable of lipoylating proteins or replicating in all conditions tested due to the blockade of the lipoate *de novo* biosynthesis pathway (Figure 11). As anticipated, Δ*lipM* Δ*lplA1* and Δ*lipM* Δ*lplA2* mimicked the results observed for the lipoate ligases in the background of a Δ*lipA* when media was supplemented with lipoate (Figure 9B and Figure 10B). LplA2 function, as assessed via Δ*lipM* Δ*lplA1*, showed no role for salvage of octanoate. In contrast, the growth phenotype and lipoylation profile of Δ*lipM* Δ*lplA2* revealed that LplA1 is involved in incorporating octanoate *in vitro* (Figure 11C).

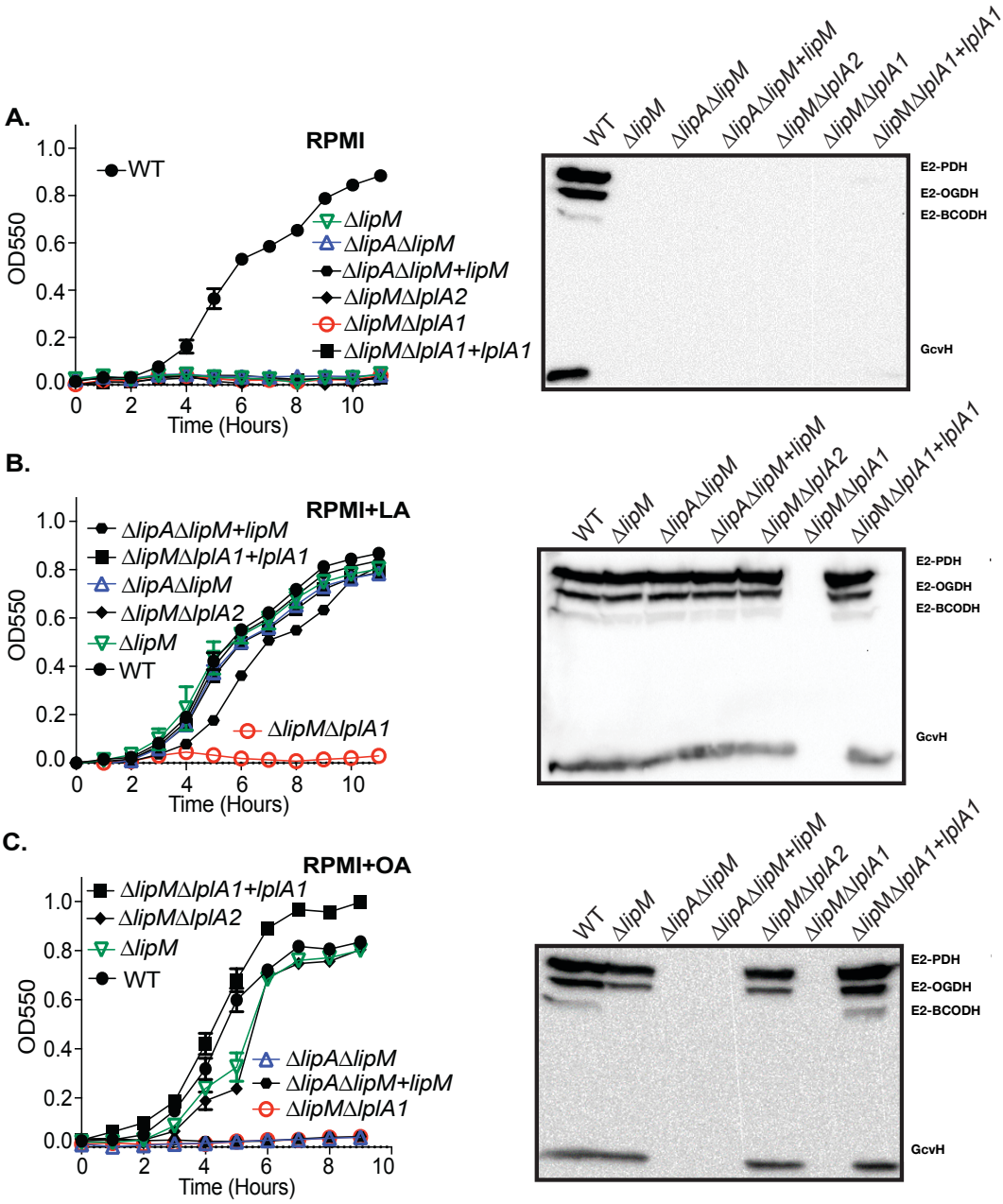

Figure 11: Assessment of lipoate ligases in the background of Δ*lipM*

LplA2 is capable of ligating octanoate through the salvage pathway. Growth curves (left) and immunoblots detected with anti-lipoic acid antibody (right) of whole cell lysates of strains grown in RPMI+BCFA (A), RPMI+BCFA+Lipoate (B), RPMI+BCFA+Octanoate (C). All strains were able to replicate in RPMI+BCFA (Fig. 18 C- Appendix II). Coomassie Blue stained protein patterns are shown in Figure 20- Appendix II.

This information was confirmed by the Δ*lipM* Δ*lplA1+lplA1* strain, for which we observed restoration of a Δ*lipM* mutant protein lipoylation and growth phenotype. Taken together, these data suggest that LplA1 can provide octanoylated proteins to LipA, independently of the enzymatic activity of LipM.

Assessment of the requirement for lipoate salvage *in vivo*

We have demonstrated that biosynthesis of lipoate is required for heart infection in mice during sepsis. In contrast, the enzymatic function of LipA was dispensable for kidney infection. Therefore, we tested the hypothesis that the salvage of lipoate supports efficient infection in the renal tissue. Additionally, we tested whether or not LplA2 play a role in lipoate salvage during infection. We infected mice with either the single deletion mutants of the lipoate ligases; with the Δ*lplA1* Δ*lplA2* double mutant or with the complemented strains Δ*lplA1* Δ*lplA2* + *lplA1* and Δ*lplA1* Δ*lplA2* + *lplA2.* Our results show that in the kidneys, Δ*lplA1* and Δ*lplA2* had bacterial burden levels equivalent to those detected in both the wild type strain and the *lipA* deletion mutant, indicating that neither LplA1 nor LplA2 are essential to colonize this tissue (Figure 12). Remarkably, kidney infection with a Δ*lplA1* Δ*lplA2* double mutant was severely compromised in the host, showing a 4-log reduction in bacterial burden compared to the wild type strain. This suggests that both lipoate ligases are required for efficient infection of the renal tissue. Interestingly, bacterial seeding after infection with Δ*lplA1* Δ*lplA2* was not completely eliminated, supporting the notion that lipoate *de novo* biosynthesis contributes to some degree during infection of the kidneys.

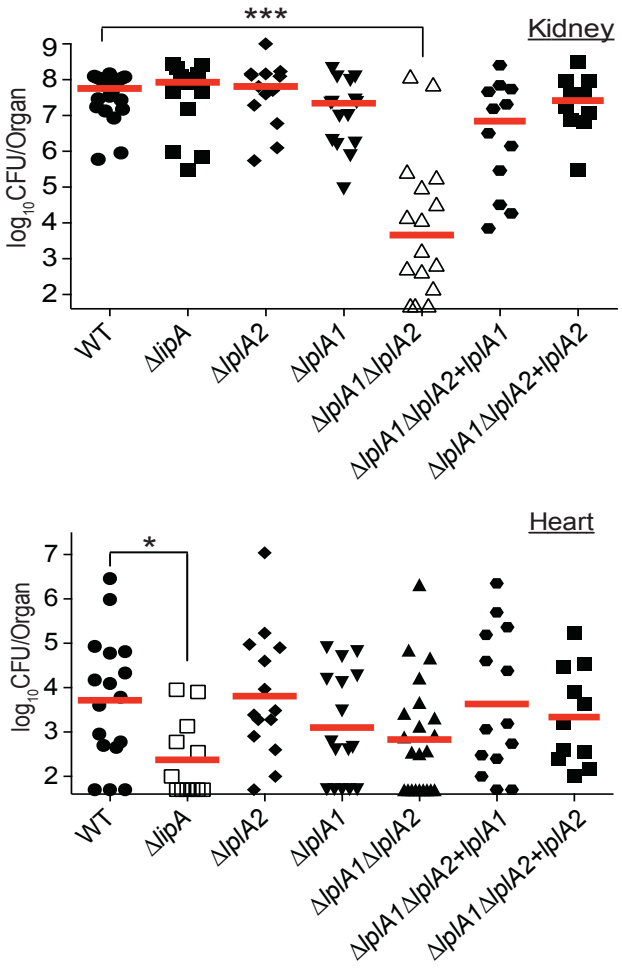

Figure 12: Assessment of the requirement for lipoate salvage *in vitro*

Lipoate ligases LplA1 and LplA2 are individually required for efficient infection of the kidney. Bacterial burden displayed as $\log_{10}$CFU in kidneys and heart at 96 hours post bloodstream infection with 1.0 x 10^7 CFU of WT (N=20), Δ*lipA* (N=15), Δ*lipA1* (N=15), Δ*lipA2* (N=14), Δ*lipA1*Δ*lipA2* (N=16), Δ*lipA1*Δ*lipA2* + *lplA1* (N=12) and Δ*lipA1*Δ*lipA2* + *lplA2* (N=12). Median is expressed as a measure of central tendency. Statistics were calculated via nonparametric 1-way ANOVA and statistical significance was evaluated using Krustal-Wallis multiple group comparisons. Analysis of the statistical significant differences in CFU showed *P*<0.0001 for Δ*lipA1*Δ*lipA2* compared to WT, Δ*lipA* and Δ*lplA2* (****) and *P*<0.05 for Δ*lipA1*Δ*lipA2* compared to

Δ*lipA1* and Δ*lipA1*Δ*lipA2* + *lplA2* (*). In the heart, a $P<0.05$ value of statistical difference was observed for WT compared to Δ*lipA*.

Additionally, bacterial burden levels for Δ*lplA1* Δ*lplA2* + *lplA1* or Δ*lplA1* Δ*lplA2* + *lplA2* were near those found for the wild type strain. Taken together, these data suggest that both lipoate ligases can compensate for one another to facilitate infection of the kidneys (Figure 12).

The results for heart infection revealed that bacterial infection in this tissue is dependent on the *de novo* biosynthesis of lipoate. When we infected mice with Δ*lplA1,* Δ*lplA2,* or with Δ*lplA1* Δ*lplA2*, no significant alterations in bacterial burden were detected. This indicates that lipoylation through the salvage pathway does not contribute significantly to pathogenesis of the heart (Figure 12).

In sum, we can conclude that *de novo* lipoate biosynthesis is essential for *S. aureus* survival in the heart during sepsis. Additionally, *S. aureus* pathogenesis in the kidneys relies upon the lipoate salvage pathway, a process that is supported by both lipoate ligases LplA1 and LplA2.

Evaluation of the role of the lipoyl transferase LipL in de novo biosynthesis and salvage of lipoate

Our previous experiments showed that LipL transfers lipoyl groups to E2-PDH and E2-BCODH; nevertheless, our assessments were insufficient to define which enzyme is responsible for the lipoylation of E2-OGDH and GcvH. To gain knowledge on this matter, we generated a set of mutant strains constructed in a Δ*lipL* background and performed immunoblots of Δ*lipA* Δ*lipL*; Δ*lipM* Δ*lipL*; Δ*lipL*

Δ*lplA1*; Δ*lipL* Δ*lplA2*; and Δ*lipL* Δ*lipA* Δ*lipM*. We did not execute growth curves in RPMI supplemented with lipoate or octanoate for this mutant strains due to the known replication phenotype found for *lipL* deletion mutant (Figure 6). Nevertheless, cells were capable of replicating in BCFA-RPMI, as shown in Figure 18A, Appendix II for Δ*lipL* (results for the rest of the mutants described in this section are not shown).

In the absence of supplements, Δ*lipA* Δ*lipL,* Δ*lipM* Δ*lipL* and Δ*lipL* Δ*lipA* Δ*lipM* failed to lipoylate proteins (Figure 13). In the same conditions a Δ*lipL*Δ*lplA2* double mutant mimicked a Δ*lipL* single deletion mutant, indicating that LplA2 is not involved in lipoylating E2-OGDH or GcvH. Interestingly, this was not the case for Δ*lipL*Δ*lplA1*, for which we detected lack of lipoylation for E2 - OGDH. This result implies that LplA1 is capable of transferring a lipoyl group from GcvH to the E2-OGDH without lipoate or octanoate supplementation independently of LipL.

The supplementation of lipoate restores the Δ*lipL* protein lipoylation phenotype in all tested mutants with the exception of Δ*lipL*Δ*lplA1*, for which the E2-OGDH lipoylated band remains absent (Figure 13B). Lipoate supplementation for Δ*lipL* Δ*lipA* Δ*lipM* also indicates that LplA1 is efficient at ligating lipoate to E2-OGDH and GcvH, and it also reveals that this process can occur trough a mechanism that is independent of the enzymatic activity of LipA, LipM and LipL.

Furthermore, GcvH gets lipoylated in both Δ*lipL*Δ*lplA1* and Δ*lipM* Δ*lipL* mutant strains after octanoate supplementation, yet E2-OGDH remains

undetectable (Figure 13C). In contrast, lipoylation of GcvH does not occur in Δ*lipA* Δ*lipM* Δ*lipL*. These results indicate that both LplA1 and LipM can attach octanoate to GcvH, but also, that LplA1 is incapable of ligating octanoate onto E2-OGDH (Figure 13C).

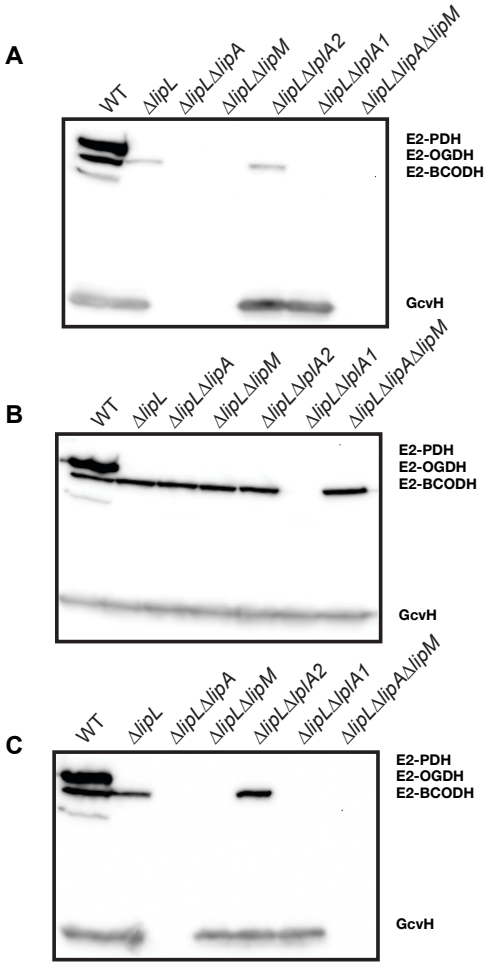

Figure 13: Lipoylation profiles of mutant strains in a Δ*lipL* background

LplA2 is responsible for lipoylation of OGDH and GcvH. Growth curves (left) and immunoblots detected with anti-lipoic acid antibody (right) of whole cell lysates of strains grown in RPMI+BCFA (A), RPMI+BCFA+Lipoate (B), RPMI + BCFA + Octanoate (C). Coomassie Blue stained protein patterns are shown in Figure 21- Appendix II.

Taken together, these data support the hypothesis that protein lipoylation initiates through octanoylation of GcvH, either via the enzymatic activity of LipM or the function of LplA1. Additionally, it implies that lipoate *de novo* biosynthesis occurs in sequential steps in *S. aureus.* This process initiates through lipoylation of GcvH and continues with the transfer of the lipoyl group to E2-PDH and E2-BCODH via the action of LipL. In contrast, lipoylation of E2-OGDH via LplA1 seems to occur exclusively through ligation of free lipoate.

Evaluation of *S. aureus* requirement for lipoate during infection and the individual contribution of lipoate ligases *in vivo*

Efficient infection of the renal tissue during sepsis depends on lipoate scavenging. When we tested Δ*lplA1* Δ*lplA2* in this infection model we observed a significant decrease in bacterial burden compared to the wild type. Nevertheless, we could still detect bacterial colonization, meaning that the *de novo* biosynthesis pathway, which is functional in this mutant, was possibly enabling infection in this tissue. To determine whether or not *S. aureus* requires lipoate acquisition in any capacity to survive during sepsis we used a Δ*lipA* Δ*lplA1* Δ*lplA2* triple mutant that lacks the ability to engage in both *de novo* biosynthesis and salvage of lipoate. This mutant was completely avirulent *in vivo* (Figure 14), indicating that *S. aureus* requires lipoic acid *in vivo* and cannot switch to other metabolic pathways (anaerobic metabolism) to bypass this requirement. Infection with the complementation strains Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA1* and Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2* confirmed our previous results; both lipoate ligases can incorporate lipoate

in vivo, promoting bacterial replication in the host. In sum, these results allowed us to validate and further characterized the *de novo* biosynthesis and salvage pathway but, more importantly, provided insight into one of the mechanisms used by *S. aureus* to better colonize its host.

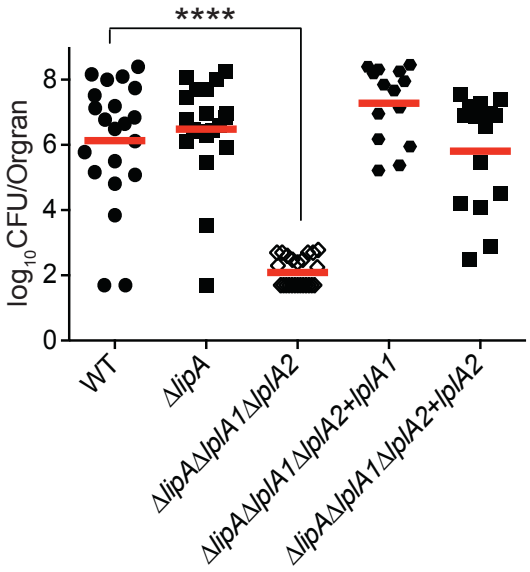

Figure 14: Assessment of the requirement for lipoate acquisition

S. aureus requires acquisition of lipoate to infect the kidney. Either LplA1 or LplA2 suffice to restore infection in the renal tissue. Bacterial burden displayed as $\log_{10}$CFU in kidneys and heart at 96 hours post bloodstream infection with 1.0 x 10^7 CFU of WT (N=21), Δ*lipA*Δ*lipA1*Δ*lipA2* (N=20), Δ*lipA*Δ*lipA1*Δ*lipA2* + *lplA1* (N=14) and Δ*lipA*Δ*lipA1*Δ*lipA2* + *lplA2* (N=15). Median is expressed as a measure of central tendency. Statistics were calculated via nonparametric 1-way ANOVA and statistical significance was evaluated using Krustal-Wallis multiple group comparisons. Analysis of the statistical significant differences in CFU showed *P*<0.0001 (****), *P*<0.001 (***) and *P*<0.05 (*) value of statistical difference was observed for WT compared to Δ*lipA*. .

CHAPTER FOUR

DISCUSSION

The central hypothesis of this work was that lipoate acquisition mechanisms are capable of promoting *S. aureus* infectivity. In order to test this hypothesis, we first needed to elucidate the mechanisms by which *S. aureus* lipoate acquisition occurs. We used bacterial genetics techniques to generate mutant strains containing deletions in the five lipoate biosynthesis and salvage genes of *S. aureus.* The mutants were later assessed for their ability to lipoylate proteins and to replicate *in vivo* and *in vitro* in the presence or absence of lipoic acid or octanoic acid. A working model for the lipoate *de novo* biosynthesis and salvage pathway in *S. aureus* is shown in Figure 15.

During the course of this work, we confirmed that *S. aureus* has four lipoylated enzyme complexes (PDH; OGDH; BCODH and GCS) that require a lipoate modification on one of its subunits in order to become active. GcvH-L is an additional protein that bears lipoate but was undetected under the conditions tested in our work. This protein is contained in an operon that is presumed to be upregulated under oxidative

stress conditions, which could explain why we were unable to detect it in our immunoblots (53). Our work also determined that *S. aureus* uses two mechanisms to incorporate lipoate onto these enzyme complexes, the *de novo* biosynthesis and the salvage pathway. Additionally, we have characterized the gene products involved in this pathway and found unexpected results that might suggest a potential role of lipoate biosynthesis enzymes in regulation of metabolic processes in *S. aureus* and in resistance to host defenses.

After elucidation of the lipoate *de novo* biosynthesis and salvage pathway in *S. aureus,* we evaluated the roles of lipoate metabolism *in vivo* using a mouse model of sepsis. We measured the ability of mutant strains to colonize host tissues and we found that, during sepsis, *S. aureus* relies either on lipoate *de novo* biosynthesis or salvage to infect the host. This process occurs in a tissue-dependent manner and confirms our hypothesis that mechanisms of lipoate acquisition contribute to *S. aureus* fitness and pathogenesis. Importantly, we were able to confirm that lipoate metabolism and, by extension, aerobic metabolism, is essential for *S. aureus* pathogenicity.

Lipoate *de novo* biosynthesis and salvage pathway

LipA and LipM are lipoate *de novo* biosynthesis enzymes

As shown in Figure 6, we were able to confirm the hypothesis that LipA is the lipoate synthetase in *S. aureus*, since a Δ*lipA* mutant could only replicate and

lipoylate proteins after lipoate supplementation. LipM was identified as the octanoate transferase after observing restoration of growth and protein lipoylation to wild type levels when lipoate or octanoate were supplemented in the culture medium. The contribution of LipA and LipM to *de novo* biosynthesis of lipoate was further confirmed after the assessment of the double deletion mutant Δ*lipA* Δ*lipM* and the complemented strain Δ*lipA* Δ*lipM* + *lipM*, both of which could not be rescued by octanoate supplementation Figure 11.

Only LplA1 is required for *in vitro* utilization of lipoate

In silico analysis predicted that *S. aureus* encodes two lipoate ligases involved in salvage of lipoate, LplA1 and LplA2. The combined information, provided by the deletion mutants of the lipoate ligase genes and the mutants constructed in the background of Δ*lipA* revealed that only LplA1 is involved lipoate scavenging *in vitro.* This was evident in the Δ*lipA* Δ*lplA1* mutant*,* for which LplA2 was incapable of facilitating growth and protein lipoylation regardless of lipoate supplementation Figure 9.

Previous research has shown that *S. aureus* LplA2 lipoylates GcvH-L, a glycine cleavage system H-like protein that is encoded in the same operon as LplA2 (53). We were not able to detect lipoylation of GcvH-L during the course of this thesis, probably due to reduced expression of this operon *in vitro* (52). Yet, a few additional hypotheses that are not mutually exclusive could explain the observed lack of observable activity for LplA2 *in vitro*.

First, LplA2 might be less efficient at ligating free lipoate compared to LplA1. This was the case for differences observed between *L. monocytogenes* lipoate ligases (19, 61). Another hypothesis is that *S. aureus* LplA2 uses a different substrate in place of free lipoate, such as peptide-bound lipoate. This is supported by the investigations conducted by *Rack et al.*, in which *S. aureus* LplA2 was shown to lipoylate the non-canonical GcvH using lipoamide, a lipoic acid derivative (53). Finally, *lplA2* may not being expressed under the conditions tested in this thesis.

Support for the last hypothesis comes from results observed after complementing back the *lplA2* gene in the triple mutant Δ*lipM* Δ*lplA1* Δ*lplA2* (Figure 12). The Δ*lipM* Δ*lplA 1*Δ*lplA2* + *lplA2* strain displayed partial lipoylation of proteins, a result most likely explained by the nature of the complementation construct, which expresses *lplA2* constitutively.

Additional support for the regulation hypothesis is derived from the fact that *lplA2* is known to be encoded in an operon that seems to be upregulated under oxidative stress conditions (52, 53). We could directly test this by generating a transcriptional reporter fusion to *gfp* followed by evaluating GFP expression under redox stress conditions. Such stress conditions are easily induced *in vitro* using oxidative stressors capable of promoting formation of disulfide bonds, such as H_2O, the redox cycling drug menadione, or diamide, a compound specific for thiol oxidation (5). Oxidative conditions can be assessed *in vivo* by co-culturing *S. aureus*

with purified macrophages and neutrophils. If the *lplA2* operon is induced by oxidative stress, we expect GFP fluorescence to increase in the presence of the reactive oxygen intermediates (71).

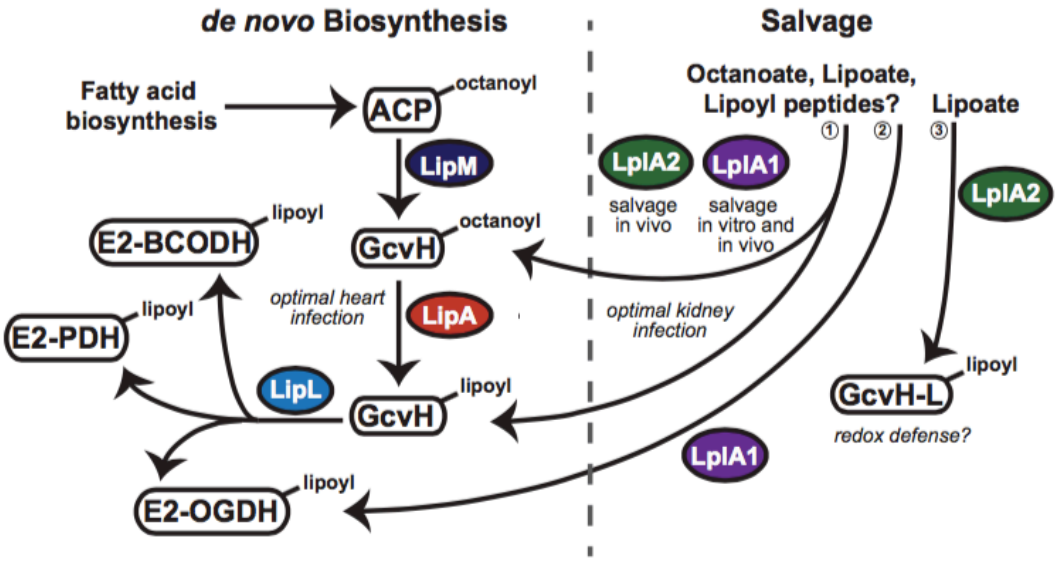

Figure 15: Model pathway for lipoate acquisition in *S. aureus*

S. aureus synthetizes lipoate through the *de novo* biosynthesis pathway and incorporates free lipoate from the environment via the salvage pathway. We propose this model pathway for *S. aureus* based in the combination of our results and data from other authors (16, 53, 59, 61). In the salvage pathway, LplA1 is capable of lipoylating GcvH both *in vivo* and *in vitro*. In contrast, we could only determine salvage of lipoate *in vivo* for LplA2, supporting the hypothesis that lplA2 containing operon is upregulated during oxidative stress conditions, as stated by Rack et al (52). These authors showed that LplA2 is also involved in salvage of lipoate in vivo by being capable of lipoylating both GcvH and GcvH-L, although the latter with grater affinity. Both LplA1 and LplA2 were shown to be required for optimal renal infection in the kidneys. We have also

shown that LplA1 was capable of attaching octanoate to GcvH and lipoate to E2-OGDH, although we consider this to be secondary to its main role as a salvage enzyme involved in lipoylation of GcvH. The biological importance of these observations is currently under investigation in our laboratory

LplA1 can ligate free octanoate

Assessing the putative lipoate ligases in the background of a Δ*lipM* mutant increased our knowledge of the contribution of these enzymes to the salvage of octanoate. As shown in the immunoblots and growth curves of Δ*lipM* Δ*lplA2*, Δ*lipM* Δ*lplA1 and* Δ*lipM* Δ*lplA1* + *lplA1* LplA1 was capable of ligating the lipoate precursor, octanoate, bypassing Δ*lipM* (Figure 11). Lipoate then proceeds via the lipoate synthetase LipA, which would convert the octanoyl group to lipoate. Whether LplA1 is capable of using both free and peptide-bound octanoate is unknown. In contrast to what was observed after the complementation of LplA2 in a Δ*lipA* Δ*lplA2* + *lplaA2* mutant strain, the constitutive expression of *lpla2* in Δ*lipM* Δ*lplA2* did not restore growth or protein lipoylation, suggesting that LplA2 cannot catalyze octanoate ligation under our experimental conditions.

Interestingly, lipoate ligase-mediated octanoylation was previously observed for LplA1 of *L. monocytogenes* as well as LplJ of *B. subtilis* (54, 61). This argues in favor of a lipoate ligase-dependent pathway for protein octanoylation that is independent of LipM. From our knowledge of the lipoate biosynthesis and salvage pathway in other species and our own results, we postulate that the octanoyl group is being attached to GcvH via

LplA1 and, subsequently, LipA inserts two sulfur atoms, converting the octanoyl moiety to a lipoyl moiety.

A deep assessment of the octanoylation capacity of LplA1, as well as LipM is complicated due to the endogenous biosynthesis of octanoate. Nevertheless, octanoylation of GcvH could be tested through enzymatic studies *in vitro* or by generating a triple mutant of the lipoate ligases and the lipoate synthetase in the background of a fatty acids auxotroph strain.

LipL is required for efficient protein lipoylation in *S. aureus*

Based on bioinformatics analyses and similarities to *B. subtilis* LipL, the product of the *0571* gene, LipL, is predicted to encode a lipoate transferase. Growth assessment of a Δ*lipL* mutant showed that this strain was incapable of replicating even after supplementation with lipoate or octanoate. Additionally, we observed lipoylation defects in a Δ*lipL* mutant, such that only lipoylated GcvH and E2-OGDH could be detected.

Replication of Δ*lipL* in RPMI BCFA medium was normal (Figure 18A Appendix II), indicating that this strain was metabolically competent and only the absence of functional E2-PDH and E2-BCODH was responsible for growth arrest. We suspect Δ*lipL* replication arrest could be explained by the lack of a functional BCODH, which causes alterations in membrane fluidity due to defects in short branched-chain fatty acid biosynthesis and incorporation into the lipid bilayer (11, 54, 59), although this remains to be tested.

Importantly, the results observed in Figure 13 were consistent with the findings published on *B. subtilis* LipL, which showed abrogated growth *in vitro* and partial protein lipoylation in a *lipL* deletion mutant (59), as well as with the role of LipL as a lipoyl-transferase in *L. monocytogenes*. Although we do not have direct evidence of LipL transferring lipoylated groups to E2-OGDH, based on the role of LipL in other species we presume this protein is capable of attaching a lipoyl molecule to this subunit. We demonstrated that LplA1 is capable of lipoylating E2-OGDH when LipL is absent, but we suspect this is not the predominant lipoylation mechanism for this subunit under normal conditions. Most likely, lipoylation of E2-OGDH is secured via LipL. Therefore, we postulate that LipL is a lipoyl transferase in *S. aureus* and is the sole enzyme involved in modifying the E2 subunits of lipoylated enzyme complexes in *S. aureus*.

GcvH is the first protein to be lipoylated during *de novo* biosynthesis

The literature argues that GcvH is the first protein to get lipoylated in the lipoate biosynthesis and salvage pathway (11, 61, 70). From an evolutionary perspective, GcvH is believed to be an ancestral protein bearing the first lipoyl domain that arose in nature (11). This notion is based on the conserved structure and role of the glycine cleavage complex, which catalyzes a process that is virtually essential throughout nature. This contrasts with the TCA cycle or branched chain fatty acid

biosynthesis, which are known to be dispensable in many organisms (11, 72). The hypothesis that GcvH is the first intermediate in lipoate acquisition mechanisms is supported by the fact that Achaea and Bacteria species that lack the glycine cleavage complex (GCS) can bypass the requirement for lipoylated enzyme complexes such as PDH, OGDH or BCODH, and do not encode genes involved in lipoate metabolism (11, 72). Additionally, in eukaryotic microorganisms, such as *P. falciparum* and *T. gondii,* GcvH is almost always exclusively localized in the mitochondria and its lipoylation occurs through lipoate scavenging. This connection between the compartmentalization of GcvH in this endosymbiotic organelle and its lipoylation via salvage of lipoate is very interesting and supports the notion of GCS as the first lipoylated system in nature (16, 60, 62).

Several lines of evidence in our work also support the notion that *de novo* biosynthesis and salvage of lipoate is initiated with GcvH. The first evidence comes from the immunoblots of the Δ*gcvH* strain grown in the absence or presence of lipoate. When GSC is not functional, lipoylation appears to be blocked for E2-PDH, E2-OGDH and E2-BCODH subunits but when lipoate salvage is permitted al E2 subunits get lipoylated, indicating that GcvH lipoylation is a requirement for *de novo* biosynthesis of lipoate. Furthermore, the Δ*lipL* Δ*lipA* strain, which lacks the lipoate synthetase, is unable to lipoylate GcvH when grown without supplements

despite having LipM and LplA1 present. The same results are seen for a Δ*lipL* Δ*lipM* double mutant in the absence of supplements. In this case, LplA1 is present, but there is no exogenous lipoate available. We only see lipoylation of GcvH in a Δ*lipL* Δ*lplA1* mutant, which indicates that LipM is transferring octanoate from the ACP to GcvH.

When we supplement lipoate, we see lipoylation of GcvH in Δ*lipL* Δ*lipA,* Δ*lipL* Δ*lipM* and Δ*lipL* Δ*lplA1* strains. The fact that OGDH cannot be lipoylated in a Δ*lipL* Δ*lplA1* mutant under this condition supports the idea that LipM can only attach octanoate to GcvH, and that transfer of the lipoate group to the remaining 2-Oxoacid dehydrogenases cannot proceed when LipL is absent. The only exception to this case is direct lipoylation of E2 OGDH using free lipoate, which can be mediated by what appear to be accessory functions of LplA1. Supplementation of octanoate further supports the notion that *de novo* biosynthesis of lipoate initiates exclusively through GcvH. If we compare Δ*lipL* Δ*lipM* in RPMI alone and after octanoate supplementation we observe that LplA1 must ligate exogenous octanoate to GcvH because lipoyl-GcvH is detected by immunoblot. Additionally, a Δ*lipL* Δ*lplA1* mutant grown under lipoate restriction reveals that LipM exclusively transfers endogenous octanoate from an acyl carrier protein to GcvH. Our findings are consistent with prior work demonstrating the *B. subtilis* octanoyl transferase LipM and *E. coli* LipB catalyze the transfer of an octanoyl moiety from octanoyl-ACP to the

H subunit of the glycine cleavage system (54, 55, 57). Additionally, *B. subtilis* lipoate ligase LplJ has been shown to use free octanoate for GcvH octanoylation (11). Furthermore, GcvH has also been shown to be the first protein subunit lipoylated in *L. monocytogenes* via LplA1 (61).

Thus, based on support from the literature and from our own findings, we propose that *S. aureus* LipM is using ACP-bound octanoyl groups to modify GcvH and that LplA1 is capable of using octanoate for the same purpose. Importantly, we cannot yet determine with absolute certainty that LipM only uses ACP-derived octanoate or whether it is capable of attaching free octanoate to GcvH. Purifying this enzyme and testing if it is capable of transferring the octanoyl group from an ACP to other protein subunits in the absence of exogenous sources of octanoate could serve to determine the precise octanoylation capacity of LipM. Moreover, constructing a strain deficient in the biosynthesis of octanoate could help us answer this question. Alternatively, expressing *S. aureus* LipM in an *E. coli* Δ*fabA* mutant could conceivably allow us to determine whether free octanoate can serve as a substrate for LipM in the absence of endogenous synthesis of octanoic acid.

Importance of LipL in transfer of lipoyl moieties

Previous research revealed that LipL in both *B. subtilis* and *L. monocytogenes* catalyzes the transfer of lipoyl moieties from GcvH to the E2 subunits of the 2-Oxoacid dehydrogenases (59, 61). Based on this

information and our results, we are inclined to consider that under normal conditions, LipL is the central enzyme responsible for transferring the lipoyl group to all E2 subunits of the 2-Oxoacid dehydrogenases (E2-PDH, E2-OGDH and E2-BCODH).

We postulate that LplA1, albeit capable of directly lipoylating E2-OGDH using free lipoate, has no role in transfer of lipoate among enzyme complexes. We still need to directly test this hypothesis, but our model is supported by the data presented in Figure 5, which shows that a Δ*gcvH* mutant grown in the absence of supplements is incapable of lipoylation of E2 subunits. If the lipoate ligase LplA1 were capable of lipoate transfer, we should observe all three E2 bands in Δ*gcvH* mutant background, and that is not reflected in our results.

Of note, *in vitro* and *in vivo* research in *B. subtilis* asserts that LipL is capable of catalyzing the transfer of both octanoyl and lipoyl moieties from GcvH to all E2 subunits (54, 61). We cannot arrive to the same conclusion with the results presented in this thesis, but future research using purified LipL in lipoylation and octanoylation assays would provide a definitive assessment.

Lipoate metabolism in regulation and pathogenesis

Upon assessing the organization of lipoate biosynthesis and salvage genes in the *S. aureus* genome (Figure 3), we noted that the genes are dispersed randomly throughout the chromosome. Few

conclusions could be drawn from this observation, except that the lipoate biosynthesis and salvage genes are genetically unlinked and may not be regulated in a concerted manner. From an evolutionary perspective, this dispersion may also imply that the biosynthesis and salvage genes could have been acquired through different mechanisms or at different moments during evolution.

Remarkably, two genes involved in *S. aureus* lipoate metabolism appear to have novel genetic linkages; the gene encoding the lipoate ligase LplA2 belongs to a large operon that contains genes involved in post-translational modification of protein (52, 53), and the gene for the lipoyl transferase LipL is located in a putative operon next to the gene that encodes the *S. aureus* phosphotransacetylase, Pta (this work). Information obtained from the literature and our own results suggest that this genetic arrangement is likely to have a direct impact in regulation of metabolic processes and possibly redox stress in pathogenesis in *S. aureus* (53).

The gene coding for LplA2 is located in a previously identified operon downstream *gcvH-L* and next to two genes that encode a macrodomain protein and a recently discovered sirtuin, SirTM, which has ADP-ribosyltransferase activity. ADP-ribosylation is a post-translational modification, often involved in regulation of essential metabolic processes such as the cell cycle and synthesis and repair (53). Despite not being the

central focus of their work, *Rack et al.*. defined for the first time the role of LplA2 in lipoylating GcvH-L, a non-canonical glycine cleavage system H protein. Furthermore, their research established that LplA2-dependent lipoylation is required for the subsequent ADP-ribosyl modification of GcvH-L (53).

Previous investigation showed that the entire operon was upregulated under conditions of oxidative stress (53). This information, along with the known antioxidant properties of lipoate, lead Rack et al. to propose that the observed crosstalk between lipoylation and ADP-ribosylation is involved in modulating the response of microbial pathogens to host-induced oxidative stress (53). In their model, the up-regulation of GcvH-L lipoylation would serve to increase the availability of lipoate for anti-redox purposes. Beyond this conclusion, the requirement for lipoylation prior to ADP-ribosylation of GcvH-L is a central piece of information, since it also reveals a potential role for protein lipoylation in the regulation of essential metabolic processes in S. aureus.

The arrangement of lipL in the same operon that pta in the S. aureus genome could also have important implications in terms of potential regulation of metabolic processes. Figure 7 describes the different complementation strategies that we used to achieve restoration of protein lipoylation and growth of a ΔlipL mutant to WT levels. Strikingly, successful complementation could only be attained after restoration of the

entire putative operon, suggesting a unique interaction between lipL and pta.

The pta gene codes for the phosphotransacetylase (Pta), the enzyme responsible for converting acetyl coenzyme A and phosphate into the phosphodonor acetyl-phosphate during nutrient-rich growth conditions. Acetyl-phosphate can be later used to generate ATP and acetate via Ack, the acetate kinase enzyme (4). Our data suggest that deletion of the LipL gene may be interfering with the function of Pta, which could potentially affect the Pta-Ack pathway leading to redirected carbon flux towards gluconeogenesis, with lactate and pyruvate accumulation (70, 73).

Sadykov et al. showed that a pta mutant had decreased intracellular levels of acetate and acetyl phosphate and increased levels of acetyl coenzyme A (70). Inactivation of a lipL pta would additionally block the pathway to the level of pyruvate, preventing its decarboxylation and generation of acetyl coenzyme A. When accumulated, this intermediate is redirected to glucose synthesis or to the TCA cycle (70, 73)

Interestingly, both acetyl coenzyme A and acetyl phosphate seem to be involved in regulation of metabolic processes in bacteria. In E. coli, acetyl coenzyme A acts as an acetyl donor to lysine residues during protein acetylation, and acetyl phosphate has been shown to interact with two-component signal transduction pathways acting as a phosphoryl donor (74, 75). This interaction leads to diverse downstream effects, from

alterations on gene expression and regulation of different cellular processes, such as organelle generation, biofilm formation, and cell cycle regulation (74-76).

With this in mind, our observations of a potential interaction between LipL and Pta suggest a possible role for lipoate in regulation of the Pta-AckA pathway. Control of the concentrations of acetyl coenzyme A and acetyl phosphate could influence expression of genes, post-translational modifications of proteins, and regulation of several virulence factors, which could have a major impact on S. aureus fitness and pathogenesis.

If we consider the role of LipL as a lipoyl transferase and the importance of Pta in S. aureus metabolism and survival, the requirement for both pta and lipL genes when complementing a ΔlipL mutant is intriguing. We could speculate that this novel genetic arrangement might imply a potential role for LipL-dependent protein lipoylation in the regulation of essential metabolic activities, with an impact on S. aureus survival in diverse nutritional conditions. Further investigation designed to study LipL and Pta interactions are currently under investigation in our laboratory.

The role of lipoate metabolism in *S. aureus* pathogenesis

Elucidation of the lipoate *de novo* biosynthesis and salvage pathway in *S. aureus* allowed us to investigate the importance of lipoate

metabolism during infection. We hypothesized that, due to its critical role in maintaining metabolic flux, lipoate acquisition mechanisms affect *S. aureus* pathogenesis. Collectively, the data generated in this work supports this hypothesis and revealed that the source of lipoate during sepsis defines infection outcome in the host.

We used a mouse model of sepsis to study the requirement for lipoate acquisition mechanisms *in vivo*, and measured bacterial burden in tissues of mice infected with different mutant strains. The evaluation of the requirement for *de novo* biosynthesis of lipoate during sepsis showed that the endogenous lipoylation of proteins is required for *S. aureus* infection of the heart in mice. In contrast, *S. aureus* replication in the kidneys required the action of the lipoate ligases, but not lipoate biosynthesis functions (Figure 8).

The individual contribution of the lipoate ligases LplA1 and LplA2 to pathogenesis *in vivo* was determined by comparing the infection capacity of the single deletion mutant strains to Δ*lplA1* Δ*lplA2* double mutant complemented with either *lplA1* or *lplA2* in mice (Figure 12). This study allowed us to conclude that efficient seeding of *S. aureus* of the kidneys relies upon the activity the lipoate ligases. This was confirmed by the experiments conducted on Δ*lipA* Δ*lplA1* Δ*lplA2* complemented with either *lpla1* or *lpla2* (Figure 14), demonstrating that both lipoate ligases, LplA1 and LplA2, could restore wild type infection phenotype.

Our observations differ from the role reported for *L. monocytogenes* LplA1 and LplA2, which exhibited a robust salvage activity *in vitro*. However, while *L. monocytogenes* LplA1 is dispensable for growth in medium containing lipoate, is required for intracellular replication and virulence (15, 19, 61). In contrast, *S. aureus* LplA2 cannot ligate lipoate *in vitro* under the conditions tested during this work, yet either lipoate ligases is sufficient to infect the host, indicating that LplA2 is capable of lipoylating proteins in the host. A possible experiment to test the role of LplA2 in protein lipoylation *in vivo* could be done by isolating colonies from absences in the hearts and kidneys after infection with Δ*lipA* Δ*lplA1* Δ*lplA2* + *lplA2,* and evaluating the presence of lipoylated proteins via mass spectroscopy.

Remarkably, we were able to demonstrate *in vitro* lipoylation activity for LplA2 only after introduction of a complementation construct in the triple mutant Δ*lipA* Δ*lplA1* Δ*lplA2*, in which the P_{HELP} promoter constitutively drives the expression of *lplA2*. Although we could not detect a role in salvage of lipoate during broth culture, *lplA2* expression was sufficient to promote sepsis *in vivo* to levels equivalent to that of LplA1, suggesting that this gene could be upregulated in the host. This was observed after comparing infectivity in mice of the single deletion mutants Δ*lplA1* and Δ*lplA1* to the double mutant Δ*lplA1* Δ*lplA2* followed by complementation with *lplA1* or *lplA2* (Figure 12). Another possible explanation for this result, besides a potential upregulation of *lplA2* expression, is that the induction of tissue damage in the host lead to an

increase on the availability of different lipoylated substrates preferred by LplA2 *in vivo* that were not supplied in broth culture. In other words, LplA2 might have alternative preferred substrates, such as peptide-bound lipoate, instead of free lipoate. Conceivably, LplA2 could be interacting with a lipoamidase in vivo or have lipoamidase activity itself, which would facilitate the release of lipoate moieties from proteins and peptides available *in vivo.* An alternative explanation could be that LplA2 only functions to lipoylate GcvH-L, and, as mentioned above, the operon that contains the pair LplA2/GcvH-L is active *in vivo* and not *in vitro* (70, 73).

Regardless of the mechanism by which LplA2 is functioning *in vivo*, it is remarkable that *S. aureus* has the ability to use two lipoate ligases to scavenge this essential cofactor during infection. This is a novel feature of lipoate acquisition in pathogenic bacteria and most likely facilitates *S. aureus* adaptation to nutrient-limited environments during infection. Additionally, it is known that lipoate is a potent antioxidant compound and the efficient scavenging of this cofactor from tissues could constitute a strategy aimed to counter the oxidative stress response induced by the host during infection (11, 12, 16, 53).

Consistent with our *in vitro* findings, the assessment of the triple mutant Δ*lipA*Δ*lplA1*Δ*lplA2 in vivo* established that *S. aureus* relies on lipoate acquisition for pathogenesis. In other words, *S. aureus* is incapable of using other metabolic strategies, such as anaerobic

metabolism, to infect the host. In sum, our data revealed that bacterial-generated lipoate is required for infection in the heart, but host-derived lipoate is necessary to infect the kidneys.

The reason for the striking division of labor among enzymes involved in lipoate salvage is currently unknown, but not uncommon in nature. A few malarial parasites showed organelle-specific acquisition of lipoate, with lipoate biosynthesis supplying this cofactor in the apicoplast, and environmentally acquired lipoate used in the mitochondrion (14, 18, 62). Additionally, the malarial parasite *P. falciparum* has two lipoate ligases, pfLplA1 and pfLplA2 that reside differentially in those organelles; pfLplA is located the mitochondrion, the site for lipoate *de novo* biosynthesis and the organelle in which PDH is synthetized. In contrast, pfLplA2 can be found in the mitochondrion as well as in the apicoplast, in which GcvH and BCDH are lipoylated.

Finally, differences in lipoate concentration or lipoate sources between the heart and the kidney could potentially explain these observations. In fact, Akiba *et al*. showed that albeit the levels of free lipoate are generally low in all mammal tissues the kidneys had the highest content of total lipoate compared to other tissues (49). Unfortunately, we lack more precise information on lipoylated substrate availability in mammalian tissues in order to draw firm conclusions on substrate accessibility. Likewise, we lack information on the ability of *S.*

aureus to incorporate protein/peptide-bound lipoate from infectious sources. Future directions aimed at shedding light on these remaining problems, include: (1) Characterize the preferred sources of lipoate used by the enzymes involved in lipoate *de novo* biosynthesis and salvage; (2) conduct enzymatic assays to determine the affinity for different lipoylated substrates and; (3) evaluate the expression of the enzymes involved in lipoate acquisition both *in vitro* and *in vivo*. Together, these future studies will clarify the mechanisms used by *S. aureus* to promote optimal replication in the host and might help identify potential drug targets to fight *S. aureus* infections.

Final remarks

In this thesis, we have proposed a model for the *de novo* biosynthesis and salvage pathway of lipoate in *Staphylococcus aureus* and demonstrated the critical role of lipoate acquisition in *S. aureus* pathogenesis. Furthermore, we have identified a previously overlooked genetic arrangement of lipoate *de novo* biosynthesis and salvage genes in the *S. aureus* genome that suggests a potential role for protein lipoylation in the regulation of key metabolic processes and defense mechanisms against host oxidative burst. We have also established the biological function of each gene product involved in lipoate metabolism and determined the contribution of the lipoate acquisition mechanisms to *S. aureus* infection. As a result, we propose that *S. aureus* is capable of

utilizing bacterial and host-derived lipoate during infection in a tissue-specific manner, thereby promoting survival in a wide variety of nutrient-restricted environments. The significant defects in virulence seen after blocking *de novo* biosynthesis and salvage of lipoic acid during sepsis offer potential for the development of novel therapeutics that target lipoate *de novo* biosynthesis and salvage enzymes in the treatment of *S. aureus* infection.

APPENDIX I

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Figure 16: Authorization to republish

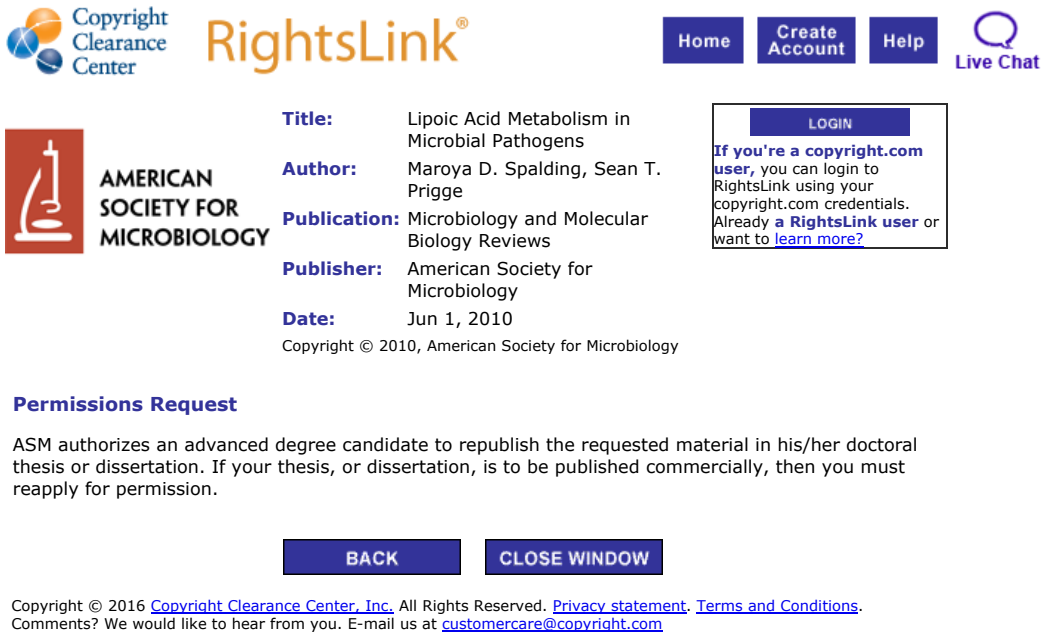

Figure 1

APPENDIX II

SUPPLEMENTARY FIGURES

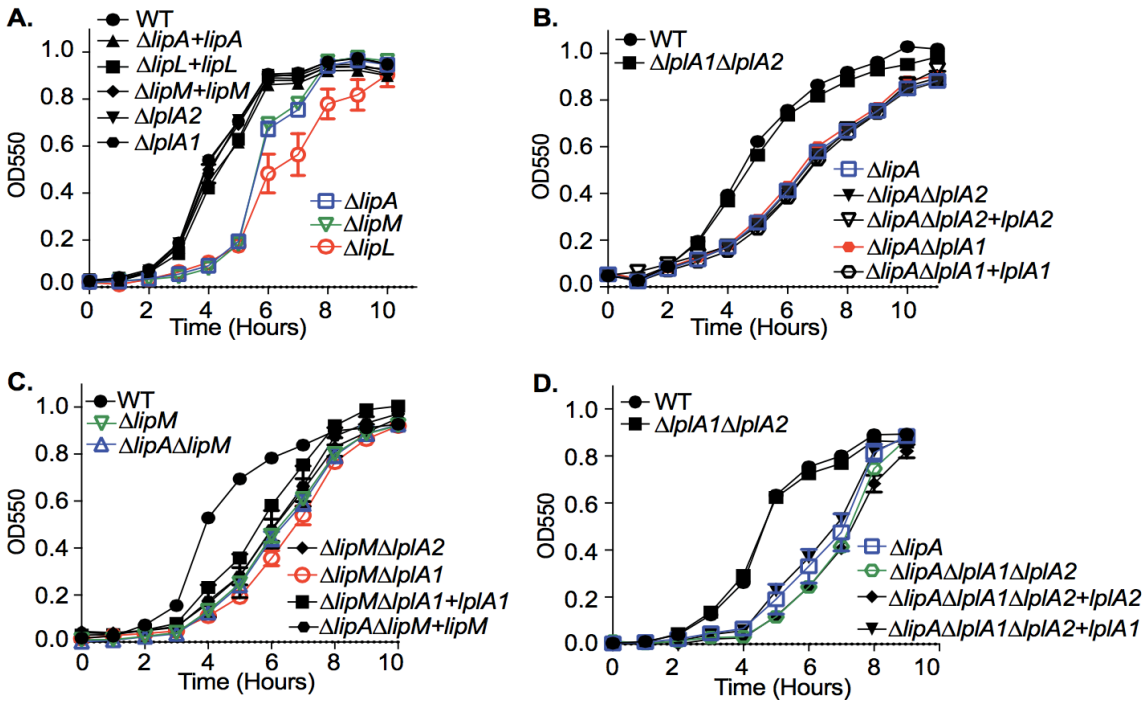

Figure 17: Growth curves of mutant sets in RPMI BCFA

Growth curves in RPMI BCFA of the indicated mutant strains sets: A) Single deletion mutants (ref. Fig. 5), B) Double deletion strains constructed in the background of a *lipA* deletion mutant (ref. Fig. 8), C) Double deletion strains constructed in the background of a *lipM* deletion mutant (ref. Fig. 10) D), Triple deletion strains of the *de novo* biosynthesis and salvage pathway enzymes (ref. Fig. 9).

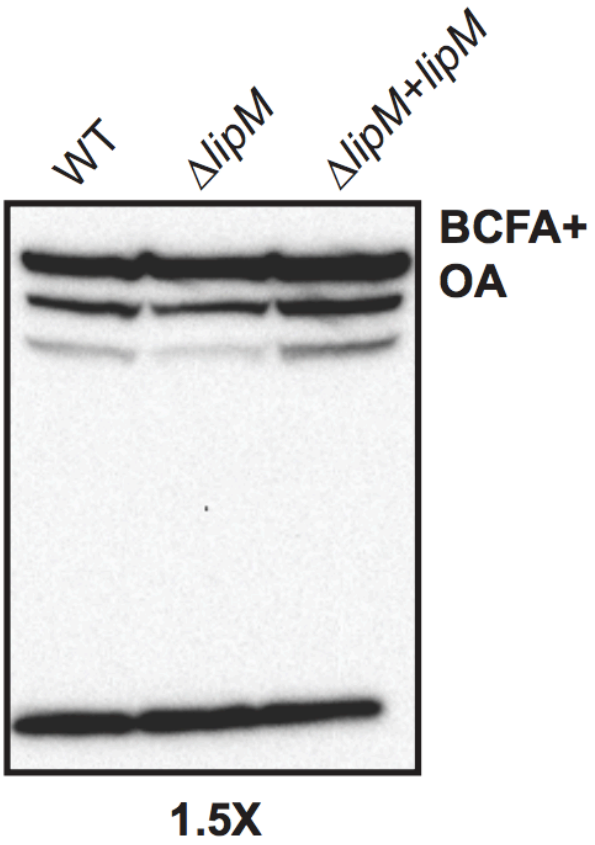

Figure 18: LipM lipoylation profile

LipM is capable of protein lipoylation to E2-OGDH. Immunoblots of whole cell lysates of the indicated mutant strains grown in RPMI + BCFA + Octanoate, detected with anti-lipoic acid antibody (C). This experiment was performed as previously described but the amount of sample loaded in each well was increased by a 1.5 factor.

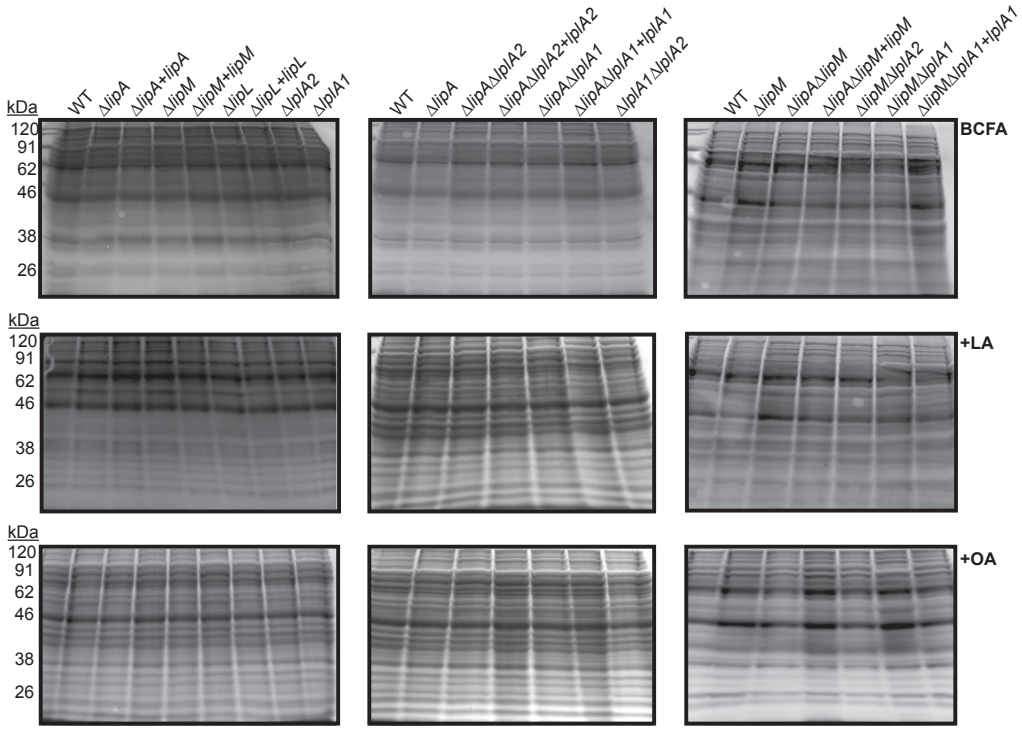

Figure 19: Whole cell protein patterns I

Proteins from whole cell lysates were resolved in 12% polyacrylamide gels and stained using GelCode Blue stain reagent (Thermo). The molecular weight of the proteins was marked using Precision Plus Protein Ladder™ (Thermo). The rows organize samples grown in RPMI+BCFA media (BCFA), RPMI+lipoate (+LA) or RPMI+octanoate (+OA). Columns organize the set of mutants tested: the first column depicts the protein patterns of the set of mutants used in figure 6. The column at the center reflects the protein patterns of mutants used for figure 9. Protein patterns of mutant strains tested in figure 11 are shown on the left column.

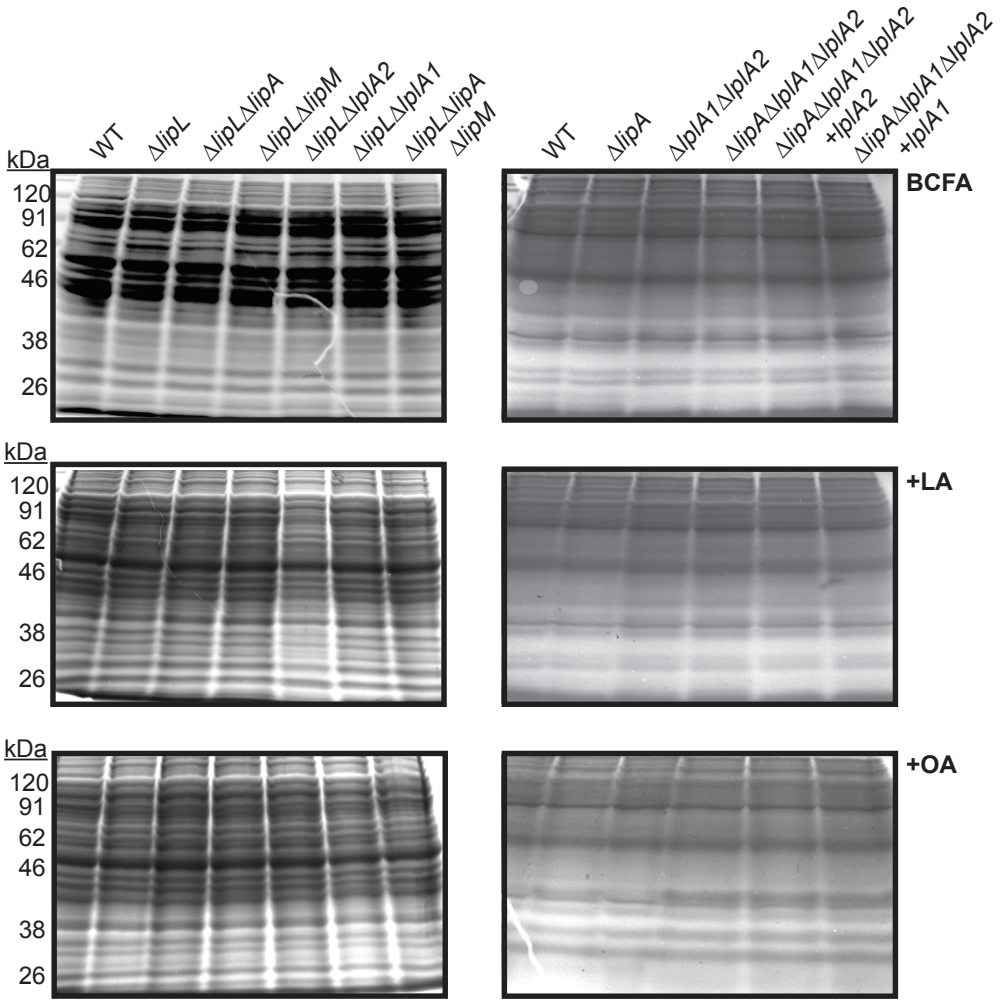

Figure 20: Whole cell protein patterns II

Proteins from whole cell lysates were resolved in 12% polyacrylamide gels and stained using GelCode Blue stain reagent (Thermo). The molecular weight of the proteins was marked using Precision Plus Protein Ladder™ (Thermo). The rows organize samples grown in RPMI+BCFA media (BCFA), RPMI+lipoate (+LA) or RPMI+octanoate (+OA). The right column depicts the protein patterns of the set of mutants used in figure 13. The protein patterns shown on the left belong to the mutant strains used in figure 10.

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VITA

Azul was born in San Carlos de Bariloche, Rio Negro, Argentina on October 24th, 1981 to Adriana Maria Robles and Alberto Pedro Zorzoli. She obtained a degree in Biochemistry from Universidad Nacional de Tucumán in December 2008. During her undergraduate studies, Azul worked as a laboratory technician for the Argentinian National Scientific and Technical Research Council.

In 2013, Azul was awarded the Argentine Presidential Scholarship in Science and Technology supported by the Fulbright Commission to pursue a Master in Sciences in the United States. In 2014, she joined the Microbiology and Immunology Department at Loyola University Chicago. Since January 2015, Azul has been investigating the role of the lipoate biosynthesis and salvage pathways in *Staphylococcus aureus* pathogenesis under the supervision of Dr. Francis Alonzo.

Upon earning her M.Sc. Degree, Azul will relocate to Scotland to pursue a Ph.D. at the University of Dundee, where she will be focusing on studying the cell wall biogenesis in *Streptococci* under the supervision of Dr. Helge Dorfmueller.