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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

A MECHANISTIC INVESTIGATION OF LIPOYL SYNTHASE FROM ESCHERICHIA
COLI AND STAPHYLOCOCCUS AUREUS

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ABSTRACT

The lipoyl cofactor plays an integral role in many metabolic processes. The second step of the *de novo* biosynthesis pathway of the lipoyl cofactor is catalyzed by the radical *S*-adenosyl-*L*-methionine (SAM) enzyme, lipoyl synthase (LipA). Radical SAM enzymes contain at least one [4Fe-4S] cluster, which is used to reductively cleave SAM into *L*-methionine and a 5'-deoxyadenosyl 5'-radical (5'-dA•). LipA contains two [4Fe-4S] clusters; one cluster is used for the SAM cleavage, while the other is sacrificed as a sulfur source for the production of lipoic acid. Two equivalents of 5'-dA• are used to abstract hydrogen atoms from the C6 and C8 positions of an octanoyl-lipoyl carrier protein (LCP) substrate, and hydrogen atoms are replaced with sulfide ions. *In vitro* studies show that LipA is restricted to only one turnover due to the requirement for the destruction of its auxiliary cluster, but it is currently unknown what enzymes are required to regenerate the catalytic form of this enzyme.

Previous studies have implicated Nfu and SufT in the regeneration of the auxiliary cluster of LipA in *Staphylococcus aureus*. SufT is hypothesized to reconstitute the cluster in *S. aureus*, as it has been proposed to aid in the maturation of iron-sulfur (Fe-S) proteins. Nfu is an Fe-S cluster carrier protein that has been shown to act synergistically with SufT. In this work, LipA was isolated and shown to be active and to contain two [4Fe-4S] clusters, while Nfu was shown to coordinate one [4Fe-4S] cluster. The activity of Nfu and LipA was monitored using liquid chromatography coupled to mass spectrometry (LC-MS). These results showed that Nfu alone has no effect on the activity of LipA.

A sequence alignment of twelve *lipA* genes revealed that the auxiliary cluster of LipA is coordinated by three cysteine residues in a CX₄CX₅C motif and a serine residue in a conserved

RSSY motif. To determine the importance of each amino acid residue in LipA catalysis, site-directed mutagenesis was used to create single amino acid substitutions. Using activity assays, it was determined that the arginine (R) and two serine (S) residues were necessary for catalytic activity, while the tyrosine (Y) is dispensable.

Finally, the *lip* locus contains two genes, *ybeD* and *ybeF*, which have yet to be characterized. As the remainder of the genes in the locus are implicated in lipoic acid biosynthesis, it was hypothesized the two proteins were involved with lipoic acid biosynthesis. In order to study them, *ybeD* was overexpressed and purified, and LipA activity assays were performed in the presence of YbeD. The addition of YbeD to the reaction had no effect on LipA catalysis.

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Chapter 1

Introduction to Radical *S*-adenosyl-*L*-methionine Enzymes

SAM and Radical SAM Enzymes

Methylation is fundamental to many biological processes, such as post-translational modifications, changes in gene expression, and the biosynthesis of many biomolecules. *S*-adenosyl-*L*-methionine (SAM) is nature's universal methylating agent, and is used to methylate a wide assortment of molecules, including DNA, RNA, proteins, carbohydrates, lipids, and myriad small-molecule metabolites.^{1,2} SAM-dependent methylations proceed through a polar S_N2 displacement mechanism, yielding the methylated product and *S*-adenosylhomocysteine (SAH) as a SAM-derived byproduct.³

Nature also uses SAM for other purposes, including the generation of a potentially oxidizing radical that initiates difficult and kinetically challenging enzymatic transformations. These reactions are catalyzed by Radical SAM (RS) enzymes. RS enzymes contain at least one [4Fe-4S] cluster, which supplies a requisite electron used for the reductive cleavage of SAM into *L*-methionine and a 5'-deoxyadenosyl 5'-radical (5'-dA•) (**Fig. 1.2**).⁴ The 5'-dA• is a highly reactive species, which abstracts hydrogen atoms from the target substrate within an enzyme's active site.⁵ The RS superfamily contains more than 115,000 unique sequences, which represent over 85 distinct reactions. Moreover, at least 50% of the sequence space of RS enzymes is unannotated.^{3,6} Indeed, RS enzymes catalyze methylation and methylthiolation of unactivated carbon or phosphinate phosphorus centers, oxidation of hydroxyl or thiol functional groups, 1,2-cross-migrations of functional groups, epimerizations, sulfur insertion, carbon-carbon bond formation, key steps in the biosynthesis of metallocofactors, and a variety of complex rearrangements,

amongst others.⁷ Despite this variety, RS enzymes share distinct features in their primary structures, such as a highly conserved CX₃CX₂C motif in which the three cysteine residues coordinate three of the iron ions of the [4Fe-4S] cluster.⁴ The fourth “unique” Fe ion is ligated by the SAM amino and carboxylate groups in a bidentate fashion.^{8,9}

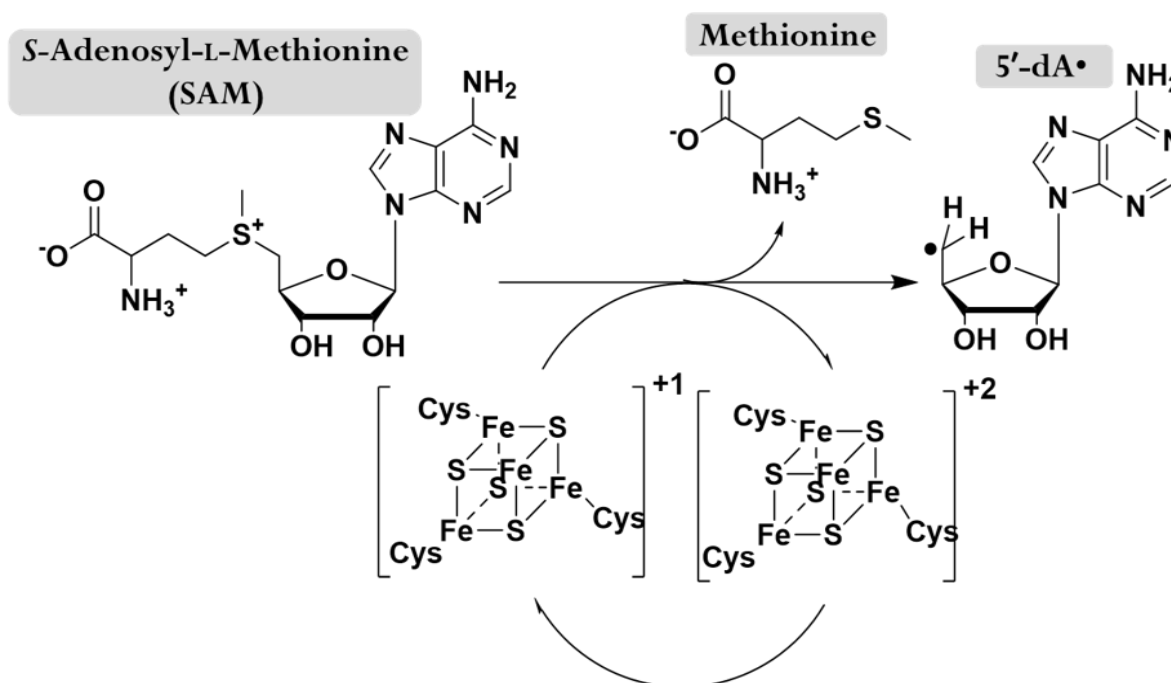


Figure 1.1. Mechanism of SAM Reductive Cleavage.⁴

Radical SAM (RS) enzymes catalyze a diverse array of reactions and play critical roles in a variety of biological processes and in the biosynthesis of numerous biomolecules.⁴ RS enzymes contain a [4Fe-4S] cluster which, in its reduced state, injects an electron into the sulfonium of SAM, fragmenting it into L-methionine and a 5'-deoxyadenosyl radical (5'-dA•).⁴ The 5'-dA• is a potent oxidant capable of performing difficult chemistry, such as hydrogen abstraction from unactivated carbon centers.⁴

A subset of RS enzymes contains one or more additional iron-sulfur (Fe-S) clusters, known as auxiliary clusters, or an additional cobalamin or pyridoxal 5'-phosphate (PLP) cofactor, which expand the diversity of reactions within the superfamily. One known role for auxiliary clusters is

as a sulfur source, which necessitates that the cluster be degraded and cannibalized. To date, two RS enzymes that catalyze sulfur insertion (lipoyl synthase and biotin synthase) have been characterized in detail, while others, such as RimO and MiaB, are presumed to use this catalytic strategy, but still lack conclusive experimental evidence. In contrast to LipA, biotin synthase, or BioB, contains a [2Fe–2S] auxiliary cluster. Using a sulfur atom derived from the auxiliary cluster, BioB generates a thioether bond between carbons 6 and 9 of dethiobiotin in the biosynthesis of the biotin cofactor.⁷

Lipoyl synthase, or LipA, coordinates a [4Fe–4S] auxiliary cluster, which it uses to insert two sulfur atoms at C6 and C8 of a protein-bound *n*-octanoyl chain to generate the essential molecule lipoic acid⁷

Lipoic acid is an essential cofactor across biology.¹⁰ Defects in lipoic acid biosynthesis or the enzymes that require it are typically fatal. Lipoic acid is most notably used as a central cofactor in several multienzyme complexes that are components of primary metabolism: the pyruvate dehydrogenase complex, α -ketoglutarate dehydrogenase, branched-chain α -keto acid dehydrogenase, acetoin dehydrogenase complexes, and the glycine cleavage system.¹⁰ In its biologically relevant form, lipoic acid is covalently attached to a lipoyl carrier protein (LCP) through an amide linkage with the ϵ -amino group of a target lysyl residue. This appendage acts as a long “swinging arm” to allow for the shuttling of intermediates between successive active sites of the multienzyme complexes that require it for activity.¹⁰ Additionally, lipoic acid is a redox active cofactor, existing in a reduced form, in which the sulfur atoms exist as free thiols, or in an oxidized form, in which the sulfur atoms form a disulfide bond rendering a dithiolane ring.¹⁰

The role of lipoic acid is not confined to its ability to function in primary metabolism. It has also been used therapeutically for a variety of clinical conditions. Most notably, lipoic acid has been found to serve as an intracellular antioxidant and has been shown to alleviate the symptoms of conditions associated with oxidative stress, such as diabetes. In addition, it has been shown to modulate various signaling pathways, including insulin and NF- κ B.¹⁰

Pathways for The Biosynthesis of Lipoic Acid

Lipoic acid is an eight carbon straight-chain fatty acid with sulfhydryl groups at C6 and C8. Very little free lipoic acid exists in nature; rather, it is appended covalently through an amide linkage to a target lysyl residue on an LCP. (**Fig. 1.2**).

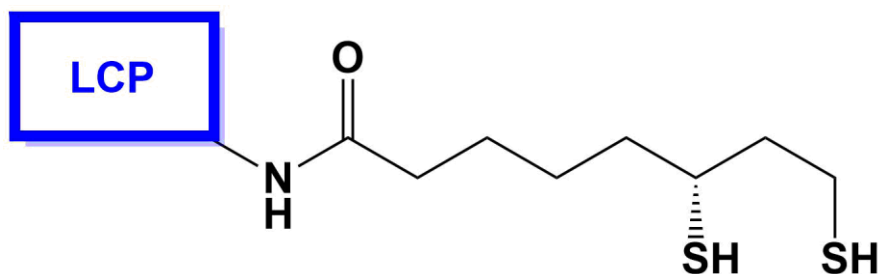


Figure 1.2. Structure of Lipoic Acid.

Lipoic acid contains two sulfur atoms that are attached at C6 and C8 of an octanoyl chain.

While variations of the biosynthetic pathway exist among different organisms, the process is well-understood in *E. coli*, in which there are two distinct pathways. In the exogenous pathway, free lipoic acid is scavenged and activated through adenylation by a protein known as lipoate protein ligase A (LplA).¹¹ This same protein then transfers the lipoyl group from lipoyl-AMP to the LCP to generate the lipoyl cofactor. In the endogenous pathway, the fatty acyl backbone of lipoic acid

is derived from type II fatty acid biosynthesis, which is followed by two key steps. In the first step, the octanoyl transferase, LipB, transfers an octanoyl fatty acyl chain from an octanoyl-acyl carrier protein (ACP) to produce an octanoyl-LCP. In the second step, LipA appends two sulfur atoms onto the octanoyl-LCP by a radical-mediated process.^{10, 12} The two pathways for lipoic acid biosynthesis in *E. coli* are shown below in Figure 1.3.

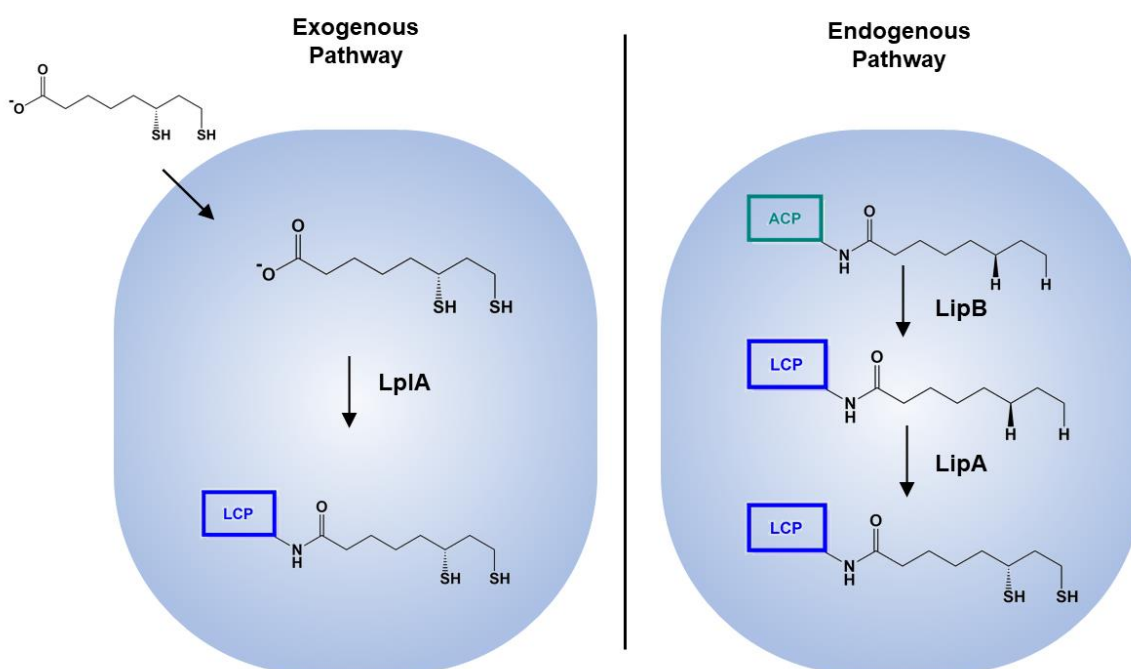


Figure 1.3. Pathways for Lipoic Acid Biosynthesis.

In the exogenous pathway, free lipoic acid is scavenged and ATP-activated by lipoyl protein ligase A (LplA). LplA then transfers the lipoyl group from lipoyl-AMP to the lipoyl carrier protein to generate the lipoyl cofactor. In the endogenous pathway, lipoic acid is derived from type II fatty acid biosynthesis and is followed by two enzyme-catalyzed steps. In the first step, the octanoyl transferase, LipB, transfers an octanoyl fatty acyl chain from an octanoyl-acyl carrier protein (ACP) to an octanoyl-lipoyl carrier protein (LCP). In the second step, LipA appends two sulfur atoms onto the octanoyl-LCP by a radical-mediated process.

Proposed Mechanism of LipA

LipA contains a second tri-cysteine (CX₄CX₅C) motif.¹⁰ This motif is universally conserved in LipA and serves as a strong recognition sequence for identifying these proteins.¹⁰ The cysteines in the motif coordinate an auxiliary [4Fe-4S] cluster, which is degraded during turnover to provide the source of the sulfur atoms in lipoic acid.¹³ The currently proposed mechanism of LipA is shown in Figure 1.4. LipA appends two sulfur atoms onto the octanoyl-LCP by a radical-mediated process. One sulfur atom is stereo-selectively inserted at C6, affording the *R*-enantiomer, and one is inserted at C8. (**Fig 1.4**).^{10, 12} LipA uses the 5'-dA• produced by the reductive cleavage of SAM to abstract the hydrogen atoms from the carbons where the sulfur atoms are introduced. Because this cluster is degraded during turnover, LipA can only catalyze one

turnover in *in vitro* reactions. The mechanism by which the cluster is reinstalled after turnover to reactivate the protein is not readily understood.

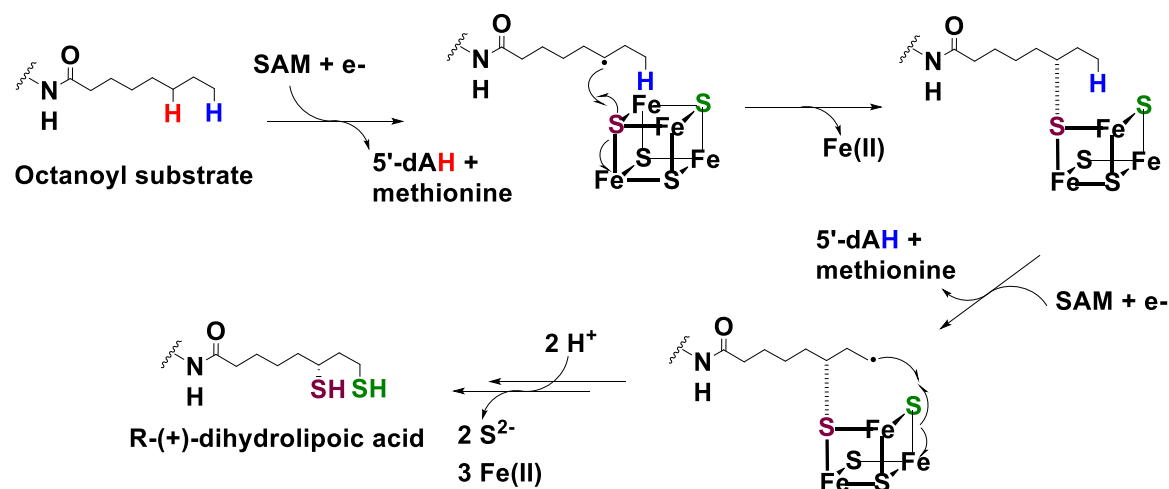


Figure 1.4. Proposed Mechanism of LipA.¹²

LipA contains two [4Fe–4S] clusters, one of which is sacrificed during turnover. Catalysis proceeds by reductive cleavage of SAM to render a 5'-deoxyadenosyl radical (5'-dA•), which abstracts the C6 *pro-R* hydrogen atom (H•) of a pendant *n*-octanoamide chain on a lipoyl carrier protein (LCP). The resulting C6 substrate radical attacks one of the sulfide ions of the auxiliary cluster, which is followed by loss of an Fe²⁺ ion to afford a [3Fe–3S–1(6S)-thio-octanoamide]LCP intermediate. A second reductive cleavage of SAM generates a second 5'-dA•, which abstracts an H• from C8 of the thio-octanoamide protein intermediate. The resulting C8 substrate radical attacks a second sulfide ion of the auxiliary cluster, which is followed by the addition of two protons and the loss of three Fe²⁺ ions and two S²⁻ ions to generate the lipoyl group in its reduced form.¹⁰ The mechanism by which the auxiliary cluster is regenerated is the current area of research.

Iron-sulfur cluster biosynthesis and trafficking

It seemed likely that an unknown system existed that could regenerate LipA's auxiliary cluster for catalysis. Most organisms contain one or more established pathways dedicated to the biosynthesis of Fe-S clusters. The three general pathways are: (1) the iron sulfur cluster (Isc) system, (2) the sulfur formation (Suf) system, and (3) the nitrogen fixation (Nif) system.¹⁴ The three pathways are used differently between different organisms, and it is suggested that the different pathways are used based on the needs of the cell.¹⁴ Though variations between organisms and pathways exist, they share the same common framework. Cluster assembly begins with a cysteine desulfurase, which uses free cysteine to acquire sulfur atoms through a PLP-dependent mechanism.¹⁵ Iron is supplied from a still-debated source, while electrons are inputted from a ferredoxin/ferredoxin reductase. The Fe-S cluster is assembled on a central scaffolding protein.¹⁵ Cluster transfer is then mediated by an adapter protein or protein complex to its apo-protein target. The mechanism of how Fe-S clusters are trafficked in the cell is a poorly understood process.

Disruption of the pathways for Fe-S cluster assembly result in a number of notable diseases, such as Friedrich's ataxia and multiple mitochondrial dysfunction syndrome (MMDS).^{4,16} MMDS is a rare, autosomal recessive disorder that presents during infancy.⁴ Symptoms of MMDS include early onset death, respiratory failure and impaired neurological development.¹⁷ Several clinical reports described fatal disorders presented in infants that were attributed to mutations in the gene encoding NFU1.¹⁸ Intriguingly, western blots performed using anti-lipoic acid antibodies revealed that the fibroblasts of the patients contained significantly reduced levels of lipoic acid. At the time, the biological role of NFU1 was unknown; however, from this observation, it was hypothesized that this protein could play a role in lipoic acid biosynthesis, which accounts for the MMDS

phenotypes that are associated with debilitated energy metabolism.⁴ To test the role of NFU1 in lipoic acid biosynthesis, the *E. coli* homologue of NFU1, NfuA, was overproduced and isolated.

It is known that LipA degrades its auxiliary cluster in order to produce the lipoyl cofactor, and *in vitro* studies show that LipA is restricted to only one turnover due to this strategy for sulfur mobilization.¹⁹ Studies linked the proteins NFU1 and BOLA3 to lipoyl cofactor biosynthesis in yeast and mammalian cells via catalytic deficiencies in LipA.²⁰ NfuA was used to determine that the carrier protein can restore the catalytic function of LipA in *E. coli*.¹⁷ Moreover, it was demonstrated that through its A-type domain, NfuA is able to recognize and interact with LipA.²¹

Project Objectives

The overarching objective of this research was to characterize lipoyl synthase by its activity *in vitro* and iron content and further understand the biosynthetic pathway for regenerating its auxiliary cluster. The following specific aims were used to address the overarching goal.

- Aim 1:* to characterize the proteins involved in the *de novo* biosynthesis of the lipoyl cofactor in *Staphylococcus aureus*.
- Aim 2:* to determine the significance of the conserved RSSY motif through the characterization of the variants of *Escherichia coli* LipA.
- Aim 3:* to further understand whether *ybeD* and *ybeF*, which are part of the *lip* operon in *Escherichia coli*, are involved in lipoic acid biosynthesis.

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Chapter 2

Characterization of Proteins Involved in the de novo Biosynthesis of the Lipoyl Cofactor in *Staphylococcus aureus*

Introduction

Infections caused by the pathogenic organism *Staphylococcus aureus* have been rampant across United States hospitals for the past few decades.¹ With the rise of methicillin-resistant *Staphylococcus aureus*, or MRSA, researchers have been charged with further uncovering the complexities of the physiology of the bacterium to discover novel methods to combat these infections.

Lipoyl synthase (LipA) catalyzes the last step in the biosynthesis of the lipoyl cofactor, which is the attachment of sulfur atoms at carbons 6 and 8 of an octanoyl group that is covalently tethered to a lipoyl carrier protein (LCP). During catalysis, one of its two [4Fe-4S] cluster cofactors is degraded to supply the sulfur atoms, limiting the enzyme to only one turnover.^{2,3,4,5} The degraded Fe-S cluster must be regenerated in order for this enzyme to catalyze more than one turnover. It was hypothesized that two proteins in *Staphylococcus aureus*, SufT and Nfu, may be involved in the regeneration of this cluster and the biosynthesis of the lipoyl cofactor in this organism.^{6,7}

In Δ sufT strains of *S. aureus*, the primary phenotypes were a decrease in lipoic acid production and a decrease in the activity of enzymes that utilize lipoic acid as a cofactor.⁷ *S. aureus* SufT is an auxiliary protein involved in the maturation of many proteins that require Fe-S clusters, such as aconitate hydratase A and 3-isopropylmalate dehydratase.⁷

In *Escherichia coli*, cluster regeneration can be carried out by NfuA, an intermediate Fe-S cluster carrier protein.⁵ It was hypothesized that *S. aureus* Nfu, which is homologous to *E. coli*

NfuA, could fulfill a similar function in *S. aureus*, though their sequence similarity is limited to the C-terminal domain. The ultimate goal of this research was to clone the genes that encode SufT, Nfu, and LipA from *S. aureus*, isolate the corresponding proteins, and assess whether Nfu and SufT, individually or in combination, can render LipA catalytic.

Materials and Methods

Materials — Restriction enzymes and materials for cloning were obtained from New England Biolabs (Ipswich, MA). DNA isolation kits were purchased from Machery-Nagel (Dueren, Germany). Deoxynucleotides for PCR amplification were from Denville Scientific Corporation (South Plainfield, New Jersey). Kanamycin, arabinose, isopropyl β -D-thiogalactopyranoside (IPTG), tris (2-carboxyethyl) phosphine (TCEP), dithiothreitol (DTT), and ampicillin were from Gold Biotechnology (St. Louis, MO). Protein calibration standards, FeCl₃, L-cysteine, DNase I, β -mercaptoethanol (BME), pyridoxal 5'-phosphate (PLP), and sodium dithionite were purchased from Sigma Co. (St. Louis, MO). Ni-NTA resin for protein isolation was obtained from Qiagen. HEPES (sodium salt) and potassium chloride were from Dot Scientific (Burton, MI). Imidazole and lysozyme were from Alfa Aesar (Haverhill, MA). Coomassie brilliant blue dye and Bradford reagent were from Amresco (Fountain Parkway Solon, OH). Unlabeled Na₂S and bovine serum albumin (BSA) used for the Bradford standard were from Thermo Fisher Scientific (Waltham, MA). Materials used in liquid chromatography-mass spectrometry (LC-MS) assays, including the lipoyl, octanoyl, and 6-thiooctanoyl peptides, SAH nucleosidase, external standard peptide, and SAM have been previously described.^{8,9} All chemicals and reagents were of the highest grade available.

General procedures — UV-visible spectra were recorded on a Varian Cary 50 spectrometer (Walnut Creek, CA) using the WinUV software package. The Polymerase Chain Reaction (PCR) was performed using a Bio-Rad S1000 Thermal Cycler. High-performance liquid chromatography (HPLC) with detection by mass spectrometry (LC–MS) was conducted on an Agilent Technologies (Santa Clara, CA) 1200 system coupled to an Agilent Technologies 6410 QQQ mass spectrometer. The system was operated with the associated MassHunter software package, which was also used for data collection and analysis. Iron and sulfide content of proteins was measured using previously described methods.^{10,11}

Expression and Purification of Staphylococcus aureus LipA (Sa LipA), Nfu (Sa Nfu), and SufT (Sa SufT) —A single colony was used to inoculate a 200 mL starter culture of *Escherichia coli* BL21(DE3) cells containing pDB1282 and the desired gene construct (*S. aureus lipA*-pET28a, *S. aureus nfu*-pET28a, or *S. aureus sufT*-pET28a). Expression was performed in 16 L (4 × 4 L) of M9 Minimal Media, preincubated at 37 °C. The cells were grown at 37 °C with shaking at 180 rpm. At an OD₆₀₀ of 0.3, *L*-(+)-arabinose was added to a final concentration of 0.2% to induce expression of genes on plasmid pDB1282. At an OD₆₀₀ of 0.6, FeCl₃ and IPTG were added to the cultures to final concentrations of 50 μM and 200 μM, respectively. The flasks were then chilled on ice for at least 30 min, and shaken by hand every 5 min. The cultures were incubated overnight at 18 °C with shaking at 180 rpm. The following day, the cells were harvested by centrifugation at 7,000 × *g* for 15 min, and the resulting cell paste was flash-frozen in liquid nitrogen. Samples from each flask to be used for sodium-dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE)

were removed just before addition of arabinose, just before addition of IPTG and FeCl₃, and just before harvesting.

Proteins were purified anaerobically (<1 ppm O₂) in a Coy (Grass Lake, MI) anaerobic chamber with the exception of centrifugation steps. The cell pellet was re-suspended in 200 mL lysis buffer (50 mM HEPES, pH 7.5, 300 mM KCl, 20 mM imidazole, 10 mM 2-mercaptoethanol (BME), 1 mM *L*-cysteine, 0.1 mM PLP, 5 mM MgCl₂, 5 mM ATP), containing lysozyme (1 mg/mL), and DNase I (0.1 mg/mL). This mixture was stirred at room temperature for 30 min before lysing the cells by sonic disruption. The lysate was loaded into centrifuge bottles, which were tightly sealed before they were removed from the anaerobic chamber, and the lysate was centrifuged at 45,000 × *g* for 1 h and 4 °C. The supernatant was applied to a column of nickel-nitrilotriacetic acid (Ni-NTA) resin equilibrated in lysis buffer.¹² The column was washed with 200 mL wash buffer (50 mM HEPES, pH 7.5, 300 mM KCl, 40 mM imidazole, 10 mM BME, 10% glycerol) before eluting the protein with elution buffer (50 mM HEPES, pH 7.5, 300 mM KCl, 250 mM imidazole, 10 mM BME, 10% glycerol). Fractions were collected based on brown color, and the pooled fractions were concentrated using an Amicon centrifugal filtration device fitted with a 30 KDa molecular weight cut-off (MWCO) membrane. The protein was then exchanged into storage buffer (100 mM HEPES pH 7.5, 300 mM KCl, 1 mM DTT, 20% glycerol) using a PD-10 column (GE Healthcare Life Sciences). The protein was aliquoted and flash-frozen in liquid N₂ and stored in liquid N₂ until further use. Samples of the pellet, supernatant, flow-through, wash and elution were analyzed by SDS-PAGE to assess protein purity. Protein concentration was determined using the Bradford method with BSA as a standard.¹³

Chemical Reconstitution of LipA—Wild-type LipA was chemically reconstituted under anaerobic conditions to ensure full [4Fe–4S] cluster incorporation. A reaction containing 100 μ M LipA was slowly stirred on ice. DTT (5 mM final) was added in three increments every 20 min, and the solution was incubated for 1 h. Next, 500 μ M FeCl₃ was added in five increments every 5 min, and the solution was incubated for 30 min. Finally, 500 μ M Na₂S was added in five increments every 30 min, and the reaction was incubated on ice overnight. The following day, aggregates were removed by centrifugation at $14,000 \times g$ for 10 min, and the protein was concentrated to 2.5 mL. The protein was then further purified by size-exclusion chromatography on a HiPrep 16/60 Sephacryl HR S-200 column (GE Health Sciences) equilibrated in storage buffer (flow-rate of 0.5 mL/min) and connected to an ÄKTA protein liquid chromatography system (GE Health Sciences) housed in an anaerobic chamber.

LipA activity determinations—LipA activity was determined under anaerobic conditions in reaction mixtures containing the following in a final volume 210 μ L: 50 mM HEPES, pH 7.5, 300 mM KCL, 10% glycerol, 300 μ M peptide substrate (Glu-Ser-Val-[N⁶-octanoyl]Lys-Ala-Ala-Ser-Asp), 200 μ M *S. aureus* LipA, 0.5 μ M S-adenosylhomocysteine (SAH) nucleosidase, 1 mM SAM, and 400 μ M *S. aureus* Nfu when appropriate. Reactions were performed at room temperature, and were initiated by addition of sodium dithionite to a final concentration of 2 mM. Reactions were quenched at 0, 1, 2, 4, 8, 15, 30, 60, 90, 120, and 150 min in a final concentration of 100 mM H₂SO₄/4 mM TCEP/100 μ M tryptophan/20 μ M AtsA peptide (internal standards). Detection of substrates and products was performed using LC-MS.

Results

Expression and Purification of Staphylococcus aureus LipA and Nfu

Genes encoding *S. aureus* LipA, Nfu and SufT were all cloned into the *Nde*I site of pET28a expression vectors, which affords proteins with N-terminal hexahistidine tags to be used for purification by immobilized-metal affinity chromatography (IMAC). Two of the proteins, LipA and Nfu, were overexpressed at reasonably high levels, while the expression of SufT was significantly more modest. As shown in **Fig. 2.1A** and **2.1C**, bands migrating at the appropriate molecular masses for LipA (37.2 kDa) and Nfu (10.9 kDa) became apparent after induction with IPTG. By contrast, as shown in **Fig. 2.1B**, the band for SufT was significantly more modest after addition of IPTG. Attempts to purify SufT resulted in no significant amounts of pure protein.

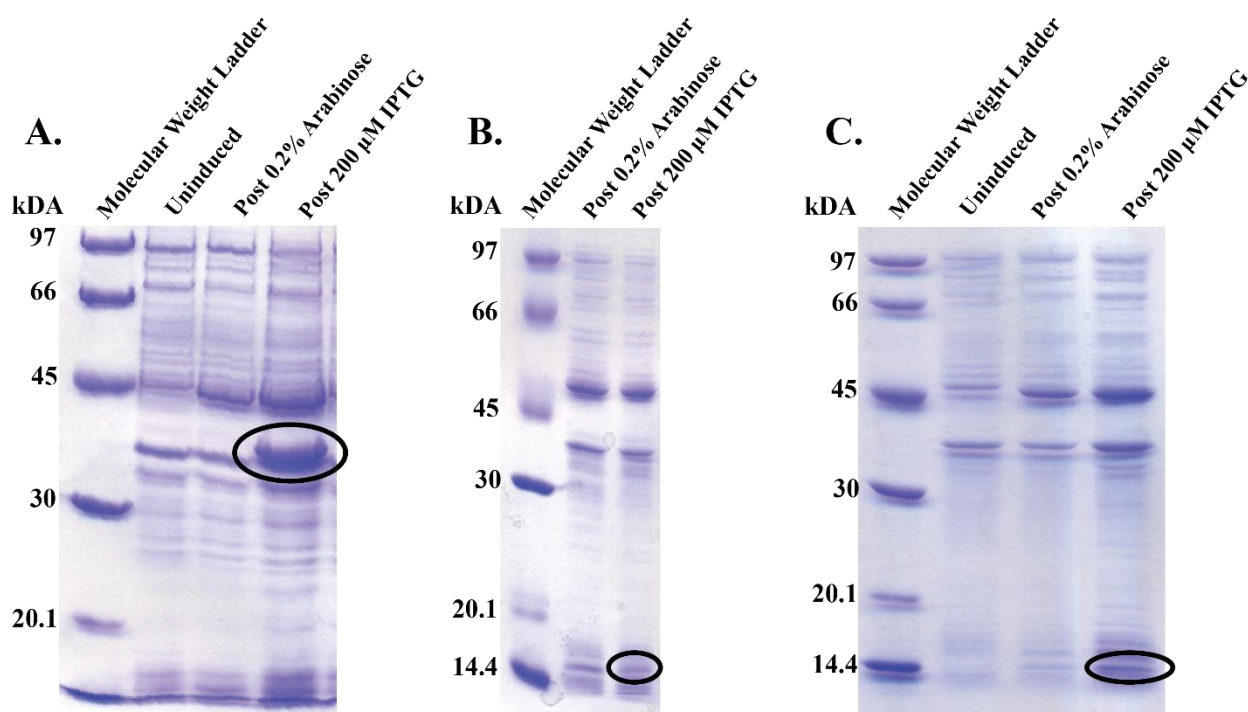


Figure 2.1. Overexpression of *Sa* LipA, *Sa* SufT, and *Sa* Nfu.

A. Overexpression of *Sa* LipA (37.2 kDa) **B.** Overexpression of SufT (11.8 kDa) **C.** Overexpression of Nfu (10.9 kDa).

LipA and Nfu were further purified to $\geq 95\%$ homogeneity by IMAC with yields of 180 mg (LipA) and 54 mg (Nfu) from 16 L of culture (**Figures 2.2A and 2.2B**). As can be seen in **Fig. 2.2A**, a significant amount of LipA was present in the pellet, meaning that it was overproduced in inclusion bodies. By contrast, Nfu appeared to be largely soluble.

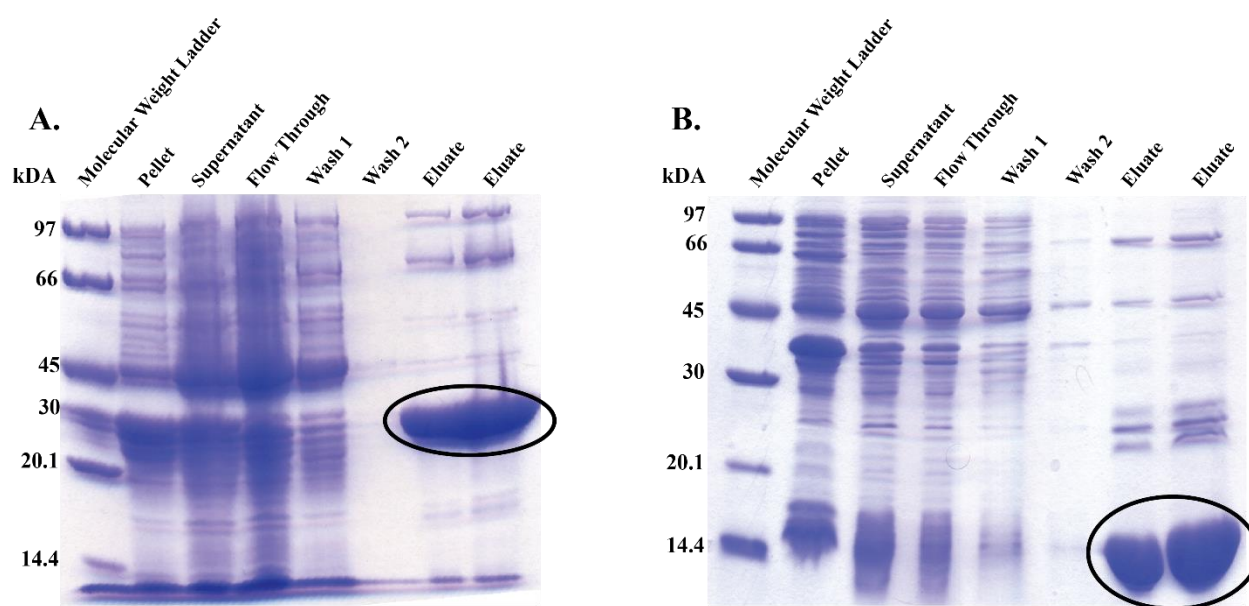


Figure 2.2. The purification of *Sa* LipA and *Sa* Nfu.

A. Purification of *Sa* LipA. The last two lanes show the purified protein sample. About 180 mg were recovered.

B. Purification of Nfu. The low abundance of other bands in the pure samples indicated that the sample was $\geq 95\%$ pure. There was little indication of Nfu in the lanes from the samples collected from the pellet, supernatant, flow-through, and wash.

Characterization of Staphylococcus aureus LipA

Iron and sulfide analysis of as-isolated *Sa* LipA indicated that the protein binds 3.9 ± 0.6 iron ions and 5.0 ± 0.8 sulfide ions, indicating sufficient iron and sulfide only for one [4Fe–4S] cluster. Theoretically, *Sa* LipA should contain two [4Fe–4S] clusters. The sulfur content was also about half of the expected amount. Because the iron content was low, the protein was subjected to a chemical reconstitution procedure by incubating it with iron and sulfide under reducing conditions to increase the stoichiometry of [4Fe–4S] clusters. The reconstituted protein, however, only contained 5.1 ± 0.78 iron ions and 4.6 ± 0.68 sulfide ions, which is similar to that obtained for the as-isolated protein. Without a Bradford correction factor for the *Sa* LipA, the number of iron and sulfide ions per protein is inaccurate.

After measuring the Fe content of the protein, the UV-visible absorption spectrum of LipA was recorded, which revealed the characteristic features of a protein containing a [4Fe–4S] cluster (**Fig. 2.3**). The peak at 280 nm derives from absorption of tryptophans in the protein, while the hump at 440 and the shoulder around 320 nm are features that indicate the presence of at least one [4Fe–4S] cluster. (**Fig. 2.3**). The spectra of both the protein before and after reconstitution are shown below. The reconstituted protein absorbed slightly more at 320 and 440 nm.

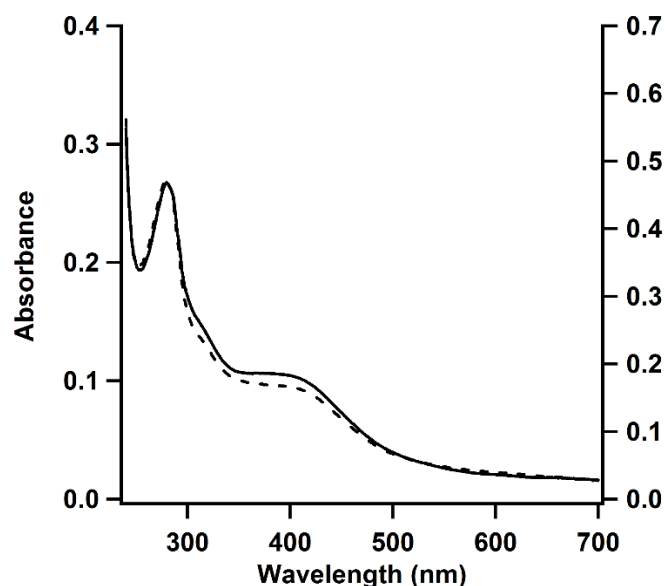


Figure 2.3. UV absorption spectrum of *Sa* LipA.

The peak at 280 is characteristic of all proteins. The hump at 440 nm and the shoulder around 320 nm are indicative of at least one [4Fe-4S] cluster. The dashed line is the spectrum of LipA as-isolated and the solid line is the spectrum of the reconstituted LipA.

Characterization of Staphylococcus aureus Nfu

Sa Nfu has been reported to coordinate a [4Fe-4S] cluster. However, iron and sulfide analysis of *Sa* Nfu isolated herein indicated that it binds 0.6 ± 0.1 iron ions and 0.5 ± 0.14 sulfide ions per protein, which is not sufficient to coordinate even one [4Fe-4S] cluster. It is possible that the low iron and sulfide numbers are due to the lack of a correction factor to properly extract the protein concentration from Bradford analysis.

The UV absorption spectrum of Nfu, however, is consistent with the presence of an Fe-S cluster, although it is not clear what type (**Fig. 2.4**). The spectrum appears to have absorption peaks at 320 nm and 420 nm, but it does not have the defining shoulder and hump of a [4Fe-4S] cluster,

so it is likely that this is another form of Fe-S cluster. Further studies will be needed to determine the cluster species.

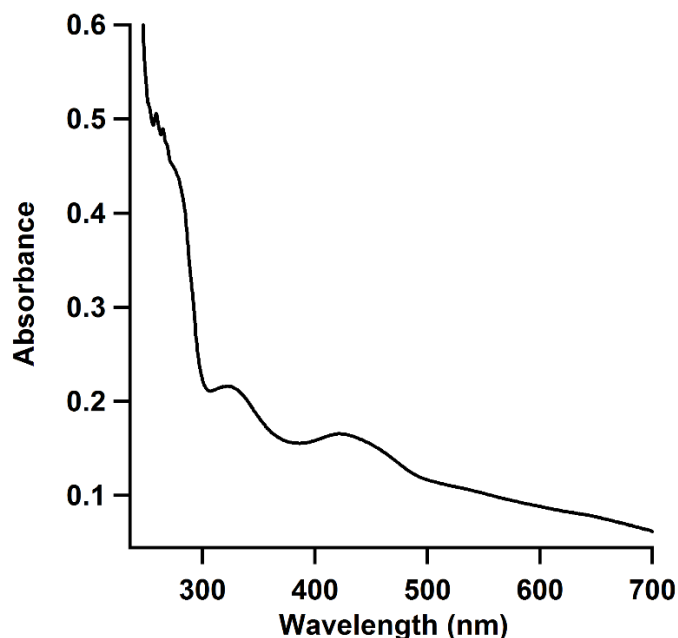


Figure 2.4. UV absorption spectrum of *Sa* Nfu.

The absorption at 280 is characteristic of all proteins. The peaks at about 420 nm and 320 nm are indicative of a [4Fe-4S] cluster.

Biochemical assays indicate that Nfu has no effect on the regeneration of the LipA auxiliary cluster

Under single turnover conditions, 200 μ M of as-isolated *Sa* LipA catalyzed the formation of about 30 μ M of product (**Fig. 2.5**). Because LipA was unable to regenerate its auxiliary cluster, it was unable to participate in further reactions, causing the formation of product to rapidly increase then plateau. The decreasing levels of the intermediate reflect the formation of product (**Fig. 2.5**).

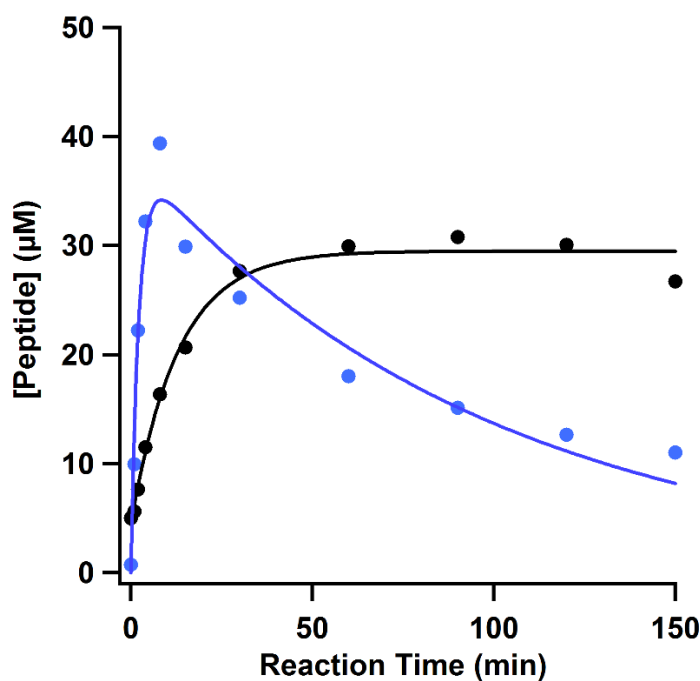


Figure 2.5. Intermediate and Product formation by *Sa* LipA As-Isolated.

200 μM of *Sa* LipA was reacted with 300 μM of peptide substrate and product/intermediate concentration versus time was plotted. Formation of the product (black line) rapidly increases, then levels out, producing about 30 μM of product, which is consistent with one turnover. The levels of the intermediate (blue line) reflect the product formation.

After the reconstitution of *Sa* LipA, product formation increased about 5-fold, further indicating the importance of reconstituting the Fe-S cluster (**Fig 2.6**). Both of the curves begin to plateau after about 50 minutes.

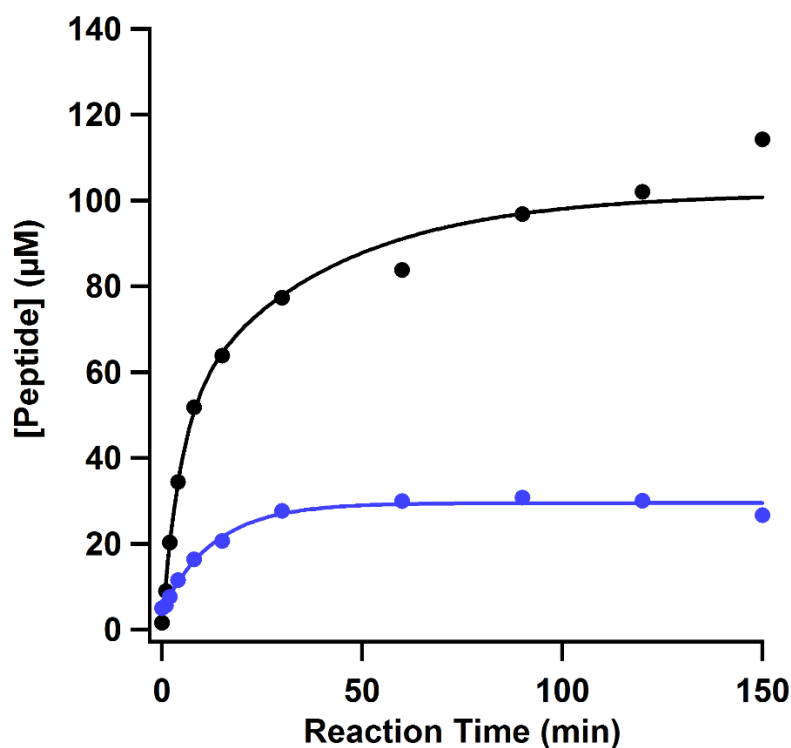


Figure 2.6. *Sa* LipA AI and RCN controls.

Under single turnover conditions, 200 μM of as-isolated *Sa* LipA (blue) and 200 μM of reconstituted *Sa* LipA were reacted with 300 μM of peptide substrate and product concentration versus time was plotted. The reconstituted enzyme (black line) makes about 5-fold more product than the as-isolated protein (blue line). After 2.5 hours, the reconstituted protein made about 115 μM peptide, while the as isolated protein only made about 25 μM protein.

Once the amount of turnover was established in the absence of Nfu, a reaction was performed in the presence of Nfu to assess its effect on formation of the lipoyl group. If product formation was greater than the amount produced in the control assay, then it would indicate that Nfu facilitates cluster regeneration, which was measured by the ability to restore catalytic function to LipA. However, upon addition of Nfu, an almost identical product curve to that of the reaction

in the absence of Nfu was observed demonstrating that the addition of Nfu had no effect on product formation (**Fig. 2.7**).

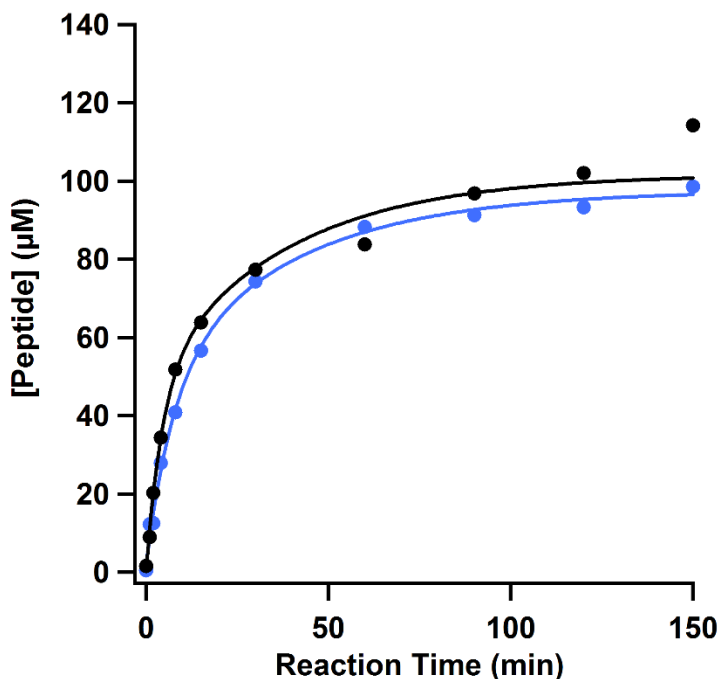


Figure 2.7. *Sa* LipA activity control.

200 μM of reconstituted *Sa* LipA were reacted with 300 μM of peptide substrate without (black line) and with 400 μM *Sa* Nfu (blue line), and product concentration versus time was plotted. Both reactions made about the same final concentration of product. After 2.5 hours, the reconstituted protein made about 115 μM peptide, while LipA in the presence of Nfu made about 100 μM protein. The addition of Nfu to the reaction made no difference in product formation.

Discussion

Through these experiments, it was demonstrated that *S. aureus* LipA is active *in vitro*, but because it has not been determined what proteins are needed to regenerate the auxiliary cluster, the enzyme is limited to one turnover. The iron and sulfide analyses as well as the UV vis spectrum

indicated that *S. aureus* LipA coordinates at least one [4Fe-4S] cluster, while the Nfu Fe-S cluster species remains unknown. Most importantly, it can be concluded that *S. aureus* Nfu alone has no effect on the activity of LipA.

Because SufT was unable to be overexpressed and purified, it is possible that Nfu and SufT must work in concert to regenerate the auxiliary cluster in *S. aureus* LipA. In order to test this hypothesis, SufT would need to be purified. To resolve the issues of expression, *sufT* and *nfu* could be co-expressed and purified, possibly allowing *sufT* to be more stably expressed. The effects of the two proteins on LipA's turnover would then need to be measured. It would also be valuable to determine the structures of *S. aureus* SufT, Nfu, and LipA, which may provide further information on how the three proteins may hypothetically interact with one another.

Determining this mechanism of regeneration of the Fe-S cluster will provide vital knowledge that may provide the scientific community with further understanding of the mechanisms of Fe-S cluster formation and a novel target for drug development. At this time, it is still unclear what proteins are needed to regenerate the auxiliary cluster of lipoyl synthase in *S. aureus*. An alternative route that may provide further information about this mechanism may be co-immunoprecipitation studies or transcriptome analyses of strains deficient in the ability to generate endogenous lipoic acid.

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Chapter 3

The significance of the LipA RSSY motif in *Escherichia coli*

Introduction

The auxiliary cluster of *Escherichia coli* lipoyl synthase (LipA) is coordinated by three cysteine residues in a CX₄CX₅C motif and a serine residue in a conserved RSSY motif.¹ In 1968, it was found that three mutant strain of *E. coli* were unable to grow on lipoic acid deficient media. All three strains had mutations that mapped to the *lipA* locus, and one was shown to convert Ser308 (the second serine in the RSSY motif) to Phe.^{2,3} This phenotype indicated the importance of this serine to catalysis. Crystal structures show that this serine coordinates the iron ion of the auxiliary cluster.¹ The arginine of this motif has been hypothesized to be involved in the protection of the auxiliary cluster during substrate binding and in the absence of the substrate.⁴ The functions of the final two amino acids in the motif appear to aid in the positioning of the C-terminal tail.¹ The crystal structure of LipA does not immediately indicate the importance of these four residues. Therefore, the experiments described herein leverage activity assays on RSSY variants assess the importance of amino acids in this motif in formation of lipoic acid under single turnover and multiple turnover conditions.

Materials and Methods

Materials – Restriction enzymes and materials for cloning were obtained from New England Biolabs (Ipswich, MA). DNA isolation kits were purchased from Machery-Nagel (Dueren, Germany). Deoxynucleotides for PCR amplification were from Denville Scientific Corporation

(South Plainfield, New Jersey). Kanamycin, arabinose, isopropyl β -D-thiogalactopyranoside (IPTG), tris (2-carboxyethyl) phosphine (TCEP), dithiothreitol (DTT), and ampicillin were from Gold Biotechnology (St. Louis, MO). Protein calibration standards, FeCl₃, L-cysteine, DNase I, β -mercaptoethanol (BME), pyridoxal 5'-phosphate (PLP), and sodium dithionite were purchased from Sigma Co. (St. Louis, MO). Ni-NTA resin for protein isolation was obtained from Qiagen. HEPES (sodium salt) and potassium chloride were from Dot Scientific (Burton, MI). Imidazole and lysozyme were from Alfa Aesar (Haverhill, MA). Coomassie brilliant blue dye and Bradford reagent were from Amresco (Fountain Parkway Solon, OH). Unlabeled Na₂S and bovine serum albumin (BSA) used for the Bradford standard were from Thermo Fisher Scientific (Waltham, MA). Materials used in LC-MS assays, including the lipoyl, octanoyl, and 6-thiooctanoyl peptides, SAH nucleosidase, external standard peptide, and SAM have been previously described.^{5,6} All chemicals and reagents were of the highest grade available.

General procedures — UV-visible spectra were recorded on a Varian Cary 50 spectrometer (Walnut Creek, CA) using the WinUV software package. The polymerase chain reaction (PCR) was performed using a Bio-Rad S1000 Thermal Cycler. High-performance liquid chromatography (HPLC) with detection by mass spectrometry (LC-MS) was conducted on an Agilent Technologies (Santa Clara, CA) 1200 system coupled to an Agilent Technologies 6410 QQQ mass spectrometer. The system was operated with the associated MassHunter software package, which was also used for data collection and analysis. Iron and sulfide content of proteins was measured using previously described methods.⁵

Creation of the variants — In a previous experiment, a former graduate student used site specific mutagenesis was used to create four different variants of *E. coli* LipA: R306K, S307A, S308A, and Y309F.¹

Expression and Purification of Escherichia coli LipA variants (R306K, S307A, S308A, and Y309F)

— A single colony was used to inoculate a 200 mL starter culture of *Escherichia coli* BL21(DE3) cells containing pDB1282 and the desired gene construct (*E. coli lipA* R306K-pET28a, *E. coli lipA* S307A-pET28a, *E. coli lipA* S308A-pET28a, or *E. coli lipA* Y309F-pET28a). Expression was performed in 16 L (4 × 4 L) of M9 Minimal Media, preincubated at 37 °C. The cells were grown at 37 °C with shaking at 180 rpm. At an OD₆₀₀ of 0.3, *L*-(+)-arabinose was added to a final concentration of 0.2% to induce expression of genes on plasmid pDB1282. At an OD₆₀₀ of 0.6, FeCl₃ and IPTG were added to the cultures to final concentrations of 50 μM and 200 μM, respectively. The flasks were then chilled on ice for at least 30 min and shaken by hand every 5 min. The cultures were incubated overnight at 18 °C with shaking at 180 rpm. The following day, the cells were harvested by centrifugation at 7,000 × *g* for 15 min, and the resulting cell paste was flash-frozen in liquid nitrogen. Samples from each flask to be used for sodium-dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) were removed just before addition of arabinose, just before addition of IPTG and FeCl₃, and just before harvesting.

Proteins were purified anaerobically (<1 ppm O₂) in a Coy (Grass Lake, MI) anaerobic chamber with the exception of centrifugation steps. The cell pellet was re-suspended in 200 mL lysis buffer (50 mM HEPES, pH 7.5, 300 mM KCl, 20 mM imidazole, 10 mM BME, 1 mM *L*-cysteine, 0.1 mM PLP, 5 mM MgCl₂, 5 mM ATP), containing lysozyme (1 mg/mL), and DNase I (0.1 mg/mL). This mixture was stirred at room temperature for 30 min before lysing the cells by

sonic disruption. The lysate was loaded into centrifuge bottles, which were tightly sealed before they were removed from the anaerobic chamber, and the lysate was centrifuged at $45,000 \times g$ for 1 h and 4 °C. The supernatant was applied to a column of nickel-nitrilotriacetic acid (Ni-NTA) resin equilibrated in lysis buffer.⁸ The column was washed with 200 mL wash buffer (50 mM HEPES, pH 7.5, 300 mM KCl, 40 mM imidazole, 10 mM BME, 10% glycerol) before eluting the protein with elution buffer (50 mM HEPES, pH 7.5, 300 mM KCl, 250 mM imidazole, 10 mM BME, 10% glycerol). Fractions were collected based on brown color, and the pooled fractions were concentrated using an Amicon centrifugal filtration device fitted with a 30 KDa molecular weight cut-off (MWCO). The protein was then exchanged into storage buffer (100 mM HEPES pH 7.5, 300 mM KCl, 1 mM DTT, 20% glycerol) using a PD-10 column (GE Healthcare Life Sciences). The protein was aliquoted and flash-frozen in liquid N₂ and stored in liquid N₂ until further use. Samples of the pellet, supernatant, flow-through, wash and elution were analyzed by SDS-PAGE to assess protein purity. Protein concentration was determined using the Bradford method with bovine serum albumin (BSA) as a standard.⁹

LipA activity determinations—The activity of LipA variants was determined under anaerobic conditions in reaction mixtures containing the following in a final volume 210 µL: 50 mM HEPES, pH 7.5, 300 mM KCL, 10% glycerol, 300 µM peptide substrate (Glu-Ser-Val-[N⁶-octanoyl]Lys-Ala-Ala-Ser-Asp), 200 µM *E. coli* LipA, 0.5 µM S-adenosylhomocysteine (SAH) nucleosidase, 1 mM SAM, and 400 µM *E. coli* NfuA when appropriate. Reactions were performed at room temperature and were initiated by addition of sodium dithionite to a final concentration of 2 mM. Reactions were quenched at 0, 1, 2, 4, 8, 15, 30, 60, 90, 120, and 150 min in a final concentration of 100 mM H₂SO₄/4 mM TCEP/100 µM tryptophan/20 µM AtsA peptide (internal standards).

Detection of substrates and products was performed using liquid chromatography-mass spectrometry.

Results

LipA R306K is unable to catalyze the generation of the product.

Immobilized-metal affinity chromatography (IMAC) was used to purify the LipA R306K variant to $\geq 95\%$ homogeneity with an overall yield of 530 mg from 16 L of cell culture. As it can be seen from the purification gel, the eluted protein was pure although a significant portion of the protein remained in the supernatant (**Fig. 3.1A**). Using the Bradford analysis, the concentration of this protein was found to be 2.5 mM. Iron analyses showed that there were 6.7 ± 3.8 Fe/protein. The absorption spectrum showed a peak around 400 nm and a shoulder around 320 nm, consistent with the protein containing a [4Fe-4S] cluster (**Fig. 3.1B**).

Comparison of the product formation of the R306K variant and the variant in the presence of NfuA to that of the wildtype LipA showed that the variant made no product, as the limit of detection is 500 nM (**Fig. 3.1C**). This result shows that NfuA had no effect on the formation of production with respect to the variant.

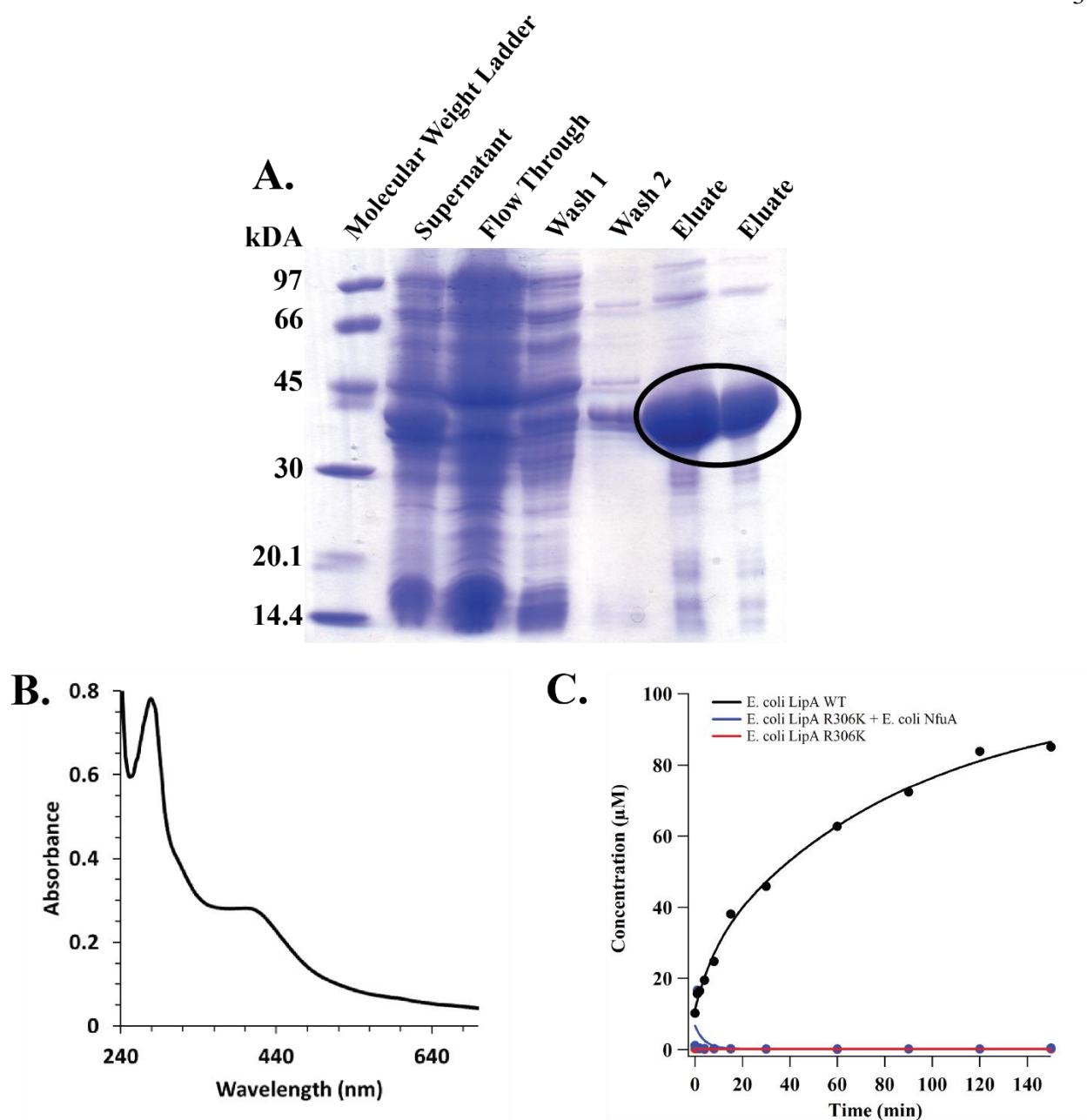


Figure 3.1. Characterization of the R306K variant of *E. coli* LipA.

A. Protein purification gel. **B.** UV visible spectrum. **C.** Activity assays of the R306K variant comparing the concentration of product made between the R306K variant, the R306K variant with NfuA, and the wildtype LipA. 200 μ M of WT and R306K LipA were reacted with 400 μ M of NfuA and 300 μ M of substrate in the respective assays.

LipA S307A was inactive.

Lip S307A was purified to $\geq 95\%$ homogeneity by IMAC with a yield of 599 mg from 16 L of culture. The purification gel has dense bands at 37.2 kDa, indicating LipA in the eluate, but it also shows the presence of unknown proteins at 27 and 15 kDa (**Fig. 3.2A**). As with previous LipA purifications, there was evidence of a significant amount of LipA S307A remaining in the pellet, indicating that this protein readily forms inclusion bodies. The concentration of this variant was determined to be 2.7 mM by Bradford analysis. Iron analysis found that the protein contained 3.7 ± 0.6 Fe/protein. The UV absorption spectrum of this protein also showed the characteristics of a [4Fe-4S] cluster (**Fig. 3.2B**). LipA S307A was almost completely inactive. This variant made no product, which could be due to the amino acid change, or the presence of the 15 kDa and 27 kDa protein contaminants in the protein solution (**Fig 3.2C**).

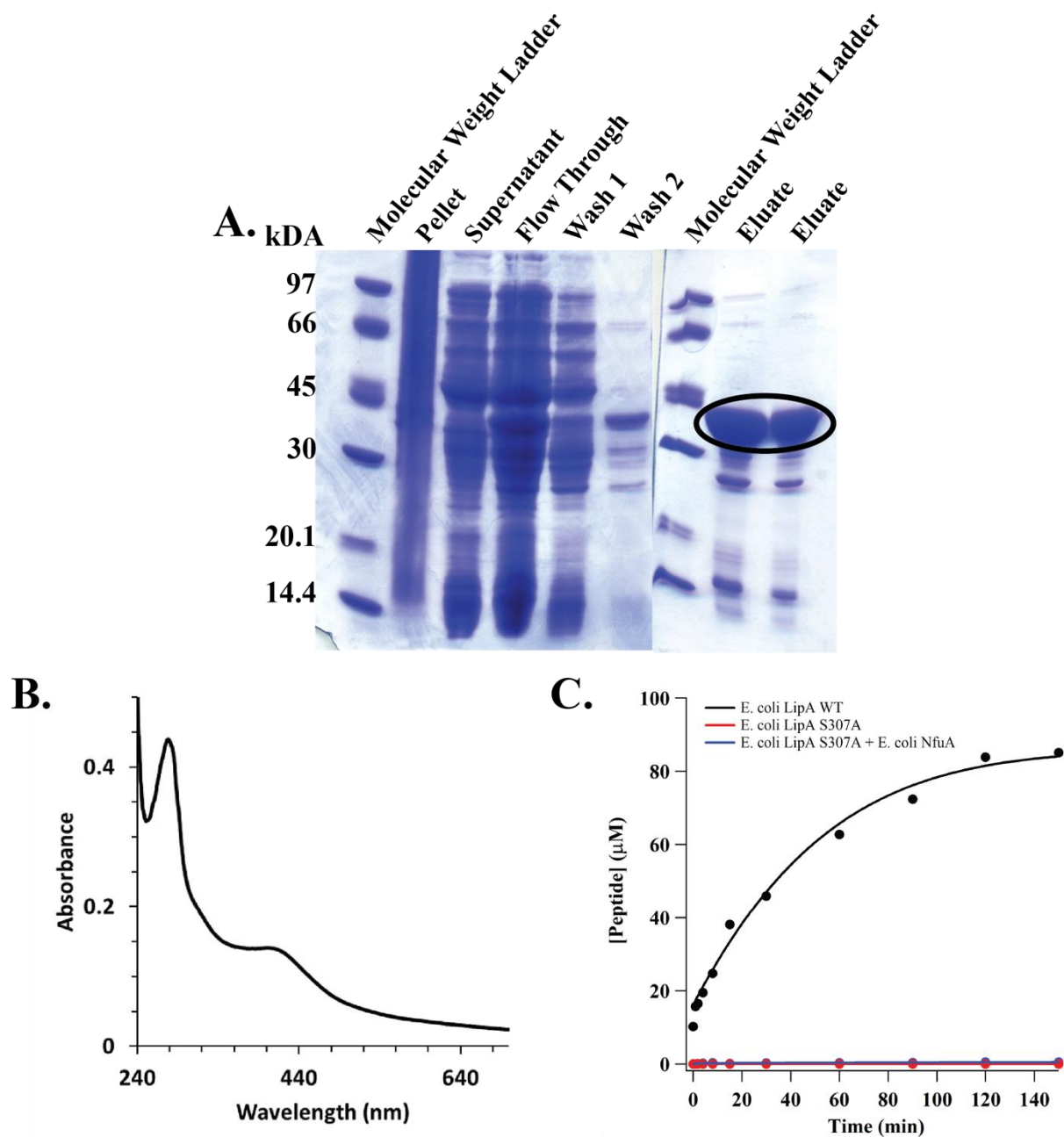


Figure 3.2. Characterization of the S307A variant of *E. coli* LipA.

A. Protein purification gel. **B.** UV visible spectrum. **C.** Activity assays of the S307A variant comparing the concentration of product made between the S307A variant, the S307A variant with NfuA, and the wildtype LipA. 200 μ M of WT and S307A LipA were reacted with 400 μ M of NfuA and 300 μ M of substrate in the respective assays.

LipA S308A variant made low quantities of product.

Using IMAC, the LipA S308A variant was purified to $\geq 95\%$ homogeneity with a yield of 114 mg from 16 L of cell culture. This yield was about 5-fold lower than those of the previous two variants. Bradford analysis revealed that the protein concentration was 664 μM . Although the concentration was very low, the eluate was very pure (**Fig. 3.3 B**). The UV absorption spectrum indicated that the variant had a [4Fe-4S] cluster, as the characteristics of the spectrum included a shoulder at 320 nm and a hump at 400 nm (**Fig. 3.3C**). This protein had lower absorbances than the other variant, which might reflect a lower stoichiometry of [4Fe-4S] clusters. LipA S308A only had 2.5 ± 0.7 Fe/protein, as determined by iron analysis. With only 2.5 Fe atoms of LipA's normal 8, it was assumed that this protein would be less active. The S308A variant made 0.85 μM product alone and 4.5 μM product with NfuA; the wild type LipA made about 17 times more product (**Fig. 3.3D**).

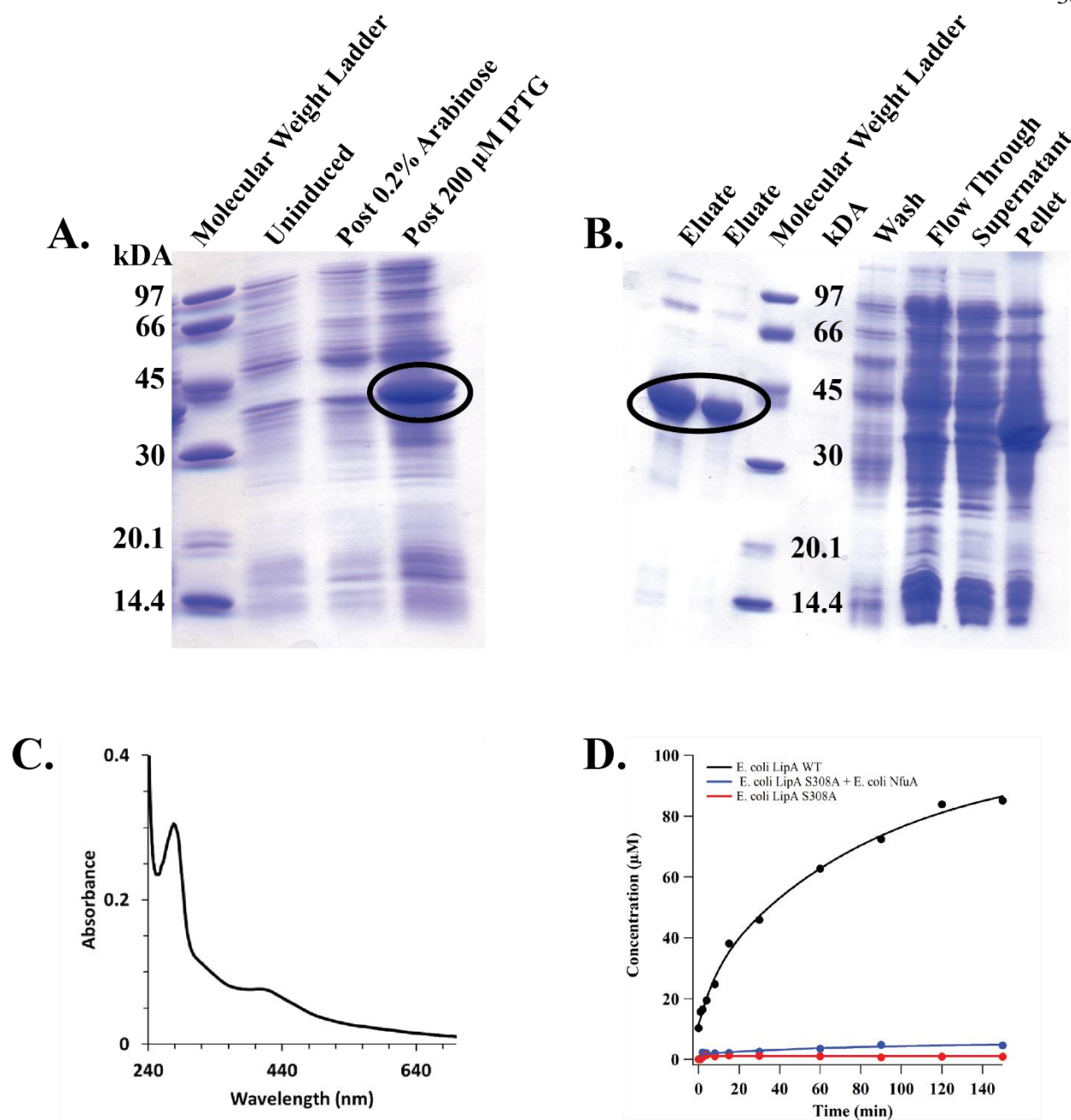


Figure 3.3. Characterization of the S308A variant of *E. coli* LipA.

A. Protein expression gel. **B.** Protein purification gel. **C.** UV visible spectrum. **D.** Activity assays of the S308A variant comparing the concentration of product made between the S308A variant, the S308A variant with NfuA, and the wildtype LipA. 200 μ M of WT and S308A LipA were reacted with 400 μ M of NfuA and 300 μ M of substrate in the respective assays.

LipA Y309F retained its catalytic function.

The purification of the Y309F variant to $\geq 95\%$ homogeneity by IMAC yielded 521 mg from 16 L of cell culture. Bradford analysis was used to determine that the concentration of the protein was 3.5 mM. As shown in the gel, overexpression occurred, but the purification gels demonstrates the impurity of the as-isolated LipA Y309F (**Fig. 3.4A and B**). The protein had the key characteristics of a [4Fe-4S] cluster, as shown by its UV-vis spectrum (**Fig. 3.5A**). Iron analysis the presence of 3.4 ± 0.5 Fe/protein.

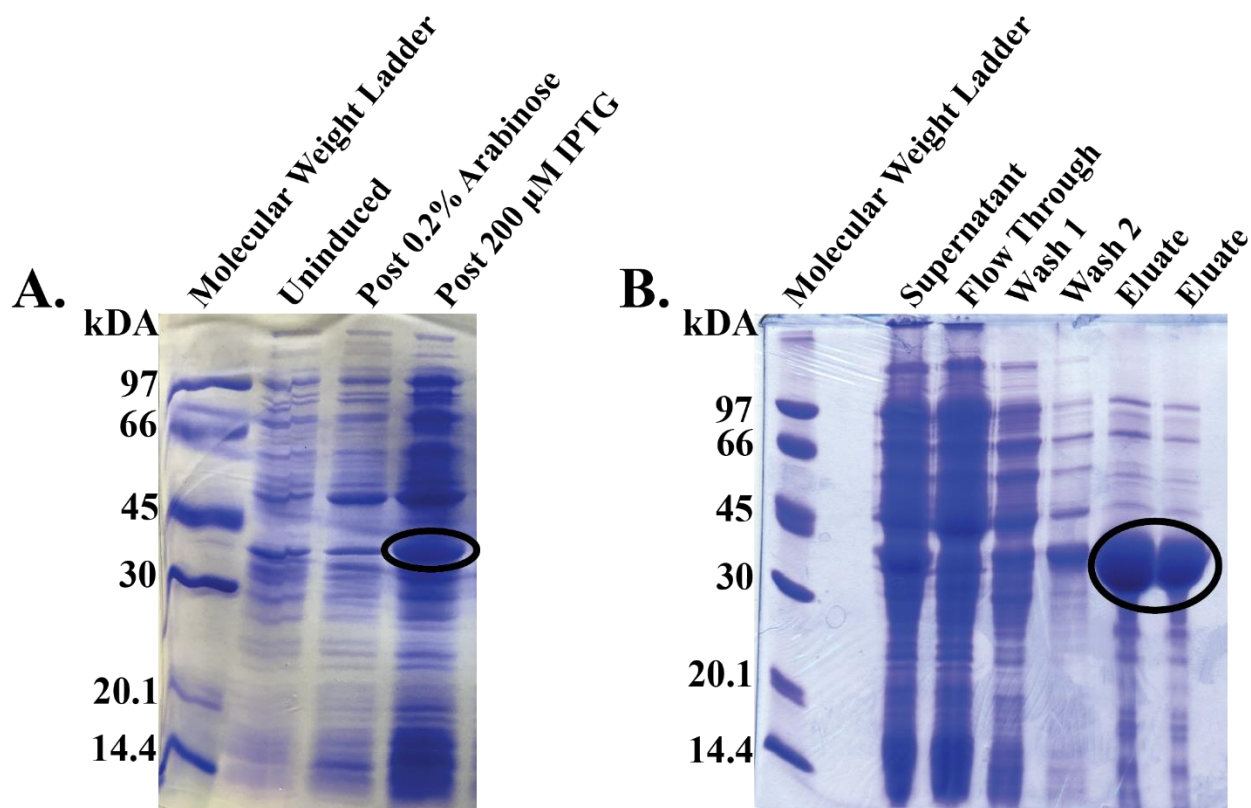


Figure 3.4. Overexpression and purification of the Y309F variant of *E. coli* LipA.

A. Protein expression gel. **B.** Protein purification gel.

The Y309F variant was the most active, the only variant to retain its catalytic function in the presence of NfuA (**Fig. 3.5B**). Without NfuA, the protein produced 12 μM product, but made ~ 120 μM product in the presence of NfuA. This result was unique to the LipA Y309F variant, so this assay was repeated and showed that neither of the two reaction conditions yielded more than ~ 1 μM product (**Fig. 3.5C**). These conflicting results could indicate a human error and would require further testing to be able to draw conclusions from this data.

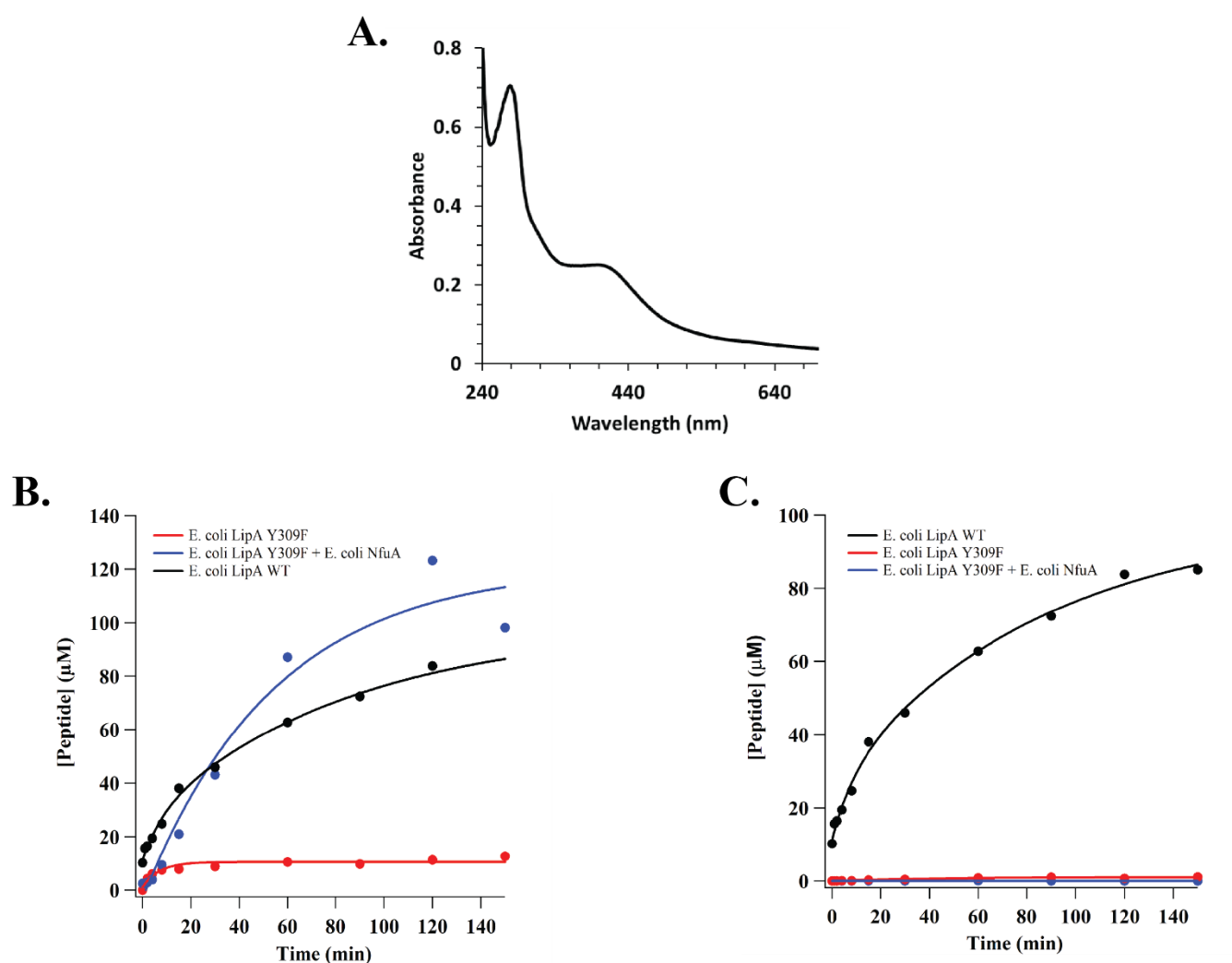


Figure 3.5. Characterization of the Y309F variant of *E. coli* LipA.

A. UV visible spectrum. **B.** Activity assays of the Y309F variant comparing the concentration of product made between the Y309F variant, the S308A variant with NfuA, and the wildtype LipA. **C.** Repeat of the Y309F activity assays. 200 μ M of WT and Y309F LipA were reacted with 400 μ M of NfuA and 300 μ M of substrate in the respective assays.

Discussion

The RSSY motif is almost universal in lipoyl synthases, where R306 and S308 are strictly conserved. To understand the importance of these residues to turnover, single amino acid substitutions were engineered into LipA and the resulting variants were tested for activity. All of the variants were inactive except the Y309F variant, which was corroborated by the results of a former graduate student.¹ The arginine at position 306 is necessary for substrate binding, while the serine at position 308 coordinates the auxiliary cluster. Theoretically, these functions are important, which could explain why the R306K and S308A variants were inactive.

Although three of the four variants did not retain enzymatic activity, it is important to note that each of the variants retained the ability to coordinate a cluster. This characteristic suggests that as long as three of the four residues are intact, the enzyme can still coordinate a cluster. Because LipA contains two [4Fe-4S] clusters, the UV vis data may be from the radical SAM cluster and not the auxiliary cluster, so it is possible that making double mutants may cause the enzyme to lose this function. This also suggests that there may be other explanations for the functions of the individual residues, as only the arginine and serine at position 308 is strictly conserved, or the possibility that the *in vitro* conditions do not provide enough biological context

to be able to observed the effects of the mutations, in terms of their previously described functions in LipA.

Despite the fact that the variants appear to coordinate [4Fe-4S] clusters, UV-vis data show that the variants did not coordinate a lot of iron. It would be interesting to examine the behavior of the proteins if their clusters were reconstituted. For further investigation into this motif, crystallization of these variants with and without NfuA could show how the variant interacts with the cluster carrier protein providing insight into the functions of the serine at position 307 and the tyrosine at 309, which remain unknown.

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6. Devasagayam TP, et al., Prevention of singlet oxygen-induced DNA damage by lipoate. *Chem Biol Interact*, 1993. **86**(1): 79 – 92.
7. Beinert H, Micro methods for the quantitative determination of iron and copper in biological material. *Methods Enzymol*, 1978, **54**: 435 – 445.
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Chapter 4

The function of the *Escherichia coli* proteins ybeD and ybeF

Introduction

The *lip* locus of *E. coli* houses genes involved in the endogenous biosynthesis of lipoic acid, such as *lipA* and *lipB*.¹ The gene for *ybeD* exists within the intergenic sequence of *dacA* and *lipB*, while that of *ybeF* is found between the *lipA* and *lipB* genes (**Figure 4.1**).¹



Figure 4.1. Map of *E. coli* Lip Operon.

The gene for *ybeD* exists within the intergenic sequence of the D-alanyl-D-alanine carboxypeptidase, *dacA*, and *LipB*, while that of *ybeF* is found between the *LipA* and *LipB* genes

YbeF is a 36 kDa protein with an N-terminal DNA-binding helix-turn-helix motif, which has led to the hypothesis that the protein is a LysR-type transcriptional regulator.² LysR-type regulators are the most numerous bacterial regulators and are involved in the regulation of virulence, motility, quorum sensing, and metabolism genes.³ These regulators are defined by their conserved N-terminal DNA-binding helix-turn-helix and C-terminal co-inducer binding domain.^{4,3} Using nuclear magnetic resonance, the structure of the 9 kDa protein, YbeD, showed structural similarity to ACT domains.² These domains have been found in proteins that regulate bacterial

metabolism, which may implicate *ybeD* in the regulation of lipoic acid biosynthesis.⁵ The sequences of both proteins are shown below in Figure 4.2.^{6,7}

A.

<i>E. coli ybeD</i>	
1	MKTKLNELLEFPFPFTYKVMGQALPELVDQVVEVQRHAPGDYTPTVKPS
51	SKGNYHSVSITINATHIEQVETLYEELGKIDIVRMVL

B.

<i>E. coli ybeF</i>	
1	MDSNNQIEPCLSRKSSEGKQIFTLRNIDLNLLTIFEAVYVHKGIVNAA
51	KVLNLTPSAISQSIQKLRFVFPDPLFIRKGGQVTP TAFAMHLHEYISQGL
101	ESILGALDIEGSYDKQRTITIATTPSVGALVLPVIYRAIKTHYPQLLLRN
151	PPISDAENQLSQFQTDLIIDNMFCTNRTVQHHVLFDTNMLICREGNPLL
201	SLEDDRETIDNAAHVLLLPEEQNFSGLRQRVQEMFPDRQINFSTYNILTI
251	AALVANSMDLAIPSRFYNLFSRCWPLEKLPFPSLNEEQIDFSIHYNKFS
301	LRDPILHGVIVIRNAF

Figure 4.2. Amino Acid Sequence of *E. coli ybeD* and *ybeF*.

A. Amino acid sequence of *ybeD*. **B.** Amino acid sequence of *ybeF*.

Both *ybeF* and *ybeD* are conserved in bacteria, but their specific functions are yet to be discovered.^{1,8} Currently, it is unclear how, and if, *ybeD* and/or *ybeF* functions in relation to the lipoic acid biosynthetic genes. In an attempt to provide more insight into the biosynthesis of the lipoyl cofactor, it became of interest to determine if these proteins have an effect on *in vitro* lipoic acid production.

Materials and Methods

Materials – Restriction enzymes and materials for cloning were obtained from New England Biolabs (Ipswich, MA). DNA isolation kits were purchased from Machery-Nagel (Dueren, Germany). Deoxynucleotides for PCR amplification were from Denville Scientific Corporation

(South Plainfield, New Jersey). Kanamycin, arabinose, isopropyl β -D-thiogalactopyranoside (IPTG), tris (2-carboxyethyl) phosphine (TCEP), dithiothreitol, and ampicillin were from Gold Biotechnology (St. Louis, MO). Protein calibration standards, FeCl₃, L-cysteine, DNase I, β -mercaptoethanol (BME), pyridoxal 5'-phosphate (PLP), and sodium dithionite were purchased from Sigma Co. (St. Louis, MO). Ni-NTA resin for protein isolation was obtained from Qiagen. HEPES (sodium salt) and potassium chloride were from Dot Scientific (Burton, MI). Imidazole and lysozyme were from Alfa Aesar (Haverhill, MA). Coomassie brilliant blue dye and Bradford reagent were from Amresco (Fountain Parkway Solon, OH). Unlabeled Na₂S and bovine serum albumin (BSA) used for the Bradford standard were from Thermo Fisher Scientific (Waltham, MA). Materials used in liquid chromatography-mass spectrometry (LC-MS) assays, including the lipoyl, octanoyl, and 6-thiooctanoyl peptides, SAH nucleosidase, external standard peptide, and SAM have been previously described.^{9,10} All chemicals and reagents were of the highest grade available.

General procedures — UV-visible spectra were recorded on a Varian Cary 50 spectrometer (Walnut Creek, CA) using the WinUV software package. The Polymerase Chain Reaction (PCR) was performed using a Bio-Rad S1000 Thermal Cycler. LC-MS was conducted on an Agilent Technologies (Santa Clara, CA) 1200 system coupled to an Agilent Technologies 6410 QQQ mass spectrometer. The system was operated with the associated MassHunter software package, which was also used for data collection and analysis. Iron and sulfide content of proteins was measured using previously described methods.¹¹

Expression and Purification of Escherichia coli ybeD and ybeF — A single colony was used to inoculate a 200 mL starter culture of *Escherichia coli* BL21(DE3) cells containing pDB1282 and the desired gene construct (*E. coli lipA ybed*-pET28a and *E. coli lipA ybef*-pET28a). Expression was performed in 16 L (4 × 4 L) of M9 Minimal Media, preincubated at 37 °C. The cells were grown at 37 °C with shaking at 180 rpm. At an OD₆₀₀ of 0.3, *L*-(+)-arabinose was added to a final concentration of 0.2% to induce expression of genes on plasmid pDB1282. At an OD₆₀₀ of 0.6, FeCl₃ and IPTG were added to the cultures to final concentrations of 50 μM and 200 μM, respectively. The flasks were then chilled on ice for at least 30 min and shaken by hand every 5 min. The cultures were incubated overnight at 18 °C with shaking at 180 rpm. The following day, the cells were harvested by centrifugation at 7,000 × *g* for 15 min, and the resulting cell paste was flash-frozen in liquid nitrogen. Samples from each flask to be used for sodium-dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) were removed just before addition of arabinose, just before addition of IPTG and FeCl₃, and just before harvesting.

Proteins were purified anaerobically (<1 ppm O₂) in a Coy (Grass Lake, MI) anaerobic chamber with the exception of centrifugation steps. The cell pellet was re-suspended in 200 mL lysis buffer (50 mM HEPES, pH 7.5, 300 mM KCl, 20 mM imidazole, 10 mM BME, 1 mM *L*-cysteine, 0.1 mM PLP, 5 mM MgCl₂, 5 mM ATP), containing lysozyme (1 mg/mL), and DNase I (0.1 mg/mL). This mixture was stirred at room temperature for 30 min before lysing the cells by sonic disruption. The lysate was loaded into centrifuge bottles, which were tightly sealed before they were removed from the anaerobic chamber, and the lysate was centrifuged at 45,000 × *g* for 1 h and 4 °C. The supernatant was applied to a column of nickel-nitrilotriacetic acid (Ni-NTA) resin equilibrated in lysis buffer.¹² The column was washed with 200 mL wash buffer (50 mM HEPES, pH 7.5, 300 mM KCl, 40 mM imidazole, 10 mM BME, 10% glycerol) before eluting the

protein with elution buffer (50 mM HEPES, pH 7.5, 300 mM KCl, 250 mM imidazole, 10 mM BME, 10% glycerol). Fractions were collected based on brown color, and the pooled fractions were concentrated using an Amicon centrifugal filtration device fitted with a 30 KDa molecular weight cut-off (MWCO). The protein was then exchanged into storage buffer (100 mM HEPES pH 7.5, 300 mM KCl, 1 mM dithiothreitol (DTT), 20% glycerol) using a PD-10 column (GE Healthcare Life Sciences). The protein was aliquoted and flash-frozen in liquid N₂ and stored in liquid N₂ until further use. Samples of the pellet, supernatant, flow-through, wash and elution were analyzed by SDS-PAGE gel to assess protein purity. Protein concentration was determined using the Bradford method with bovine serum albumin (BSA) as a standard.¹³

LipA activity determinations—LipA activity was determined under anaerobic conditions in reaction mixtures containing the following in a final volume 210 µL: 50 mM HEPES, pH 7.5, 300 mM KCL, 10% glycerol, 300 µM peptide substrate (Glu-Ser-Val-[N⁶-octanoyl]Lys-Ala-Ala-Ser-Asp), 200 µM *E. coli* LipA, 0.5 µM S-adenosylhomocysteine (SAH) nucleosidase, 1 mM SAM, and 400 µM *E. coli* YbeD and/or *E. coli* NfuA when appropriate. Reactions were performed at room temperature, and were initiated by addition of sodium dithionite to a final concentration of 2 mM. Reactions were quenched at 0, 1, 2, 4, 8, 15, 30, 60, 90, 120, and 150 min in a final concentration of 100 mM H₂SO₄/4 mM TCEP/100 µM tryptophan/20 µM AtsA peptide (internal standards). Detection of substrates and products was performed using LC-MS.

Results

E. coli YbeD protein does not increase E. coli LipA activity.

YbeD was over-expressed and purified to $\geq 95\%$ homogeneity by IMAC resulting in a very low yield of 60 mg of protein from 16 L of cell culture (**Fig. 4.3A**). Using Bradford analysis, the concentration of this protein was 1.4 mM. The UV-vis spectrum showed no features of an enzyme that coordinated an iron-sulfur cluster (**Fig. 4.3B**). The eluate was transparent, and therefore, an iron analysis was not performed. Product concentrations in assays containing YbeD were comparable to those without the addition of the protein. The activity assay revealed that YbeD did not enhance LipA's turnover (**Fig. 4.1C**).

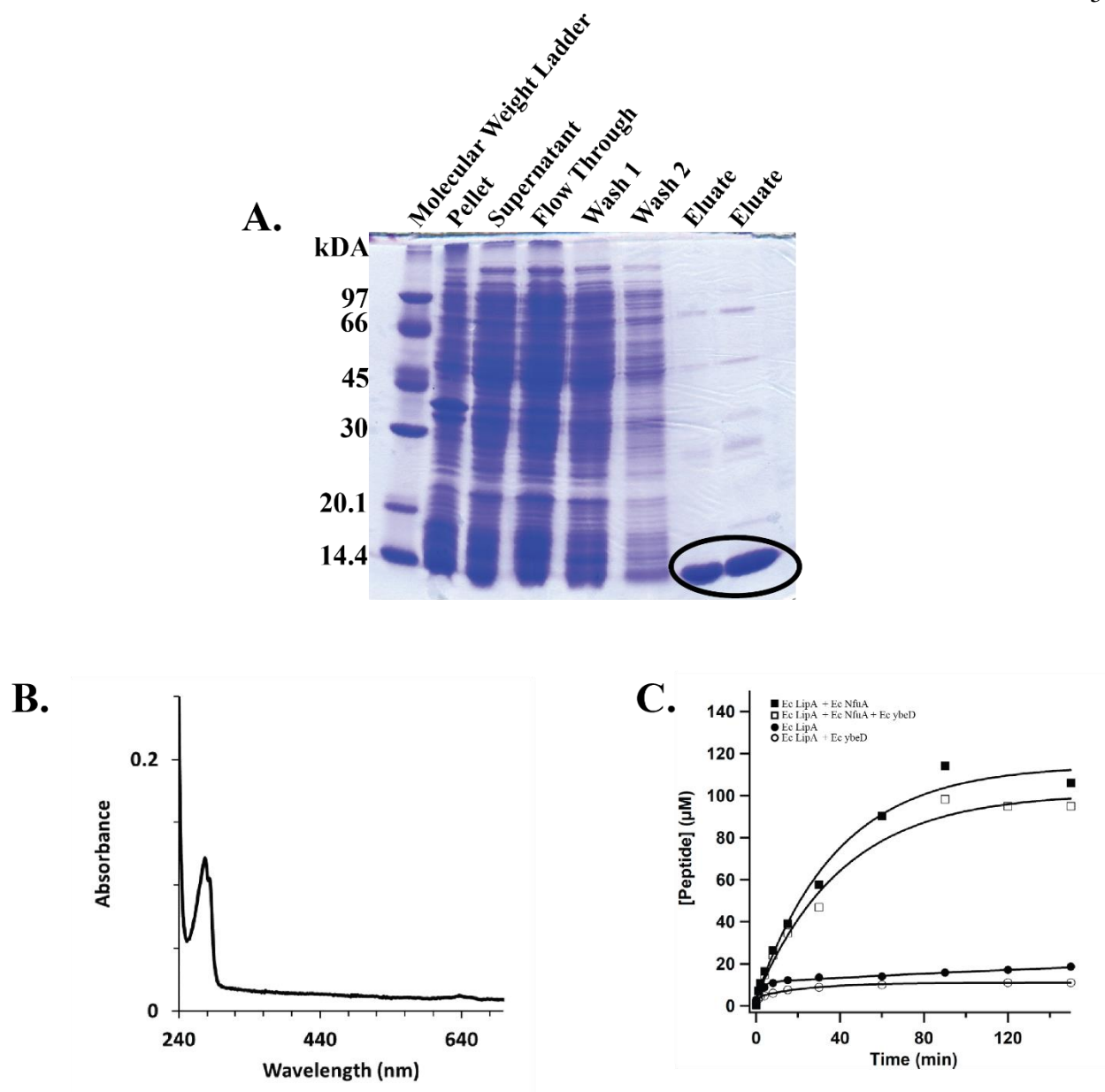


Figure 4.3. Characterization of *E. coli* protein ybeD.

A. Protein purification gel. **B.** UV visible spectrum. **C.** Activity assays comparing the concentration of product made by LipA in the presence of NufA, ybeD, or NfuA and ybeD. 200 μ M of LipA and 300 μ M of substrate were reacted with 400 μ M of NfuA and/or ybeD in the respective assays.

E. coli YbeF was insoluble.

The overexpression gel of YbeF shows an obvious increase in protein content (at the appropriate molecular weight) after the addition of IPTG, indicating that overexpression occurred (**Fig. 4.2A**). After attempting to purify the protein to $\geq 95\%$ homogeneity by IMAC, the protein was largely located in the pellet. This illustrates that the protein was insoluble and was largely contained in inclusion bodies in the cell (**Fig. 4.2B**). The effects of YbeF on *E. coli* LipA catalysis was not tested.

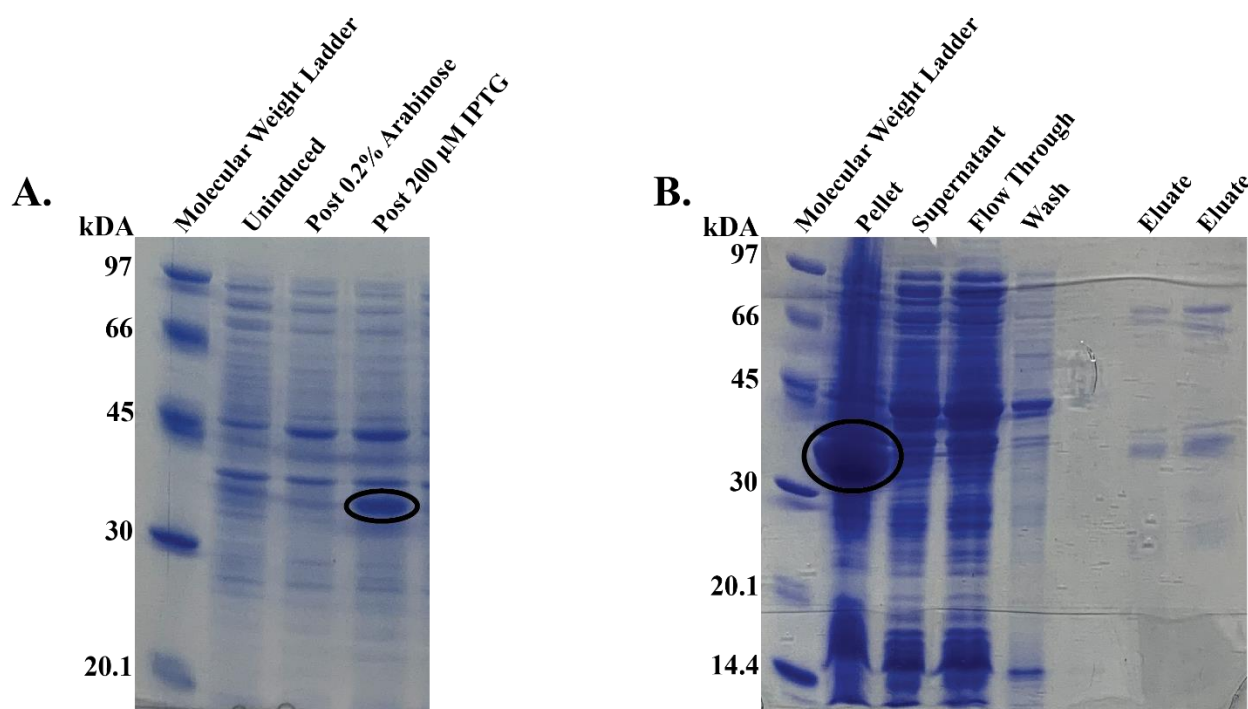


Figure 4.4. Overexpression and Purification of *E. coli* ybeF.

A. Overexpression SDS-PAGE. **B.** Purification SDS-PAGE.

Discussion

Due to the position of *ybeD* and *ybeF* in the *lipA/lipB* operon, it was thought that these proteins may be important to the function of LipA. In order to investigate the functions of the

proteins, they were characterized by their UV vis spectra and iron content to determine whether or not they are capable of coordinating a cluster. They were also tested for their ability to enhance product formation by LipA. Biochemically, it appears that the YbeD has no enhancement on LipA activity when compared to the LipA and LipA with NfuA controls. Due to issues of insolubility, YbeF was unable to be purified and therefore its effects on LipA catalysis remain unknown.

To further explore the interactions of LipA with YbeD and YbeF, it would be valuable to use the bacterial adenylate cyclase two-hybrid system (BACTH).¹⁴ This system exploits the activity of adenylate cyclase to determine if two proteins interact. Adenylate cyclase consists of two complementary domains that are needed for the enzyme's activity. To test the interaction between the two proteins, these domains are separated and fused to the genes of interests. If the two proteins interact, the two adenylate cyclase domains would be brought together and produce a functional adenylate cyclase, and subsequently produce cAMP. Cyclic AMP is then used to activate a promoter leading to the expression of a reporter gene. Additionally, molecular sieve chromatography could be used to assess whether either of the proteins, YbeD and/or YbeF, co-elute with LipA. These next directions for this project would help to uncover more about the function of *E. coli* YbeD and YbeF.

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10. Devasagayam TP, et al., Prevention of singlet oxygen-induced DNA damage by lipoate. *Chem Biol Interact*, 1993. **86**(1): 79 – 92.
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14. Olson MG, et al. A Bacterial Adenylate Cyclase-Based Two-Hybrid System Compatible with Gateway® Cloning. *Methods Mol Biol*, 2018. **1794**: 75 – 96.

ACADEMIC VITA

Education

The Pennsylvania State University

Bachelors of Science in Microbiology (May 2020)

Minor in French and Francophone Studies

Schreyer Honors College

Honors in Biochemistry and Molecular Biology

Millennium Scholars Program (Cohort 4)

Research Experience

The Pennsylvania State University

Undergraduate Researcher

University Park, PA

September 2017 – May 2020

Performing research in Dr. Squire J. Booker's Lab on the importance of strictly conserved amino acid residues on the function of the proteins LipA and NfuA in *Escherichia coli*.

BioMedRAP at Washington University in St. Louis

Undergraduate Researcher

St. Louis, MO

June 2019 – August 2019

Investigated the mechanism of action of a small molecule in *Mycobacterium smegmatis* under the supervision of Dr. Christina Stallings.

Amgen Scholar's Program at Columbia University

Undergraduate Researcher

New York, NY

May 2018 – August 2018

Conducted research in Dr. Jonathan Dworkin's Lab on the role of elongation factor P in growth and sporulation in *Bacillus subtilis*.

The Pennsylvania State University Chemistry REU

Undergraduate Researcher

University Park, PA

May 2017 – August 2017

Performed research in Dr. Squire J. Booker's Lab on the characterization of proteins involved in the *de novo* biosynthesis of the lipoyl cofactor in *Staphylococcus aureus*.

United States Department of Agriculture

Intern

Beltsville, MD

September 2015 – April 2016

Conducted research in the Food Quality Lab, with Dr. Tianbao Yang, on the effect of UV blocking membranes on the texture and quality of strawberries.

Publications

1. McCarthy, E. L., Rankin, A. N., Dill, Z. R., and Booker, S. J. (2018) The A-type domain in *Escherichia coli* NfuA is required for regenerating the auxiliary [4Fe–4S] cluster in *Escherichia coli* lipoyl synthase. *The Journal of Biological Chemistry*.

Professional Development and Presentation Experience

Annual Biomedical Research Conference for Minority Students (ABRCMS) *Anaheim, CA*
Undergraduate Poster Presenter *November 13 - 16, 2019*

Presented a poster on the mechanism of action of a small molecule in *Mycobacterium smegmatis*.

SACNAS National Diversity in STEM Conference *Honolulu, HI*
Undergraduate Poster Presenter *October 31 – November 2, 2019*

Presented a poster on the role of elongation factor P in growth and sporulation in *Bacillus subtilis*.

Washington University in St. Louis Summer Symposium *St. Louis, MO*
Undergraduate Poster Presenter *August 2, 2019*

Presented a poster on the mechanism of action of a small molecule in *Mycobacterium smegmatis*.

Grant Writing Course *St. Louis, MO*
Summer Intern *May 2019 – August 2019*

As an intern in the BioMedRAP program at Washington University in St. Louis, we were enrolled in a grant writing course where we were taught the techniques and skills needed to successfully write a grant, specifically the NSF GRFP.

Genentech Campus Engagement Day *South San Francisco, CA*
Undergraduate *November 9, 2018*

Visited Genentech, Inc. to participate in their Campus Engagement Day. Students are selected from thousands of applicants to visit Genentech and meet with hiring managers.

Annual Biomedical Research Conference for Minority Students (ABRCMS) *Phoenix, AZ*
Undergraduate Poster Presenter *November 1 – 4, 2017*

Presented a poster on the characterization of proteins involved in the *de novo* biosynthesis of the lipoyl cofactor in *Staphylococcus aureus*.

7th Annual American Chemical Society Local Section Poster Symposium *University Park, PA*
Undergraduate Poster Presenter *September 2017*

Presented a poster on the characterization of proteins involved in the *de novo* biosynthesis of the lipoyl cofactor in *Staphylococcus aureus*.

Lab Skills

Protein purification	Designing primers	Bradford analyses	Iron and Sulfide analyses
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Polymerase chain
reaction

Molecular Biology
Techniques

Microscopy

Microplate alamar blue
assay

Microbial culturing
techniques

Awards and Honors

Schreyer Honors College

Awarded: \$4,000

University Park, PA

July 2016 – May 2020

A merit-based honors college whose mission is to recruit and develop students who want to identify and solve problems, who want to make their communities and the world a better place

Penn State Millennium Scholars Program

Awarded a full scholarship to the Pennsylvania State University

University Park, PA

July 2016 – May 2020

A merit-based scholarship program designed to prepare students for the pursuit of doctoral degrees in science, technology, engineering and mathematics (STEM) disciplines.

Phi Beta Kappa Honors Society

Networking and professional development opportunities

April 26, 2020

Induction to this honors society is awarded to student who demonstrated academic excellence and outstanding leadership qualities.

Stan Latta Dedication Scholarship

Awarded: \$1,000

University Park, PA

April 24, 2020

This award provides recognition and financial assistance to an outstanding senior undergraduate student enrolled at Penn State who has demonstrated service to a student organization and the campus community, initiated programming efforts to enhance the out of class experience for students, has been actively involved in a wide array of co-curricular activities, and has displayed strong leadership behaviors and encouraged leadership development among others.

National Science Foundation Graduate Research Fellowship

Awarded: \$34,000

March 30, 2020

The program recognizes and supports outstanding graduate students in NSF-supported science, technology, engineering, and mathematics disciplines who are pursuing research-based master's and doctoral degrees at accredited United States institutions.

Penn State Biochemistry and Molecular Biology Department Microbiology Student Marshal

University Park, PA

May 2020

An honor reserved for the top graduating student in the Microbiology major. His or Her duty is to represent the Microbiology graduates at graduation.

National Association for the Advancement of Color People Penn State University Star Award

February 14, 2020

This award was given to six student, faculty, and staff members who exemplify dedication to improving the Penn State community.

Forum on Black Affairs Fannie Lou Hamer-W.E.B. DuBois Service Scholarship

Awarded: \$1,000

January 15, 2020

The Fannie Lou Hamer-W.E.B. DuBois Service Scholarship is the awarded to two students who serve the Penn State community and identify and propose a solution to a problem that exists in the local, national, or global community.

SACNAS Travel Scholarship

Awarded: \$500

October 30, 2019

This award covers the cost of housing for four days and four nights at a hotel in downtown Honolulu for the SACNAS National Diversity in STEM Conference.

Goldwater Scholarship

Awarded: \$7,500

April 26, 2019

Out of at national pool, only the top 10% of the applicants were named awardees. This scholarship is awarded to college sophomores and juniors who intend to pursue research careers in the natural sciences, mathematics and engineering and demonstrate knowledge of their field and a commitment to basic science research.

Phi Kappa Phi Honors Society

Networking and professional development opportunities

April 7, 2019

Induction to this honors society is awarded to student who demonstrated academic excellence and outstanding leadership qualities.

Evan Pugh Scholar Award

Awarded Twice

March 31, 2019 and April 15, 2018

The Evan Pugh scholars are juniors and seniors who are in the upper 0.5 percent of their respective classes and have completed at least 48 graded Penn State credits at the end of the fall semester of the academic year the award is given.

ABRCMS 2017 Presentation Award

Awarded: \$300

November 4, 2017

Recipients were presented with this award in recognition of their outstanding presentation at the Annual Biomedical Research Conference for Minority Students.

Undergraduate Conference Travel Support

Awarded: \$525

November 2017

The Eberly College of Science and the Biochemistry and Molecular Biology Department funded the costs of registration at the Annual Biomedical Research Conference for Minority Students.

ABRCMS Student Travel Award

Award Valued at \$1,500

September 2017

This award covers the cost of housing for five days and four nights at a hotel in downtown Phoenix and round-trip airfare.

Leadership Experiences

The National Council of Negro Women

President

University Park, PA

May 2019 – May 2020

Penn State's chapter of NCNW aims to increase the engagement of black women on campus by promoting success, community, and support amongst its members. NCNW's national mission is to lead, develop, and advocate for women of African descent as they support their families and communities. It is my duty to be the spokesperson for the organization, to preside at all meetings, to maintain cooperative relationships with other offices, and to oversee all Executive Board members as well as all activities and programs.

Island Fever (Caribbean Student Association Dance Team)

Captain

University Park, PA

May 2019 – May 2020

Island Fever brings the culture and traditions of the Caribbean to Penn State in a unique way through performances on and off campus, workshops, and education on the cultures of the Caribbean. The role of the captain includes running practices, coordinating events, and making administrative decisions for the success of the team.

Millennium Scholar Mentor

Mentor

University Park, PA

August 2018 – May 2020

Millennium Scholar mentors guide freshmen and sophomores through their first two years in school. Mentors offer academic and emotional support to students as they transition into college life. They provide guidance on academic research, applying to summer research experiences, academic support, and adjusting to new surroundings.

Millennium Scholars Program Tutor

Student Employee

University Park, PA

August 2017 – May 2020

As a tutor for the Millennium Scholars Program, I tutor fellow Millennium Scholars in the areas of general Chemistry and Physics, as well as Calculus.

Science Lion Pride

Tour Guide and College Ambassador

University Park, PA

September 2016 – May 2020

A student ambassador group representing the Eberly College of Science to prospective students, current students, parents, and alumni

The National Organization for the Professional Advancement of Black Chemists and Chemical Engineers

University Park, PA

Web Team Coordinator

September 2017 – May 2019

The Penn State chapter of NOBCChE aims to create a professional space for minority students in STEM. The duties of the web team coordinator include: managing the website, keeping the social media pages up to date, promote events, and design advertisements.

General Skills

French (written,
conversational, reading
comprehension)

Microsoft Office 365

Mac and PC OS Systems

