

GAINING INSIGHT INTO THE MATURATION OF IRON-SULFUR (FES) CLUSTERS IN
STAPHYLOCOCCUS AUREUS BY DETERMINING THE ROLE OF DUF59 PROTEIN, SUFT

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ABSTRACT OF THE THESIS

Gaining insight into the maturation of Iron-Sulfur (FeS) clusters in *Staphylococcus aureus* by determining the role of DUF59 protein, SufT

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Staphylococcus aureus is a serious mammalian pathogen. For *Staphylococcus aureus* to successfully inflict pathogenesis upon a host, it is imperative for it to acquire and effectively utilize iron (Fe). Once Fe is internalized, *S. aureus* utilizes the SUF system to assemble small inorganic cofactors called iron-sulfur (FeS) clusters. FeS clusters have a wide variety of functions in cells, thus, defective FeS cluster assembly results in global metabolic defects. In addition, FeS cluster synthesis and the assembly of FeS proteins is essential in *S. aureus* and a number of alternate bacterial pathogens suggesting that it is a viable antimicrobial target. Proteins containing DUF59 domains have roles in FeS cluster assembly and are found throughout Eukarya, Bacteria and Archaea. However, the function of DUF59 remains unknown in *S. aureus*. We have identified the *S. aureus* SufT, which is composed solely of the DUF59 domain and demonstrated that it has a role in the maturation of iron-sulfur (FeS) proteins.

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DEDICATION

I'm dedicating this to my family. For my mother Rita, who worked hard to provide for her children; to my brother Naman, whose always there for me; to my love Karthy, who reminded me every day to complete my thesis and got me focused to complete it. Without all of your support, this would not have existed.

PREFACE

The following two publications have been previously completed and published. The work and data presented in this thesis were conducted prior to the publications and were used as a platform to build and test hypotheses that aided in studies examining the function of SufT. The work and data presented herein describes the results of experiments conducted by myself.

1. Mashruwala AA, Bhatt S, Poudel S, Boyd ES, Boyd JM (2016). The DUF59 Containing Protein SufT is Involved in the Maturation of Iron-Sulfur (FeS) Proteins during conditions of High FeS Cofactor Demand in *Staphylococcus aureus*. PLOS Genetics 12(8): e1006233. Doi:10.1371/journal.pgen.1006233. PMID: 27517714.
2. Mashruwala AA, Roberts CA, Bhatt S, May KL, Carroll RK, Shaw LN Boyd JM (2016). *Staphylococcus aureus* SufT: an essential iron-sulphur cluster assembly factor in cells experiencing a high-demand for lipoic acid. Mol Microbiol. 102(6): 1099-1119. Doi: 10.1111/mmi. 13539. PMID: 27671355.

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I. Introduction

Staphylococcus aureus is the number one cause of hospital-associated infections in the United States and recent statistics show that about one in three individuals carry *S. aureus* as a commensal[1]. Carriers of *S. aureus* are at an increased risk for infection and are the primary mode for the spread of *S. aureus* strains[2]. *S. aureus* is one of the leading causes of endocarditis, necrotizing pneumonia and mortality worldwide, and thus, poses a major health burden[3, 4]. With an increasingly dominant pressure upon our healthcare system, it is imperative that we find a long-term resolution to control antibiotic-resistant forms of *S. aureus* from taking additional lives.

Historically, *S. aureus* was naturally susceptible to almost all antibiotics, however, increased global antibiotic usage has generated increased antibiotic resistance in pathogenic organisms such as *S. aureus* through antibiotic selection[2]. An important attribute to successful pathogenesis by *S. aureus* is the capability to rapidly acquire resistance to multiple antimicrobials. *S. aureus* is known to readily acquire mobile genetic elements that provide resistance to antimicrobials. This is most evident by the introduction of methicillin in 1961, the first of the semisynthetic penicillinase-resistant penicillins, which was shortly followed that same year by the first isolate of methicillin-resistant *S. aureus* (MRSA)[5]. MRSA established worldwide dominance within 40 years of the first documented isolation and has become a widespread cause of community infections[6]. Community-associated MRSA is known to spread rapidly among healthy individuals and it is estimated that two out of every one hundred individuals are carriers of MRSA[1].

We as humans use two ways to protect ourselves from bacterial infections: the use of antibiotics and our immune system. The innate immune system, which is the first line of defense against invading bacteria employ polymorphonuclear leukocytes (PMNs) and macrophages which utilize reactive oxygen species (ROS) produced by NADPH oxidase and myeloperoxidase (MPO) in order to kill invading microbes[7]. Macrophages also have the ability to produce reactive nitrogen species (RNS) such as nitric oxide through the inducible nitric oxide synthase (*iNOS*)[8, 9]. Nitric oxide has been shown to have lethal effects on bacteria. Humans and mice that have genetic incapacities to produce either ROS or RNS are more susceptible to *S. aureus* infections[10, 11]. ROS produced by phagocytic cells includes superoxides, hypochlorous acid (HOCl), and hydrogen peroxide (H_2O_2), which create a difficult environment for bacteria to survive[12]. The body utilizes ROS as part of the immune response because oxygen can be extremely dangerous to bacterial organisms. Once a microbe is ingested by a leukocyte, the leukocyte produces a large burst of oxygen and rapidly takes up oxygen. Oxygen is deadly to microorganisms because internal iron catalyzes the reduction of oxygen by a single electron, resulting in oxidizing radicals. Oxygen radicals then dismutate spontaneously to produce hydrogen peroxide (H_2O_2) which can oxidize iron-sulfur (FeS) clusters resulting in the liberation of iron (Fe) from FeS clusters and inactivating cellular processes that require FeS proteins[13, 14].

Fe is an essential nutrient for virtually all life on earth as it is for *S. aureus*[15, 16]. Fe acquisition is crucial for successful colonization and infection by many bacterial pathogens such as *S. aureus*[17]. Fe^{2+} is readily oxidized by dioxygen resulting in an insoluble Fe^{3+} . Because of this, Fe is not readily accessible in oxygenic environments so

bacteria like *S. aureus* must use specific uptake systems to internalize Fe. Fe, although not freely soluble within the host, can be found on iron binding proteins (IBPs) such as transferrins, lactoferrins and ferritins in addition to protoporphyrins within haem and hemoproteins[18]. *S. aureus* has few mechanisms to obtain Fe from host heme such as hemolysin-dependent lysis of erythrocytes, uptake via staphyloferrin A and B and catecholamine stress hormones[19, 20]. *S. aureus* is also known to secrete siderophores, which are high Fe-affinity chelators that contribute to overall virulence and successful pathogenesis[21]. Once the ferric iron has been taken up passed the cellular membrane, challenges still exist to protect Fe from host peroxides and chelators[22]. Ferric iron is reduced to ferrous iron within *S. aureus* and is used to either metallate proteins that require Fe atoms for function or to build Fe-containing prosthetic groups such as the inorganic FeS clusters[23]. FeS clusters serve as cofactors of enzymes involved in broad cellular functions ranging from respiration to DNA replication. Failure to assemble FeS clusters can lead to metabolic disorders, metabolic paralysis and even cell death[24-26].

Bacterial FeS cluster assembly is made possible by three known synthetic systems: the nitrogen fixation (Nif), iron-sulfur cluster (ISC) and the sulfur formation (SUF) systems[27]. These systems are essential to obtain Fe and S atoms from their locations within the cell, to assemble them into an active cubane form and transfer them to apo-proteins. Only the SUF system has been found within *S. aureus*. The SUF system has been found in many organisms to activate under the presence of oxidative stress or Fe starvation, as a complement system to the basal operations of the ISC system found in *E. coli*[28]. Without mechanisms in place to effectively build FeS clusters in stressful environments, such as the human body, not only would respiration be impractical, but

pathogenesis would also be negligible since FeS cluster metabolism is essential for *S. aureus* respiration and pathogenesis[26].

It is currently known that the SUF system varies between organisms. Briefly, the key proteins involved in [Fe-S] assimilation and transfer include the following: the cysteine desulfurase SufS provides the sulfur[29]; SufU was found to be a sulfur carrier protein capable of activating SufS[30]. SufB, SufC and sometimes SufD function together as a scaffold to build FeS clusters. SufB serves as the molecular scaffold and SufC provides energy through ATP hydrolysis[31, 32]. The A-type transporter SufA transfers completed FeS clusters from SufB to target apo-proteins[33]. In *S. aureus*, the cluster carrier, Nfu, is also involved in the transfer of FeS clusters from scaffold to target apo-proteins[26].

We discovered that a protein encoded by SAUSA300_0875 (*sufT*) is composed almost entirely of the domain of unknown function 59 (DUF59), which, we also determined had a high degree of identity with *MT1513* of *Mycobacterium tuberculosis* *H37Rv*. Interestingly, *MT1513* is located at the end of an operon encoding FeS cluster biosynthetic genes. More specifically, this operon encodes for the SUF system. The function of *MT1513* is also unknown and is also composed of the DUF59 domain. Proteins containing DUF59 are important for FeS cluster assembly in eukaryotic cells. Cia2 (also identified as Fam96a/b or AE7) contains a DUF59 and is involved in cytosolic FeS cluster assembly[34, 35].

FeS cluster synthesis and FeS protein assembly are essential in *S. aureus*. The mechanisms by which *S. aureus* matures FeS proteins is biochemically distinct from the

mechanisms by which humans mature FeS proteins suggesting that these processes are excellent antimicrobial targets. Our research focuses on further understanding mechanisms for FeS protein maturation and FeS cluster transfer in *S. aureus*. The immune mechanisms of PMNs and macrophages act to disrupt cell growth and respiration, for which FeS clusters are essential, and thus, are direct targets of the immune response. A better understanding of how *S. aureus* maintains FeS maturation under stress may lead to effective medical therapies and antimicrobials used to combat *S. aureus* and possibly other deadly pathogens.

We propose here that the unknown SAUSA300_0875 is SufT and that it has a role in FeS cluster maturation. In support of this hypothesis, we applied a reverse genetic approach and conducted enzymatic analyses of several FeS cluster requiring enzymes and determined the effect resulting from a ΔsufT strain. We show here that SufT may directly or indirectly affect the FeS cluster availability by supplementing growth with TCA cycle byproducts that are produced by the enzymes requiring FeS clusters, thus, circumventing the effects that the ΔsufT strain had on FeS cluster maturity. We show that in a ΔsufT strain there are compromised Fe pools when compared to the wild type (WT) strain which may be causing the defect in FeS cluster maturation. We also show that the growth defects of a ΔsufT strain can be corrected by the addition of exogenous Fe. The ΔsufT strain displayed enhanced susceptibility to oxidative and nitrosative stressors suggesting that the absence of SufT may be making it more difficult for the mutant strain to build and/or transfer FeS clusters. We constructed *S. aureus* mutants that lack multiple FeS cluster maturation and transport proteins to assess growth and enzymatic activity to propose a possible role for SufT. We lastly conducted a bacterial-2-hybrid assay to see

possible protein-protein interactions with other FeS cluster maturation proteins and determined a mild level of interaction with key proteins involved in FeS cluster maturation.

II. Materials and Methods

Bacterial Strains and Growth Conditions

All experiments were performed in a community-associated *S. aureus* USA300_LAC (JMB1100), which was cured of its native plasmid that possessed resistance to erythromycin and utilized as the WT strain. Unless otherwise indicated, *S. aureus* was cultured in Tryptic Soy Broth (TSB) at 37°C by shaking at 200 rpm. Overnight cultures were allowed to grow for 16 to 18 hours, before reinoculation to ~0.1 OD (A_{595}) for further experimental analyses. Plasmid selection was done with antibiotics at the following concentrations: 150 µg/mL ampicillin; 30 µg/mL chloramphenicol; 10 µg/mL erythromycin; 3 µg/mL tetracycline; 125 µg/mL kanamycin; 150 µg/mL anhydrotetracycline. For plasmid maintenance, growth media was supplemented with 5 µg/mL erythromycin or 15 µg/mL of chloramphenicol.

For growth in *Staphylococcal* Defined Media and Fe-limited conditions, we prepared 100mL diH₂O containing 1g (NH₄)₂SO₄, 4.5g KH₂PO₄, 10.5g K₂HPO₄, 110mM NaCl, 30mM KCl, 50µg nicotinic acid, 50µg thiamine, 50µg pantothenic acid, 0.3µg biotin and 2.5mg of individual amino acids. Fe supplementation was then done by the addition of 120µM of FeCl₃ in 100mL of *Staphylococcal* defined media.

Anaerobic growth was conducted in 5mL aliquots of TSB media. Oxygen in the media was reduced by bubbling with nitrogen gas. Aliquots were made in 18 x 150mm Hungate tubes containing a butyl rubber stopper with a clamped aluminum seal. Where mentioned, media was supplemented with 2mM Nitrate. Reinoculations for growth were then done using a syringe to inoculate medium to a starting optical density of ~1.0 OD (A_{595}). Cultures were then grown at 37°C with 200 rpm shaking.

E. coli strains were cultured in Luria Broth (LB) medium at 37°C and shaking at 200 rpm. For plasmid maintenance, media was supplemented with 50 µg/mL ampicillin or 100 µg/mL spectinomycin.

Materials

Restriction enzymes, quick DNA ligase kit, deoxynucleotide triphosphates and Phusion DNA polymerase were purchased through New England Biolabs. DNase I was purchased through Ambion and Lysostaphin was purchased from Ambi products. Plasmid mini-prep kit and gel extraction kit were purchased from Qiagen. Oligonucleotides were purchased from Integrated DNA Technologies. Tryptic Soy broth (TSB) was purchased from MP biomedical and was added as (30 g/L) for liquid and solid medium. Agar was purchased from Fisher Scientific and was added as 1% (w/v) for solid medium. All other chemicals were purchased from Sigma-Aldrich and were of high purity, unless specified. Acetic Acid quantification kit was purchased from R-BioPharma.

Strain and Plasmid Construction

Escherichia coli DH5α was used as a host for cloning plasmid constructions. All clones were shuttled through RN4220[36], followed by transduction via phage 80α into appropriate *S. aureus* strains[37]. All *S. aureus* mutant strains and plasmids were verified using PCR or by sequencing PCR products or plasmids. All DNA sequencing was performed by Genewiz, South Plainfield, NJ.

Creation of plasmids and mutant strains. Approximately 500 base pairs upstream and downstream of *sufT* gene (*SAUSA300_0875*) were PCR amplified with oligonucleotide pairs 0875up5EcoRI and 0875up3NheI (upstream) and 0875dwn6MluI

and 0875dwn3BamHI (downstream), using JMB1100 as template. Amplicons were then gel purified and joined together using PCR and primers 0875up5EcoRI and 0875dwn3BamHI. The PCR product was then gel purified and digested with BamHI and SalI, followed by a ligation into similarly digested pJB38 yielding *pJB38_ΔsufT*. The plasmid was then transformed into *E. coli* DH5α. Transformed colonies were selected for ampicillin, for pJB38, and were screened by PCR for correct plasmids. The plasmid *pJB38_ΔsufT* was isolated and subsequently transformed into RN4220, selecting with chloramphenicol(Cm) for pJB38 at 30°C. Correctly verified colonies of plasmid *pJB38_ΔsufT* were then transduced into JMB1100 and inoculated into 5mL of TSB containing Cm, which were then allowed to grow at 42°C overnight for recombinant selection. Single colonies were then inoculated into 5mL of TSB medium and grown overnight, followed by a dilution of 1:25,000 before plating 100μl onto TSA containing anhydrotetracycline (Atet) for loss of plasmid screening. PCR was then conducted to screen for double recombination events, as well as screening for chloramphenicol sensitivity for loss of plasmid.

The *sufT::tetM* strain was created by digesting the *pJB38_ΔsufT* plasmid with MluI and NheI and inserting a *tetM* cassette between the upstream and downstream regions of *ΔsufT*. The resulting plasmid (*pJB38_sufT::tetM*) was transformed into *E.coli*, the plasmid was electroporated into RN4220 and the mutant was constructed as described above. Both *nfu::tetM* and *sufA::tetM* were created as previously described[26, 38]. The *ΔsufT_nfu::tetM* and *ΔsufT_sufA::tetM* were created by transducing strain JMB1146 (*ΔsufT*) with the *nfu::tetM* allele and *sufA::tetM* allele, respectively, by α80 phage and selecting for tetracycline (Tet) resistance. Furthermore, strains showing resistance for Tet, but sensitive to Cm were verified for deletion of *sufT*.

Complementing plasmids were constructed by subcloning digested PCR products into respective and similarly digested vectors or by using yeast homologous recombining cloning(YRC) as previously described[39]. The genes cloned into pCM28 or pLL39 contained native promoters. Genes which were cloned into pEPSA5 contained an engineered *sodM* ribosomal binding site as previously described[39].

The plasmid pCM11 was used to make transcriptional reporter plasmids[40]. *acnA* transcriptional fusion plasmid was created by amplifying 500bp upstream of the ribosomal binding site (RBS) of *acnA* and PCR products were digested. The digested PCR products were then ligated into a similarly digested pCM11 and transformed into a chemically competent *E. coli* DH5 α .

Affinity tagged strains were created by fusing the genetic sequence encoding the FLAG affinity tag sequence to the C- or N- terminus of the gene of interest on a plasmid. Plasmids were then transduced into parent strains.

Phenotypic analyses

Auxotrophic analyses, stress assays, and complementation data was generated in a 96-well plate using a BioTek 808E visible absorption spectrophotometer at 37°C with medium shake speed. For inoculation, cultures were first grown overnight in TSB (>18 hours). Cultures were then resuspended in a 1:4 dilution of overnight culture to sterile phosphate buffer solution. These were subsequently inoculated into 96-well plates to a approximate optical density of 0.1 OD (A_{595}), in fresh *Staphylococcal* chemically defined media, or TSB, with respective supplementations or restrictions and a final volume of 200 μ L. To reduce the amount of Fe and other nutrients being carried over from

overnight cultures grown in rich media, cells were washed two times using four times the volume of sterile PBS to the original overnight taken. Concentration for stressors used in phenotypic assays are as follows: 40 μ M 2,2-Dipyridyl, 62 μ M HOCl, and 1.2 mM Sodium Nitroprusside.

Top-agar overlays were created by inoculating 4 mL of molten 0.5% TSB-agar with 100 μ L of a 1:100 dilution of overnight culture and poured on top of 1% TSA. Plates were then allowed to cool for 30 minutes to allow for solidification of top-agar. Zone of inhibition assays were conducted by spotting 30 mM methyl viologen to the center of top-agar overlays. Plates were allowed to dry for 15 minutes and subsequently placed at 37°C for 16 hours before measuring the area of the zone of inhibited growth.

Transcriptional reporter fusion assays

Transcriptional reporter plasmids were constructed and assayed using the pCM11 or pXEN-1 plasmids[40]. Strains containing pCM11 or pXEN-1 were allowed to grow overnight in TSB supplemented with erythromycin. Cultures were then diluted (1:100) into fresh TSB to a final volume of 5 mL. 200 μ L of cell culture were removed and assayed at different time intervals to assay for OD, fluorescence and luminescence using a Perkin Elmer Spectrometer HTS 7000 plus bioassay plate reader. GFP excitation and emission were set to 485nm and 510nm respectively with a gain of 60. Data was then normalized with respective OD.

Enzyme Assays and Immunoblot Analyses

Cell extracts were prepared by growing up to desired OD before resuspension in buffer (25 mM Tris-citrate, 150 mM NaCl, pH 7.4). Cells were then lysed as previously

described[26]. Lysates were obtained and stored at -80°C protein concentration was quantified by Western Blot analyses.

Protein Concentration Determination. Protein concentration was determined using a copper/bicinchonic acid based colorimetric assay that was modified for a 96-well plate[41]. Bovine serum albumin (2 mg/mL) was used as a standard.

Western-Blot Analyses. 60 µg or 80 µg of total protein was loaded of pLL39 and pEPSA5 plasmids, respectively. Protein separation was conducted on a 12% SDS-PAGE gel. Proteins were then transferred to a PVDF membrane and probed with mouse anti-FLAG primary antibody (Sigma-Aldrich) (1:2000 dilution) and subsequently HRP conjugated secondary antibody (Bio-Rad) (1:12000 dilution). The blots were developed using chemiluminescent detection (ECL kit, Pierce). The blots were then scanned as high quality TIFF images and analyzed using ImageJ software[42].

Cell free extract enzymatic assays. Aconitase (AcnA) assays were conducted as previously described[26, 43]: 3 mL of fresh TSB was subcultured to 0.1 OD (A_{585}) from cultures grown overnight for 16-18 hours in TSB. The strains with *acnA* under the transcriptional control of the native promoter were then grown for 8 hours ~8.0 OD (A_{595}) and the strain with *acnA* under the transcriptional control of *xylO* were grown to an approximately 2.0 OD (A_{595}), prior to supplementation with 1% xylose. These cells were then allowed to continue growing up to 8.0 OD (A_{585}). Both sets were then harvested by centrifugation and cellular pellets were then allowed to equilibrate for 10 minutes in an anaerobic chamber before resuspension in 400 µL of anaerobic lysis buffer (25 mM Tris-citrate, 150 mM NaCl, pH 7.4). Cells were then lysed with a combination of 4 µg Lysostaphin and 8 µg DNase, incubating at 37°C until lysis was observed

(approximately 30 minutes). Cellular lysates were then obtained after 2 minutes of high-speed centrifugation for both activity analyses and protein abundance. 20 μL of lysate was added to 680 μL of lysis buffer containing 20 mM DL-isocitrate. AcnA activity was then determined by monitoring the static conversion of isocitrate to cis-aconitate by using a Beckman Coulter DU530 UV-Vis absorption spectrophotometer (cis-aconitate $\epsilon_{240\text{nm}} = 3.6\text{mM}^{-1}\text{ cm}^{-1}$)[43].

Isopropylmalate isomerase (LeuCD) assays were conducted as previously described[26]: 3 mL of TSB was subcultured to 0.1 OD (A_{595}) from overnight cultures grown for 16-18 hours in TSB. Cells were allowed to grow to 2.0 OD (A_{595}). Cultures were then supplemented with the addition of 1% xylose, and allowed to subsequently grow for another 3 hours before harvesting. Cell pellets were similarly obtained as previously mentioned for AcnA assays. LeuCD activity was determined by the addition of 20 μL of lysate to 680 μL of buffer (50 mM Tris, pH 8.0) containing 10 mM MgCl_2 , and 10 mM DL-Threo-3-isopropylmalic acid. LeuCD activity was assayed as a functional ability to convert 3-isopropylmalate to dimethylcitrate acid spectrophotometrically (dimethylcitrateconate $\epsilon_{235\text{nm}} = 4.35\text{mM}^{-1}\text{ cm}^{-1}$), as previously described[44, 45].

Dihydroxy-acid dehydratase (IlvD) assays were conducted as previously described[26]: Cultures were grown, harvested and lysed using the same method as LeuCD activity assay. IlvD activity was determined by the addition of 20 μL of cell free extract to a buffer containing 50 mM Tris (pH 8.0) supplemented with 10 mM MgCl_2 and 10 mM D,L-2,3-dihydroxy-isovalerate. Keto acid formation from D,L-2,3-dihydroxy-

isovalerate was monitored spectrophotometrically (keto acids $\epsilon_{240 \text{ nm}} = 0.19 \text{ mM}^{-1} \text{ cm}^{-1}$) to determine the activity of IlvD as previously described[46].

pH and Acetic Acid Quantification

pH was quantified using a calibrated Fisher Scientific Accumet AB15 pH mV Meter. 5 mL samples were collected every two hours and analyzed for OD. Samples were then spun down to remove cells from the medium. The cell-free medium was then analyzed for pH. Acetic acid concentration was determined using the Boehringer Mannheim / R-Biopharma Enzymatic BioAnalysis / Food Analysis kit for acetic acid quantification (Catalog Number: 10 148 261 035). The manufacturer instructions were followed for acetic acid quantification.

Bacterial-2-hybrid

Bacterial-2-hybrid was conducted as previously described[47]. The BioTek 808E was used to obtain kinetic plots of the ONPG hydrolysis vs. time at 37°C and medium shake speed in a 96-well plate. Plates were continually read until all ONPG hydrolysis was completed. The slope of the ONPG hydrolysis, determined as OD (A_{420}) over time, was used to determine the level of interaction. Negative controls, Alpha and CI encoded genes in pBR and pAC plasmids, respectively, were used to determine if a positive interaction existed. WT interaction was determined as a benchmark for strong interaction (+++).

III. Results and Discussion

The discovery of SufT.

The community-associated *S. aureus* USA300_LAC genome differs from the annotated genome of USA300_FPR3757 only by a few single nucleotide polymorphisms[48]. All experiments were performed in USA300_LAC, however, USA300_FPR3757 genome used was analyzed for the presence of additional FeS cluster assembly components. Genes encoding SufBCDS and Nfu were identified previously[26]. We identified an open reading frame (ORF) that is often associated with the *suf* operon in all three kingdoms. A high-degree of identity in genes composed of DUF59 was found between the organisms USA300_FPR3757 and *Mycobacterium Tuberculosis H37Rv* (SAUSA300_0875 and *MT1513*, respectively.)

Although many of the surrounding genes of the operon which SAUSA300_0875 exists are unknown, we found that in *M. tuberculosis H37Rv* *MT1513* was located on an operon encoding the SUF machinery. In addition, DUF59 has previously been implicated as Cia2 in eukaryotic cells involved in cytosolic FeS cluster assembly as mentioned above, however, the function of the DUF59 had yet to be determined. The SAUSA300_0875 was denoted as *sufT* because it was often found in operons with *sufS* as subsequently analyzed in other organisms. The comparisons of the two genomic locations encoding DUF59 (*sufT*) within USA300_LAC and *M. tuberculosis H37Rv* are depicted in Figure 1. FeS clusters are commonly coordinated to apo-proteins by cysteine residues[49]. The DUF59 was found to contain a single strictly conserved cysteine. These findings led us to test the hypothesis that SufT functions in FeS protein maturation in *S. aureus*.

A Δ *sufT* strain has diminished activity of FeS cluster-requiring enzyme AcnA

AcnA is a TCA cycle protein that requires an FeS cluster as a cofactor to function[43]. AcnA is required for the isomerization of citrate to isocitrate, which is the first committed step of the TCA cycle[43]. An *S. aureus* strain lacking *nfu*, a gene encoding an FeS cluster carrier, was found to have 50% AcnA activity of the WT strain[26]. The defect in an Δ *nfu* strain directly links the diminished activity of AcnA to the inability for effective FeS cluster transfer from the SufBCD scaffold to the AcnA apoenzyme[26]. These findings are consistent with the fact that strains defective in FeS cluster maturation and transport have reduced activity of AcnA.

We hypothesized that the Δ *sufT* strain would display a similar phenotype to the Δ *nfu* strain if SufT was involved in the maturation or transfer of FeS clusters. JMB3953 (parent), mutant JMB4374 (Δ *sufT*) and complement JMB4373 (Δ *sufT* *psufT*) strains were grown in rich media. AcnA activity within Δ *sufT* strain was ~60% the activity of WT. Furthermore, returning the *sufT* gene on a plasmid to the Δ *sufT* strain restored AcnA activity (Figure 2A). Decreased AcnA activity could be attributed to a number of physiological effects, including, decreased *acnA* transcription, decreased protein abundance, or defective FeS cluster assembly upon AcnA. To address these scenarios, AcnA abundance was first examined. The strains listed above were examined for AcnA protein abundance utilizing a C-terminal FLAG tagged *acnA_FLAG* as previously described[26]. The protein abundance was determined by Western Blot analyses and AcnA protein was found to accumulate in all strains even though Δ *sufT* was ~40% of the abundance (Figure 2B). To compensate for the decrease in protein abundance, the AcnA activity was normalized for the abundance found (Figure 2C). The data presented

suggest that decreased protein abundance was not a factor in the diminished enzymatic activity of AcnA. This further confirmed that the lower AcnA enzymatic activity is caused by a defect in the maturation of FeS clusters.

In vivo studies in *E. coli* have shown that AcnA activity is decreased during oxidative stress, the stability of AcnA increases, and thus, inactivation of apo-AcnA, mediates a post-transcriptional positive autoregulatory switch leading to a more stable *acnA* transcription[50]. Although no oxidative stressors were added to this experiment, this potentially could have resulted in diminished AcnA enzymatic activity for the Δ *sufT* strain. In order to examine if *acnA* transcriptional activity was the root cause of diminished AcnA activity, *acnA* activity was examined using a fluorescent reporter plasmid pCM11 with a genetic sequence encoding green fluorescent protein (GFP) under the transcriptional control of the *acnA* promoter. Fluorescence is directly correlated to *acnA* promoter activity, and thus, represents the *acnA* transcriptional activity. The fluorescence and absorbance of the strains WT (JMB2414) and Δ *sufT* (JMB2416) were monitored. The Δ *sufT* strain displayed an increased *acnA* promoter activity when compared to the WT strain (Figure 2D). Together, these results were indicative that AcnA existed in apo-form within the Δ *sufT* strain and thus, AcnA is affected because of the reduced availability of FeS clusters in a Δ *sufT* strain.

The Δ *sufT* strain has a general defect in enzymes requiring FeS clusters.

SufT may have been participating more broadly in FeS cluster metabolism, not just specifically to AcnA activity. We therefore decided to test other enzymes which require FeS cluster cofactors such as isopropylmalate isomerase (LeuCD) and dihydroxy-

acid dehydratase (IlvD). Branched chain amino acids (BCAA) biosynthesis requires enzymes LeuCD and IlvD. LeuCD and IlvD both require FeS clusters as cofactors for enzymatic function[38]. Their products lead to downstream formation of branched chain amino acids Isoleucine (Ile) and Leucine (Leu), respectively. Activity of either of the enzymes directly correlates with the quantity of BCAA produced; thus, the decreased activity of either enzyme would result in defective growth in the absence of BCAAs.

We examined the growth of WT (JMB1886), ΔsufT strain (JMB4258) and complement strain $\Delta\text{sufT psufT}$ (JMB4265) in chemically defined media with 19 of the 20 canonical amino acids. The ΔsufT strain was unable to grow in chemically defined medium in the absence of either Leu (Figure 3A) or Ile (Figure 3B). In order to verify that strains are properly growing in chemically defined media, strains were also grown alongside the test strains in 20 amino acid media for comparative analyses (Figure 3C). The ΔsufT strain showed similar growth in 20 AA medium when compared to WT. These results confirm that the *sufT* mutant strain of *S. aureus* is auxotrophic for both Leu and Ile. These findings suggested that the diminished enzymatic activities of IlvD and LeuCD were caused by the role of SufT in FeS metabolism.

In order to confirm whether the auxotrophies observed were a result of defective IlvD and LeuCD enzymes, we conducted enzymatic assays on strains grown in rich medium. Similarly to AcnA activity, we saw a decrease in the functional activities of both IlvD with a decrease in ~50% activity (Figure 4A) and LeuCD with a decrease in ~70% activity (Figure 4B) in a ΔsufT strain, when compared to both WT and complemented strains. In order for us to confirm that the decrease in activity of these enzymes weren't a direct cause of decreased protein abundance of the enzymatic

proteins, a Western Blot analyses for LeuCD was conducted in duplicate (Figure 4B top). WT levels were assumed as 100% and activity levels of ΔsufT strain and complement were plotted relative to WT activity (Figure 4C). Normalized data obtained show large defects in LeuCD activity (~60% of WT) and suggests that the ΔsufT strain defects in IlvD and LeuCD are not a result of decreased protein abundance, but suggestive that the defect is caused by the lack of FeS cluster related enzymatic function. These data also conferred with the AcnA activity presented earlier and both AcnA and LeuCD displayed ~40% decreased activity in a ΔsufT strain.

A ΔsufT strain is defective in intracellular Fe homeostasis.

The results obtained previously suggesting SufT has a role in FeS cluster metabolism warranted further investigation on determining how SufT alters FeS cluster availability. Previous studies have shown that *S. aureus* strains lacking FeS maturation proteins have altered Fe pools, and that the Fe availability directly impacts FeS cluster requiring enzymes[26, 38]. The Fe and electron donors for the Suf system are currently unknown. The decrease in FeS cluster-required enzymatic activity could be attributed to decreased Suf available Fe pools.

Since Fe availability is crucial in the maturation of FeS clusters, we decided to test if FeS cluster maturation was impacted by a diminished availability of Suf-accessible Fe pools in a ΔsufT strain. In order to test this, we first attempted to correct the amino acid auxotrophies exhibited in Figure 3. The Ile auxotrophy was chosen as a representative to test. We hypothesized that if SufT altered Fe availability, exogenous Fe supplementation to ΔsufT strain in a chemically defined media, without Ile, would

circumvent the defects observed earlier. WT (JMB1100) and ΔsufT strain (JMB1146) were grown in chemically defined media without Ile (19AA), with or without FeCl_2 supplementation. As previously noted, the ΔsufT strain was incapable of growth in the absence of Ile, however, with the supplementation of FeCl_2 , growth was restored to near WT levels (Figure 5A). These data suggest that there are two possible scenarios in a ΔsufT strain: either there is a diminished level of intracellular Fe in a ΔsufT strain or that SufT may play a role in Fe binding, uptake, or presentation to Suf machinery. The overabundance of Fe availability presented in this experiment may be circumventing the need for SufT in the mutant strain.

The cell permeable divalent metal chelator, 2,2-dipyridyl (DIP) has specificity for Fe[51]. If a ΔsufT strain has diminished Fe pools or plays a role in Fe binding, uptake or presentation to Suf machinery, then the ΔsufT strain was hypothesized to show exasperated growth defect when DIP is introduced to the growth media. We found that the growth rate of ΔsufT strain (JMB4258) was greatly diminished. The ΔsufT strain also displayed a lower final OD in comparison to both WT (JMB1886) and complemented (JMB4265) strains (Figure 5B). These results coincided with our previous findings in Figure 5A providing further proof that SufT must have a role in FeS maturation, and further, that it might have a role in Suf Fe availability.

The *isdB* gene encodes a protein involved in iron acquisition that is under transcriptional control of the ferric uptake regulator (Fur)[52]. Fur alters and inhibits gene transcription of *isdB* when bound to Fe[53-55]. Upon treatment of DIP, *isdB* promoter activity has been shown to increase[56]. We next analyzed the promoter activity of *isdB* in the ΔsufT and WT strains using the plasmid pXEN-1_isdBp. In this

construct, the *luxABCDE* genes, encoding for luciferase, are under the transcriptional control of *isdB*. This construct allows us to quantify Fur activity by monitoring luminescence as an inverse function of Fur activity. We first found that luminescence was increased in a Δfur strain, consistent with the concept that Fur is acting as a transcriptional repressor of *isdB* (data not shown). We hypothesized that if $\Delta sufT$ strain has overall diminished levels of intracellular Fe pools, then Fur will be unable to alter or inhibit gene transcription of *isdB*. We found that the $\Delta sufT$ strain (JMB4458) has greater *isdB* transcriptional activity than the WT (JMB4457) and complemented (JMB4459) strains (Figure 5C). These data suggested that SufT has a role in Fe homeostasis. It is possible that the absence of SufT results in either the decrease in total Fe load of the cell or it may result in a decrease of Fur-accessible and Suf-accessible Fe pools with the total Fe load of the cell remaining unchanged. This would suggest a role for SufT as an Fe trafficker. Unfortunately, the total Fe load between WT and $\Delta sufT$ was not quantified, and thus, conclusions of Fe load and accessibility could not directly be made.

It has been shown that an Δnfu strain has similar intracellular Fe load to the WT, but, the relative *isdB* transcriptional activity levels were lower than WT, suggesting increased Fur-accessible Fe pools[26]. The transcriptional activity of *isdB* was monitored in the WT (JMB4457), Δnfu (JMB3949) and $\Delta sufT$ (JMB4458) strains as a comparative analysis (Figure 5D). The $\Delta sufT$ strain did behave oppositely to Δnfu strain in terms of *isdB* promoter activity replicating previous findings that the Δnfu strain has an over-abundance of Fur accessible Fe as compared to WT and also that $\Delta sufT$ strain had diminished level of Fur accessible Fe. Both are shown to affect FeS cluster maturation possibly because in the Δnfu strain there is a defect in FeS maturation, making greater

availability of unincorporated intracellular Fe for Fur-accessible pools greater; however, in a ΔsufT strain, Fe availability is diminished, not only affecting the Fur-accessible pools but also the Suf-accessible pools.

The ΔsufT strain is sensitive to nitrosative stress and oxidative stress.

An important asset to host innate immunity is the capability for the production of a nitrogen monoxide radical known as nitric oxide. Nitric oxide is a causative agent of nitrosative stress[57]. Common genes with altered expression following nitric oxide exposure often include iron-homeostasis genes under the control of ferric uptake regulator (Fur), hypoxic or fermentative metabolism, and genes under the control of Staphylococcal respiratory regulator AB (SrrAB)[11]. Oxidative and nitrosative stress have previously been shown as potent inhibitors of strains devoid of FeS cluster machinery[58-60]. Notably, the presence of oxidative or nitrosative stress can lead to intracellular ROS and RNS, causing altered or damaged FeS clusters[24, 61]. Sodium nitroprusside is known to induce nitrosative stress by production of nitric oxide, and thus, would result in growth inhibition in a strain with a defect in FeS maturation. Strains WT (JMB1886), ΔsufT (JMB4258) and complement (JMB4265) were cultured with 1.2 mM sodium nitroprusside. The ΔsufT strain was found to have diminished growth in the presence of sodium nitroprusside when compared to WT and complement strains (Figure 6A). These results show that a ΔsufT strain has defects in growth rate and final OD when grown in the presence of nitric oxide stress thereby further suggesting that SufT is involved in the maturation of FeS clusters.

We hypothesized that ROS would inhibit the growth of a ΔsufT strain.

Neutrophils are known to undergo respiratory burst in the face of invading organisms. They discharge a large quantity of superoxide and H_2O_2 . Myeloperoxidase catalyzes the conversion of H_2O_2 and Cl^- to hypochlorous acid (HOCl)[62]. HOCl was used to test whether a ΔsufT strain would exhibit diminished physiological growth defects in the presence of ROS. The growth of the WT (JMB1886), ΔsufT (JMB4258) and complement (JMB4265) strains were examined after challenging them with HOCl. The ΔsufT strain had diminished growth rate after challenge with HOCl in comparison to the WT and complement strains (Figure 6B), further suggesting that SufT must have a role in maturation of FeS clusters.

A third method for testing the function of SufT within the maturation of FeS clusters was employed by examining the effect of superoxide on the growth of the ΔsufT strain. Superoxide is known to oxidize FeS clusters. We examined the potent molecule known to generate intracellular superoxides via redox cycling, methyl viologen[63]. Methyl viologen sensitivity was examined utilizing top-agar spotting analyses to view zone of inhibition. We hypothesized that ΔsufT strain would be inhibited in growth more than WT because the defects in FeS maturation would be unable to circumvent the effects of superoxide exposure. Our results (Figure 6C) showed that the ΔsufT strain was more sensitive to methyl viologen stress. These results suggested that in the absence of SufT, superoxide exposure has more pronounced effects on FeS maturation leading to diminished growth. These results further provided proof of the involvement of SufT in FeS cluster maturation.

SufT interacts with other proteins involved in FeS cluster assembly.

To further provide proof that SufT has involvement in FeS maturation, we hypothesized that SufT would interact with other known proteins involved with cluster biogenesis or transfer. Bacterial-2-hybrid analyses were conducted to determine if there are any identifiable protein-protein interactions as previously described[64]. The 96-well plate Miller method was employed to screen a library of various strains with a combination of FeS maturation machinery in order to determine interactions. Data suggested a mild interaction of SufT with SufA, SufU and SufB (Figure 7). These data are suggestive of an interaction between the FeS maturation proteins and SufT, and support the hypothesis that SufT plays a role in FeS protein maturation. It is possible that since SufT is shown here to interact with SufA cluster carrier, SufU sulfur carrier and SufB scaffold, that SufT may also be playing the role of a carrier or activator, although to what capacity seems unclear.

SufA and Nfu have been found to have functional redundancy and both serve as FeS cluster carriers[26]. Double mutants were created with ΔsufT , additionally devoid of either ΔsufA or Δnfu , and tested the strains for AcnA and LeuCD activity levels. The $\Delta\text{nfu} \Delta\text{sufT}$ double mutant and the $\Delta\text{sufA} \Delta\text{sufT}$ double mutant strains were created with an *acnA*-FLAG affinity tagged on a plasmid under the regulation of a promoter inducible by 1% volume of exogenous xylose. The native *acnA* gene was knocked out using transposon insertion in all strains and a non-induced parent strain was included as a control. Likewise, the native *leuCD* was also knocked out in certain strains with transposon insertion. We conducted enzymatic assays for both AcnA (Figure 8A) and LeuCD (Figure 8B). Data for Δnfu and ΔsufA single and double mutant pairs coincided

with previous work[26], acknowledging that the effects on FeS cluster requiring enzymes aren't amplified in the double mutant, and thus, the proteins may be playing synergistic role, but are not redundant to one another. In both ΔsufT double mutant strains, however, the reduction in observed AcnA activity was exacerbated when compared to the single mutant strains (Figure 8A). In the LeuCD enzymatic activity test, however, the ΔsufA ΔsufT double mutant showed only marginal decrease in activity when compared to the ΔsufT single mutant counterpart, whereas, the Δnfu ΔsufT double mutant strain activity was nearly undetectable (Figure 8B). Protein abundance was accounted for, as displayed, suggestive that AcnA protein abundance accumulation was not the root cause for reduced AcnA activity. These data indicate that SufT must either be directly or indirectly interacting and impacting the function of Nfu and SufA as implied by the amplified effects of the double mutants. Furthermore, the phenotypes observed in a Δnfu ΔsufT double mutant strain suggest that Nfu and SufT display synergistic activities.

The Δnfu ΔsufT strain phenocopies a ΔacnA strain.

Only partial interruption of FeS cluster metabolism has been observed in single mutant strains of Δnfu and ΔsufT , however, the Δnfu ΔsufT double mutant strain displays a phenotype that suggests complete inactivity of FeS cluster metabolism. Interestingly, the Δnfu ΔsufT double mutant strain displayed characteristics closely resembling an ΔacnA strain. We noticed that even when the Δnfu ΔsufT double mutant strain was grown in rich TSB media it showed major growth defect and amassed to a similar final OD as the ΔacnA strain. An ΔacnA strain has previously been shown to have an inactive TCA cycle, thus, inhibiting the post-exponential growth phase catabolism of acetate and resulting in premature entry into the stationary phase[65]. We hypothesized that an

inability to mature FeS clusters would yield to the inactive apo-Aconitase resulting in the abolishment of the TCA cycle in the $\Delta nfu \Delta sufT$ double mutant strain.

To show that the $\Delta nfu \Delta sufT$ double mutant strain has an inactive TCA cycle, we determined if there was build-up of acetic acid, as acetic acid catabolism would be rendered null if a mutant strain had an inactive TCA cycle. We hypothesized that if we monitored relative pH within the $\Delta nfu \Delta sufT$ double mutant strain, that we would see a decrease in overall pH followed by an inability to re-alkalinize the medium, similar to the expected physiological observation of the Δacn strain. In comparison, we hypothesized that we would see a drastic decrease in pH in the WT and single mutant strains, followed by an increase in pH or alkalization at the culmination of the TCA cycle. All mutant strains were compared, relative to their optical densities and pH of the cell cultures, as it would relate to the acetic acid production and catabolism as the cell enters the TCA cycle (Figure 9A). Consistent with our hypothesis, the WT and single mutants were all capable of re-alkalization, whereas the $\Delta nfu \Delta sufT$ double mutant and Δacn strains remained at a pH of ~6.2 suggesting an inactive TCA cycle.

Secondly, acetic acid was quantified to verify that the lower pH in the Δnfu and $\Delta sufT$ double mutant and $\Delta acnA$ strains were in fact due to an inactive TCA cycle and not because of any other outlying source of lowered pH. Strains were grown in rich media for the duration displayed and acetic acid was quantified. Both the $\Delta nfu \Delta sufT$ double mutant and Δacn strains remained steadily similar producing ~0.8g/L acetic acid with no evident of acetic acid catabolism (Figure 9B). The WT and single mutant strains both exhibited a production of ~0.8g/L acetic acid with a sharp catabolism of acetic acid down to negligible levels suggesting that the TCA cycle began at ~6 hours, coinciding with the

decrease in acetic acid. These data once again suggested that the $\Delta nfu \Delta sufT$ double mutant has a defect in TCA cycle function.

Fur positively regulates *acnA* expression under conditions of limited Fe availability[66]. In a Δnfu strain there is diminished *acnA* promoter activity because of the decreased flux through the TCA cycle and because of the elevated Fur accessible Fe pools[26]. To study transcription of *acnA*, we assessed the promoter activity of *acnA* utilizing transcriptional reporter plasmid *pCM11_acnA* containing *gfp* under the transcriptional control of the *acnA* promoter. In the $\Delta acnA$ strain with diminished TCA cycle function there was no *acnA* promoter activity (Figure 9C). The $\Delta sufA$ single and double mutant strains exhibited minimal change to WT. The $\Delta sufT$ strain displayed elevated *acnA* promoter activity presumably because of the diminished Fur accessible Fe pools. We observed the most pronounced effect in the promoter activity of *acnA* in a $\Delta nfu \Delta sufT$ double mutant strain. Unlike the $\Delta acnA$ strain the $\Delta nfu \Delta sufT$ double mutant strain was still capable of producing AcnA with an inactive TCA cycle, even though it was low in quantity. Just as the Δnfu strain displayed lower flux through the TCA cycle and therefore displayed lower *acnA* promoter activity, the $\Delta nfu \Delta sufT$ double mutant strain also had lower flux through the TCA cycle. Unlike the Δnfu strain however, the $\Delta nfu \Delta sufT$ double mutant strain had an incredibly high relative *acnA* promoter activity. This suggests that the $\Delta nfu \Delta sufT$ double mutant strain may be extremely starved for Fe, and that Fur is positively influencing AcnA activity.

The $\Delta nfu \Delta sufT$ double mutant strain grows similar to WT anaerobically.

Growth in an aerobic environment results in major growth defects for the $\Delta nfu \Delta sufT$ double mutant strain and suggested that these defects were resultant of the

inactivation of the TCA cycle. We hypothesized that if we inactivated WT TCA cycle, it would grow similar to the $\Delta nfu \Delta sufT$ double mutant strain. Since *S. aureus* is a facultative anaerobe, we decided to examine how the $\Delta nfu \Delta sufT$ double mutant strain would grow anaerobically, with and without an exogenous terminal electron acceptor forcing the cell to ferment. In Figure 10A, we demonstrate the pronounced defect of the $\Delta nfu \Delta sufT$ double mutant strain when compared to the WT in the aerobic environment. However, the $\Delta nfu \Delta sufT$ double mutant strain grew similarly to WT anaerobically without the presence of an electron acceptor (Figure 10C). When the strains were grown in an anaerobic rich medium with nitrate, an alternate terminal electron acceptor, the $\Delta nfu \Delta sufT$ double mutant strain grew slightly better than the WT strain (Figure 10B). There are two possibilities for these results, either the $\Delta nfu \Delta sufT$ double mutant strain is strictly resulting in an inactivation of the TCA cycle by the inability to form FeS clusters, or, in aerobic conditions, the Δnfu and $\Delta sufT$ double mutant strain is unable to confer protection from the deleterious effects of oxygen resulting in the inability to mature FeS clusters. In anoxic conditions, FeS cluster cofactors are able to maintain cluster formation for utilization, and in presence of nitrate, for nitrate reduction[67]. These findings require further study to determine why nitrate addition allowed for more optimal growth in the $\Delta nfu \Delta sufT$ double mutant strain in comparison to WT.

The $\Delta nfu \Delta sufT$ double mutant strain is partially corrected by exogenous addition of lipoic acid and short-chain fatty acids.

Lipoic acid synthase (LipA) is a member of the SAM-radical family of enzymes which requires FeS clusters as cofactors for enzymatic activity, therein, synthesizing lipoic acid (LA)[68]. LA is necessary for pyruvate dehydrogenase activity, which is

necessary for carbon entry into the TCA cycle. Our data above suggested that the Δnfu $\Delta sufT$ double mutant strain has serious defects in FeS cluster maturation, and thus, we hypothesized that the organism may have negligible levels of LA production. We also hypothesized that growth limitation due to LA availability could be circumvented by exogenous addition of LA. The WT and Δnfu $\Delta sufT$ double mutant strain were grown in chemically defined media containing 20AA with and without LA. As previously observed, the Δnfu $\Delta sufT$ double mutant strain was unable to grow in chemically defined media containing 20AA. LA supplementation supported growth to a lower final OD than WT suggesting that Δnfu $\Delta sufT$ double mutant strain has a decreased production of LA.

The branched chain keto-acid dehydrogenase (Bck) is essential for the synthesis of short-chain fatty acids (SCFAs) and defects in Bck activity result in growth defects within media lacking SCFAs [69]. Bck activity is dependent upon the BCAA catabolism of Ile, Leu and Valine and requires LA for function[70]. We hypothesized that SCFA supplementation could remediate partial growth opportunity in the Δnfu $\Delta sufT$ double mutant strain in chemically defined media with 20AA. The Δnfu $\Delta sufT$ double mutant strain was unable to grow in chemically defined media with 20AA, however, with the supplementation of 100mM SCFAs, growth was partially corrected. This suggested that in a Δnfu $\Delta sufT$ double mutant strain, Bck was not active and furthermore that this defect could be bypassed by adding Bck product (SCFAs) into the chemically defined growth media.

IV. Conclusion

The underlying goal of this work was to investigate additional mechanisms involved in FeS cluster metabolism in *Staphylococcus aureus* that aid the pathogen in surviving oxidative and nitrosative stress at the host-pathogen interface. Herein, we first describe and hone in on the potential role for SAUSA300_0875 (*sufT*), and to an extension, the DUF59, in FeS protein maturation in *S. aureus*. This particular gene is located on an ORF that had no known function prior to this work. Conserved domain analyses shows that SAUSA300_0875 is closely related to PaaD family member of genes that encodes putative FeS cluster transfer and biogenesis genes. Additionally, the DUF59 domain has been linked as a requirement for the maturation of FeS cluster proteins in the cytosol and chloroplast of yeast Cia2A and Cia2B proteins and plant HCF101 protein respectively[34, 71]. Thus, based on the information available, we felt that the DUF59 in *S. aureus* played a role in FeS protein maturation. Furthermore, we felt that even though this was not the case for SAUSA300, based on the fact that DUF59 was often localized on an operon near *sufS*, that we could call the DUF59 of SAUSA300 SufT henceforth. Since the work presented in this thesis, an analysis was conducted and determined that SufT was recruited and retained in the operon multiple times throughout evolutionary history and only less than 9% of the genomes with *sufT* lacked scaffold *sufBC* genes[72].

We found that an *S. aureus* Δ *sufT* strain has deficiency in AcnA, LeuCD and IlvD. These enzymes are respectively crucial for the TCA cycle and are involved in formation of essential amino acids. Furthermore, these enzymes all require an FeS cluster cofactor for function and previous studies showing a deficiency in these three enzymes have linked their deficiency to inability for effective FeS cluster maturation[26,

38]. These results were consistent with the hypothesis that SufT participates in FeS cluster metabolism.

We utilized the ΔsufT strain and applied a reverse genetic approach to determine phenotypic disabilities. Our findings discovered amino acid auxotrophies, specifically those produced by FeS cluster requiring enzymes. Once again, confirming our hypothesis that SufT participates in FeS cluster metabolism. It also seems plausible, from this data, that SufT has redundant or conditional protein counterparts that compensate for the absence of SufT and allow a ΔsufT strain to grow under favorable conditions, but make SufT essential under nutrient limited conditions. It also seems plausible that SufT is dispensable during favorable nutrient rich conditions, and essential during nutrient limited conditions. After work presented here was concluded, the SufT was found to be conditionally required during high demand for FeS clusters, and not required when demand was low[72].

To understand reasoning behind the pronounced growth defects in a ΔsufT strain, we attempted to determine the reason for why FeS cluster biogenesis was defective in the ΔsufT strain. FeS cluster biogenesis can be linked to either an inability to assimilate Fe, the lack of Fe or S availability, or the inability to transfer FeS clusters to target proteins as seen in carrier proteins such as Nfu[26]. We discovered that phenotypic growth defects in the absence of Ile could be corrected with the addition of FeCl_3 suggesting that Suf-available Fe pools were diminished in the absence of SufT. This led us to confirm our findings by growing ΔsufT strain in the presence of Fe chelator, DIP, suggesting a heightened sensitivity to chelator, and thus, a lower availability of Fe pools. Further accessible Fe pools were also diminished in a ΔsufT strain and results were conferred

with those seen previously in Nfu[26]. It is possible that SufT directly impacts Fur-accessible Fe pools, thus, it wouldn't be exclusive to Suf metabolism. It is important to note also that an Fe donor has not yet been determined for the Suf system. Data presented herein suggests that SufT imparts some effect on the Fe pools, however, more studies would have to be conducted to determine the mechanism. Although we did not determine the overall load of Fe in a ΔsufT strain in comparison to WT, this would have helped us to determine if SufT is involved in either uptake or trafficking of Fe. If the overall load was determined to be less, SufT may have a role in total Fe load of the cell.

Defects in FeS cluster maturation have previously been linked to pronounced phenotypic effects in the presence of oxidative and nitrosative stressors[26]. Defects in growth of ΔsufT strain were observed in the presence of oxidative and nitrosative stressors. Zone of inhibition analysis was utilized to display greater inhibitory effects on a ΔsufT strain to superoxide when compared to WT and complement strains. It is important to note that not all oxidative and nitrosative stressors displayed phenotypic effects (data not shown). This suggests that further analyses must be conducted to see what classes of stressors attenuate growth of ΔsufT strain and which of those don't have an effect and to elucidate the conditionality of the role of SufT.

Our Bacterial-2-Hybrid assessment allowed us to screen and see if there were any protein-protein interactions with SufT and other proteins involved in FeS maturation. We determined that there may be a mild interaction with SufA FeS carrier, SufU sulfur carrier and SufB scaffold. Interestingly, it was found after the work in this study was completed that of the organisms found that encoded for *sufT*, <99% were recruited to the Suf operon with *sufU* and *sufB*[72]. Further work needs to be conducted to determine

exactly how SufT interacts with SufA, SufU and SufB. Although it is not conclusive, it is possible that Fe plays a role in the interaction of SufT with SufA, SufU and SufB, however, biochemical analysis would have to be conducted to determine if Fe is being transferred between proteins. Also assays pulldown and biochemical assays could be conducted to determine if SufT is interacting with SufU to increase or decrease sulfur transfer to SufB or interacting and imparting effects vice versa.

Genes which encode proteins that have functional overlap are known to display a synergistic phenotypic effect[73]. An unamplified loss in AcnA enzymatic activity was previously seen in the $\Delta nfu \Delta sufA$ double mutant strain, suggesting that they may be acting as redundant carrier proteins[26]. We analyze AcnA and LeuCD enzymatic activities in both the $\Delta sufA \Delta sufT$ double mutant and $\Delta nfu \Delta sufT$ double mutant strains based on the interaction data seen through Bacterial-2-Hybrid assessment and determined that there exhibited marginally amplified loss of enzymatic activity in the $\Delta sufA \Delta sufT$ double mutant strain but a complete loss in the $\Delta nfu \Delta sufT$ double mutant strain. We determined that the synergistic effect of $\Delta nfu \Delta sufT$ double mutant strain suggested possible functionally overlapping roles for SufT and Nfu and a requirement for one another. Considering Nfu has been proven to be an FeS cluster carrier between complex and apo-protein[26], it seems unclear from the data available, how SufT and Nfu are interacting and why they are essential together. A $\Delta sufB \Delta sufT$ double mutant strain is not possible because of the essentiality of sufB, however, it would also be interesting to see how a $\Delta sufU \Delta sufT$ double mutant strain is characterized phenotypically. A phenotypic analysis of $\Delta sufU \Delta sufT$ double mutant strain could suggest why <99% of the organisms observed with *sufT* on the Suf Operon also have *sufU* and because

bacterial-2-hybrid data suggests a mild interaction.

The ΔsufT strain grew similar to WT in rich media, however, the $\Delta\text{nfu } \Delta\text{sufT}$ double mutant strain had astounding growth defects, and it was noted to grow similar to the ΔacnA strain. The ΔacnA strain is known to have an inactive TCA cycle and we confirmed that in the $\Delta\text{nfu } \Delta\text{sufA}$ double mutant strain TCA cycle was also impaired, as determined by acetic acid catabolism. Interestingly however, we found that *acnA* promoter activity was highly active which may be caused by Fur since it positively regulates *AcnA* when Fur-accessible Fe pools are low. In order to determine if this is true, further investigation involving growth of the $\Delta\text{nfu } \Delta\text{sufA}$ double mutant strain in an Fe excess medium is monitored for *acnA* promoter activity.

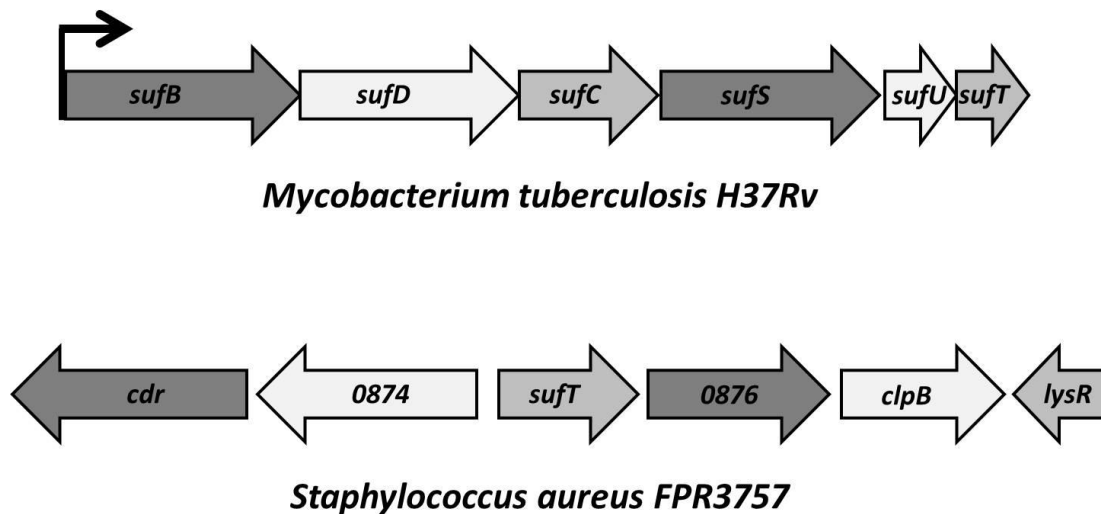
When the $\Delta\text{nfu } \Delta\text{sufT}$ double mutant strain was grown in chemically defined media, the growth defects were partially corrected by the addition of LA and short-chained fatty acids(SCFAs). As mentioned previously, Bck is required for the production of SCFAs, however it requires the catabolism of Ile, Leu and Valine as well as LA availability. Further studies should include additional Branched-Chain Fatty acids (BCFAs) to determine if the phenotypes exhibited are only corrected by exogenous addition of SCFAs. Studies conducted after the work presented in this paper showed that Bck activity was disrupted in a $\Delta\text{nfu } \Delta\text{sufT}$ double mutant strain[74]. This finding suggests that the $\Delta\text{nfu } \Delta\text{sufT}$ double mutant strain is defective in providing an FeS cluster to apo-LipA making either or both Nfu and SufT essential for LipA function. It would also be interesting to see in a future study if TCA cycle can fully be repaired by addition of other FeS cluster requiring TCA cycle byproducts.

The characterization of SufT suggests the complexity redundancy and conditional relevance of proteins within *S. aureus* FeS cluster maturation. These findings give appreciation to the complex mechanisms that pathogens engage when encountering oxidative stress brought on by host immune response. Elucidation of the function of SufT and other proteins involved in FeS cluster maturation may help us understand how pathogens persist through our immune system, and give insight into potential antimicrobial targets.

In summary, we have established the identity of a previously undescribed protein, SufT. We determined that SufT was involved in FeS protein maturation and our data suggest that it may a role in Fe uptake or trafficking to Suf for FeS cluster metabolism. Future studies beyond these data should attempt to determine exactly how SufT is involved in FeS cluster maturation and whether SufT is involved in Fe uptake or trafficking.

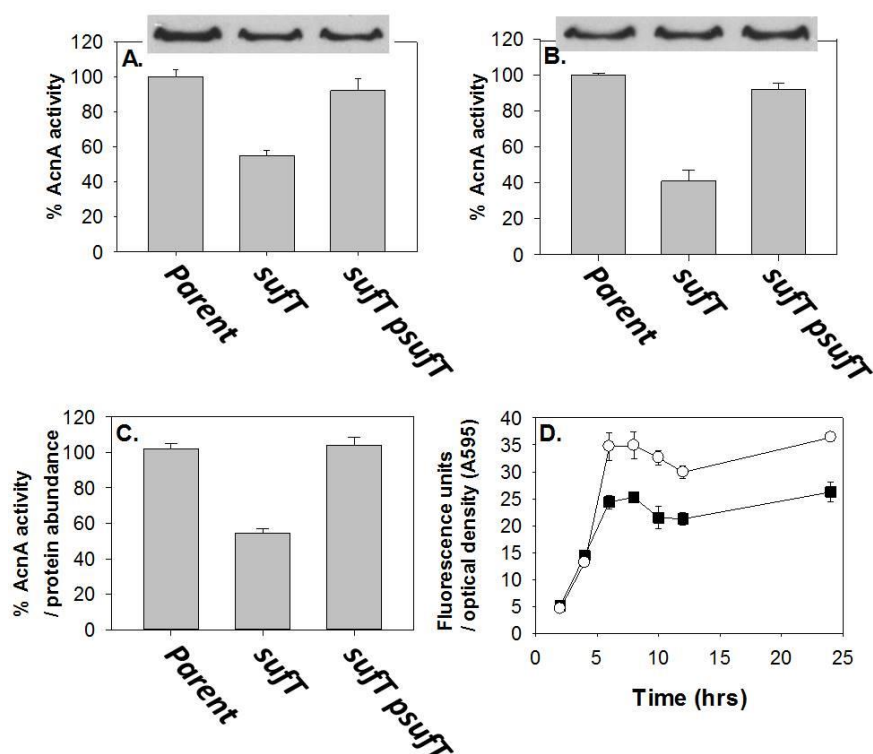
V. Figures

Figure 1: *sufT* operon figure in *Mycobacterium tuberculosis* H37Rv and *Staphylococcus aureus* USA300



Comparative sufT operon figure in Mycobacterium tuberculosis H37Rv and Staphylococcus aureus USA300. A high degree of identity exists between the gene encoding sufT in M. tuberculosis and S. aureus FPR3757. The operon encoding sufT in M. tuberculosis is presented (top) along with the genes in vicinity of sufT. The genetic vicinity of sufT in S. aureus FPR3757 is also displayed (bottom).

Figure 2: Activity levels of AcnA within a Δ *sufT* *S. aureus* strain.

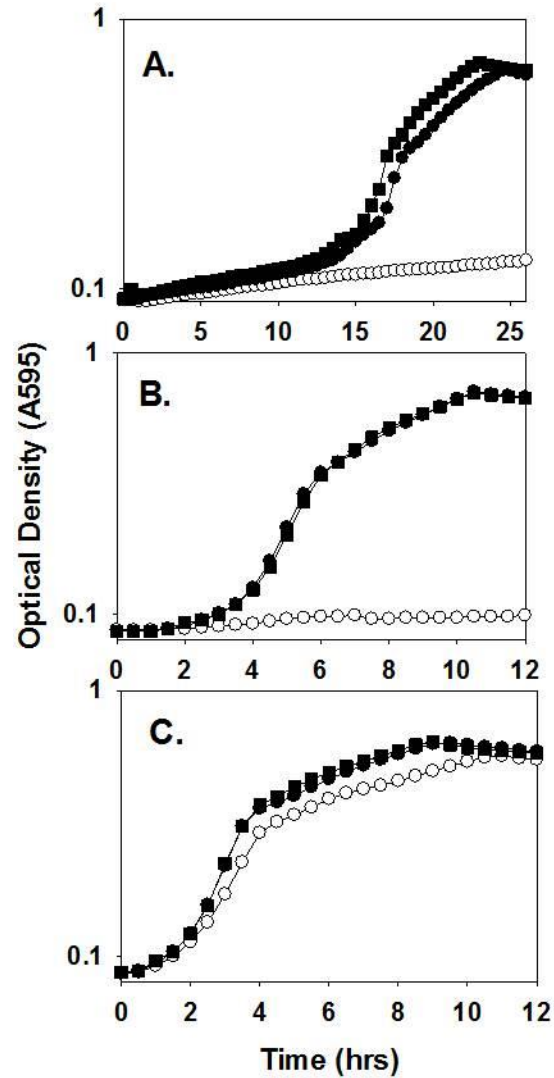


Panel A&B: Aconitase (*AcnA*) has decreased activity in a *sufT* mutant *S. aureus* strain.

Strains WT JMB3055 (*Parent*), *sufT* mutant strain, mutant JMB4202 (*sufT*), and complemented JMB4201 were assayed for enzymatic AcnA activity and are displayed as a percentage relative to WT activity. Western Blot was conducted on the samples from Panel A and B. **Panel C:** The Percent of AcnA activity was normalized for protein abundance to account for any fluctuations in total protein quantity of the sample. Data analyzed were obtained from panel B. **Panel D:** *acnA* promoter activity levels in a *sufT* mutant *S. aureus*. Strains WT JMB2414 (black squares) and mutant JMB2416 (white circles), were monitored for *acnA* promoter activity in a fluorescent reporter plasmid over

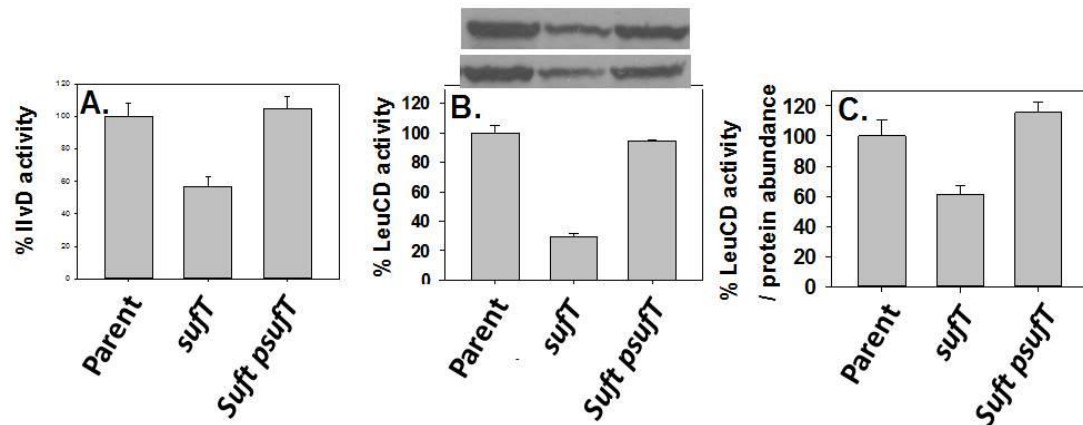
the course of 24 hours. Results are normalized to account for differences in optical density.

Figure 3: Effect of amino acid deprivation on Δ sufT *S. aureus* strain.



Strains Wildtype, JMB1886 (black square), mutant strain JMB4258 (white circle) and complement strain JMB4265 (black circle) were grown overnight in TSB and subcultured into staphylococcal chemically defined medium with 19 of the 20 essential amino acids, with either the absence of leucine (**Panel A**), isoleucine (**Panel B**) or in presence of all 20 amino acids (**Panel C**).

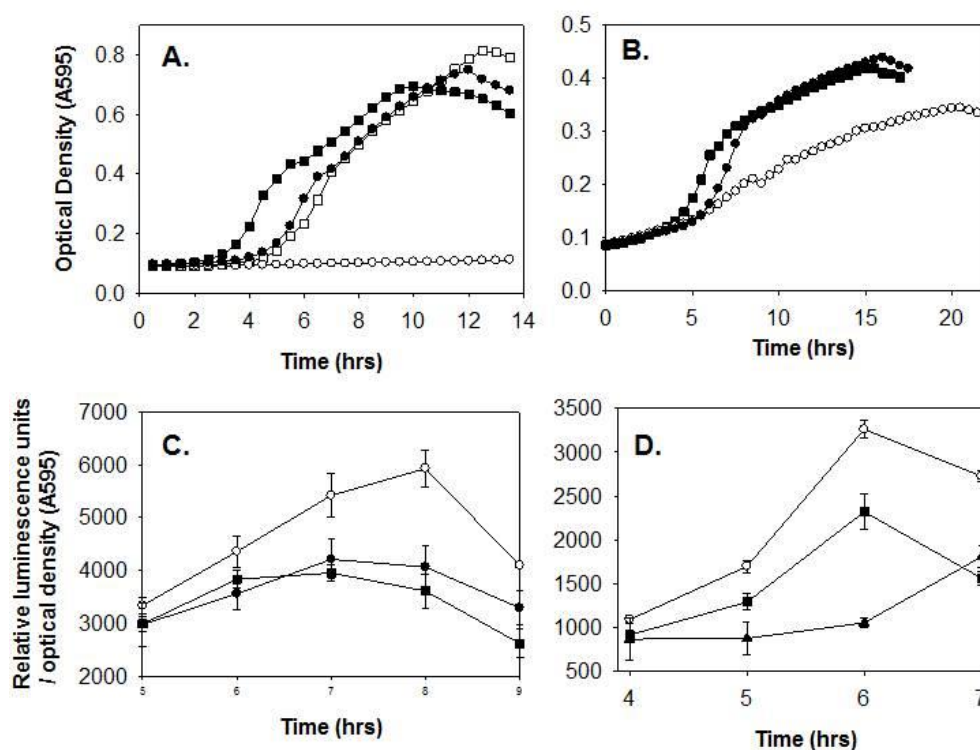
Figure 4: Diminished enzymatic activity of additional FeS cluster requiring enzymes within a Δ *sufT* *S. aureus* strain.



Panel A: Analyzing *IlvD* activity within a *sufT* mutant *S. aureus*. Strains WT JMB3966 (*Parent*), mutant JMB4376 (*sufT*), and complement JMB4375 (*sufT psufT*) cell free lysates were assayed for dihydroxyacid dehydratase (*IlvD*) enzyme activity. Activity data is displayed as a relative percent to the parent strain. **Panel B:** *Isopropylmalate isomerase (LeuCD)* activity within a *sufT* mutant *S. aureus* was diminished. Isopropylmalate isomerase (*LeuCD*) activity was assessed in cell free lysates of WT JMB3965(*Parent*), mutant JMB4383 (*sufT*), and complement JMB4382 (*sufT psufT*) Results are displayed as a percent of the Parent activity. Protein abundance from the assayed cultures were determined by Western Blot analyses as displayed above. **Panel C:** *SufT*-mutant *S. aureus* *LeuCD* activity is decreased despite *LeuCD* protein accumulation differences. Western Blot analyses was conducted, as displayed in Panel B, to obtain relative protein abundance of the sample. ImageJ protein analyses was conducted to determine relative densities of the bands of *pleuCD_FLAG* displayed. Data

displayed depicts LeuCD enzymatic activity as a ratio to the strains' protein accumulation.

Figure 5: Levels of intracellular free iron in a Δ *sufT* *S. aureus* strain.

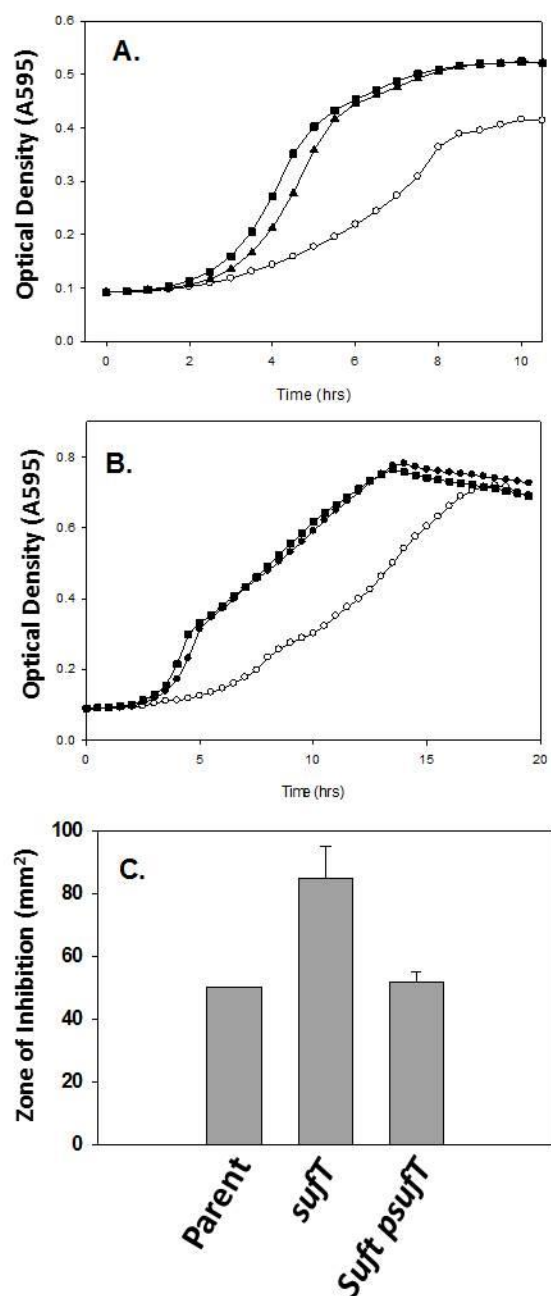


*Effect of iron supplementation on the amino acid auxotrophy of the *sufT* mutant *S. aureus**

Panel A: Strains WT JMB1100 (black square) and mutant strain JMB1146 (white circle) were grown as discussed previously in TSB, and then subcultured into *Staphylococcal* chemically defined growth media containing 19 essential amino acids, without the presence of isoleucine for the indicated amount of time, displaying the physiological growth defect of a *sufT* mutant *S. aureus*. JMB1100 and JMB1146 were also grown in similar *Staphylococcal* chemically defined media, without the presence of isoleucine, however, the strains were supplemented with 120mM FeCl_3 (white square and black circle respectively). These data show the corrective nature of Fe on JMB1146 isoleucine auxotrophy. **Panel B:** A *sufT* mutant strain has deficient 2,2-dipyridyl accessible Fe pools. WT JMB1886 (black squares), mutant JMB4258 (white circles) and

complement JMB4265 (black circles), were grown in *Staphylococcal* chemically defined media. Growth rate was monitored in the presence of 40 μ M 2,2-dipyridyl for the duration of time listed. **Panel C:** *A sufT mutant strain has diminished Fur accessible Fe pool.* Transcriptional activity of the *isdB* promoter is increased in a *sufT* mutant strain of *S. aureus*. Strains WT JMB4457 (black squares), mutant JMB4458 (white circles), and complement JMB4459 (black circle), were cultured in TSB supplemented with chloramphenicol and luminescence was monitored over the duration of time listed. Luminescence was standardized for optical density (A_{595}). Displayed values are an average of biological triplicates. Errors represent standard deviations. **Panel D:** *Fur Accessible Fe pools behave differently within a sufT mutant when compared to other known members of the SUF machinery.* Strains WT JMB4457 (black squares), Δ *sufT* JMB4458 (white circles), and Δ *nfu* JMB3949 (black triangles) were grown and assayed similarly to Panel C. Displayed values are an average of biological triplicates and errors represent standard deviations.

Figure 6: Effect of oxidative and nitrosative stress upon a Δ *sufT* *S. aureus* strain.



Panel A: Effect of Nitric Oxide Stress on a *sufT* mutant strain of *S. aureus*. Sodium Nitroprusside is known to produce Nitrosative stress in *S. aureus* and alter gene expression of genes involved in [Fe-S] cluster metabolism. Strains WT, JMB1886 (black square), Δ *sufT* strain JMB4258 (white circle), and complement strain JMB4265 (black

triangle) were grown overnight in TSB and subcultured in chemically defined media supplemented with 1.2 mM sodium nitroprusside. Optical Density (A_{595}) was taken for the listed time period. **Panel B:** *Effect of Oxidative stress on a sufT mutant strain of S. aureus.* Sodium hypochlorite is known to produce free radicals in the body and is made by human peripheral blood neutrophils. Strains JMB1886 (black square), JMB4258 (white circle), and JMB4265 (Black circle) were grown in TSB and sub cultured in chemically defined media supplemented with 62 μ M sodium hypochlorite. **Panel C:** *Effect of methyl viologen stress on a sufT mutant strain of S. aureus.* Strains JMB1886 (Parent), JMB4258 (*sufT*), and JMB4265 (*sufT psufT*) were analyzed for a zone of inhibition by the top-agar stress method. 30 mM Methyl Viologen was spotted to a top-agar overlay of the strains listed. Results are plotted as the area of growth inhibition calculated by measurement of the diameter and converted to area. Displayed values are an average of biological triplicates and errors represent standard deviations.

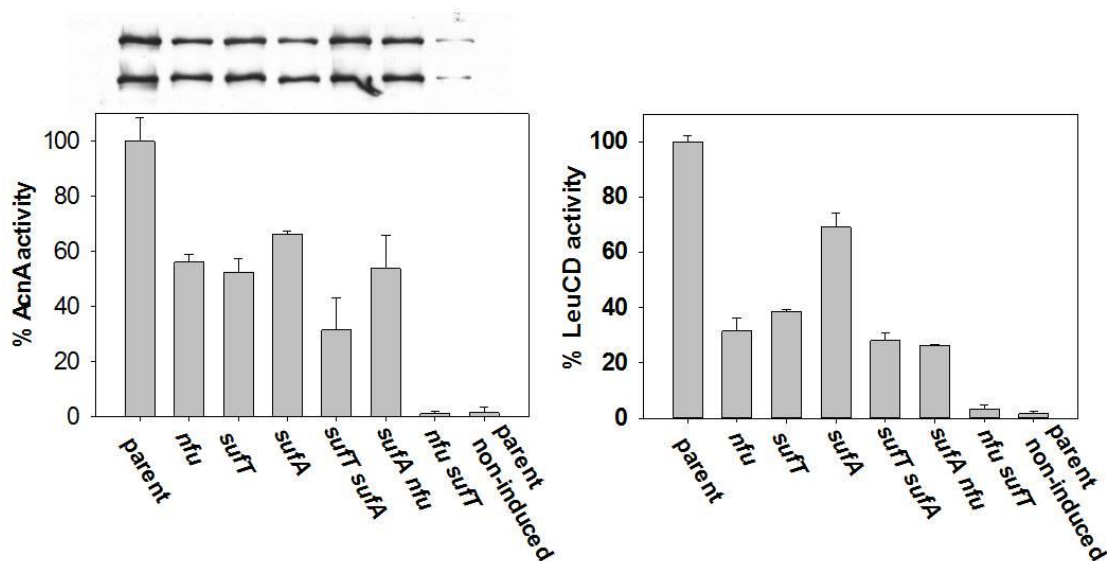
Figure 7: Bacterial-2-hybrid data suggests interactions of SufT with other proteins involved in FeS cluster maturation and transfer

		pAC										
pBR		CI	WT	SufA	SufU	SufS	SufB	SufC	SufD	SufT	Nfu	CitB
	Alpha	-	-	-	-	-	-	-	-	-	-	-
	WT	-	+++	-	-	-	-	-	-	-	-	-
	SufA	-	-	-	++	+	+	-	-	+	+	++
	SufU	-	-	++	-	+	-	-	-	+	-	++
	SufS	-	-	-	-	-	+	-	-	-	-	-
	SufB	-	-	++	+	+	-	++	+	+	+	+
	SufC	-	-	-	-	-	++	+	+++	-	++	-
	SufD	-	-	-	-	-	+++	+	-	-	-	-
	SufT	-	-	+	+	-	+	-	-	-	-	-
	Nfu	-	-	++	+	+	-	-	-	+	-	+
	CitB	-	-	+	++	-	-	-	-	+	+	-

sufT displays mild protein-protein interaction with other FeS machinery as determined by a bacterial-2-hybrid model. pACYC-derived plasmids (pAC) were encoded either CI as a negative control or any one of the proteins presented above (x-axis). pBR322-derived plasmids (pBR) either encoded the alpha as a negative control or any of the proteins presented above (y-axis). Both plasmids were maintained in the cell using culture medium with chloramphenicol and kanamycin. Strains were reinoculated from an overnight culture to a final optical density of 0.1 (A_{595}) in LB supplemented with chloramphenicol, kanamycin and IPTG and grown for 24 hours in a Biotek 808E visible absorption spectrophotometer kept at a constant 37°C set at a medium shake speed. The slope of the ONPG hydrolysis taken as optical density (A_{420}) of the exponential phase of growth was obtained for each of the samples over time. The interaction the proteins were assessed normalizing any interaction to that of of Alpha-CI as a negative control and WT-

WT as a positive control. The no interaction observed is denoted as (-); a mild interaction is denoted (+); a medium interaction is denoted (++) and a strong interaction is denoted (+++).

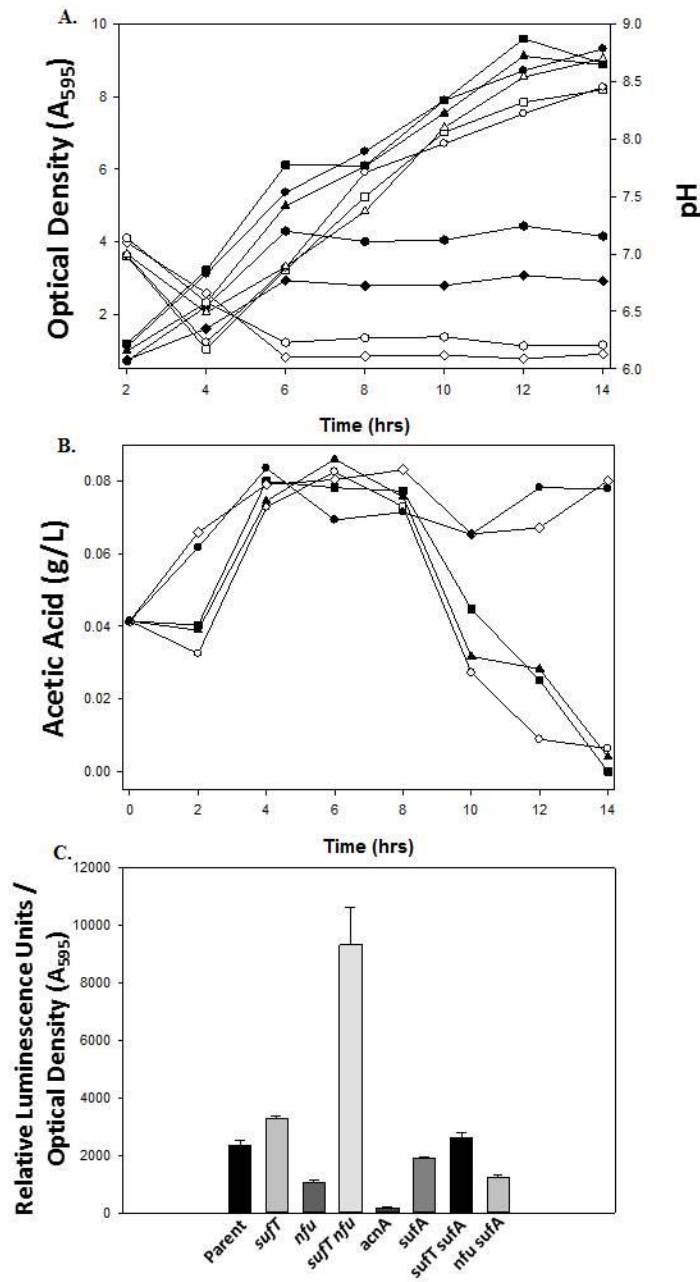
Figure 8: Enzymatic analyses of AcnA and LeuCD suggests potential interaction of SufT with other Suf maturation proteins



Panel A: A *Staphylococcal* strain mutant for both *sufT* and *nfu* has null enzymatic *AcnA* activity. All strains were initially cultured overnight and reinnoculated to a final OD of 0.1 (A_{595}). Experiments were conducted and analyzed as described. Strains JMB3522 (parent), JMB3505 (*nfu*), JMB3507 (*sufT*), JMB4261 (*sufA*), JMB3634 (*sufT sufA*), JMB3636 (*sufA nfu*), and JMB3509 (*nfu sufT*) were assayed for *AcnA* activity with the parent non-induced strain as the negative control. *AcnA* activity is plotted as the percentage activity relative to the parent strain which was set to 100% *AcnA* activity. Data obtained was normalized for relative protein abundance, as determined by Western Blot Analyses. **Panel B:** Levels of *LeuCD* activity are greatly diminished within a *S. aureus* strain mutant for both *sufT* and *nfu*. *LeuCD* activity was also analyzed as described previously. Strains JMB3521 (parent), JMB3506 (*nfu*), JMB3508 (*sufT*), JMB3597 (*sufA*), JMB3599 (*sufT sufA*), JMB3601 (*sufA nfu*), and JMB3510 (*nfu sufT*)

were assayed. Samples are depicted as the percentage activity of the parent strain when the parent is set to 100% LeuCD activity.

Figure 9: An *S. aureus* Δ *sufT* Δ *nfu* double mutant strain has amplified physiological defects.

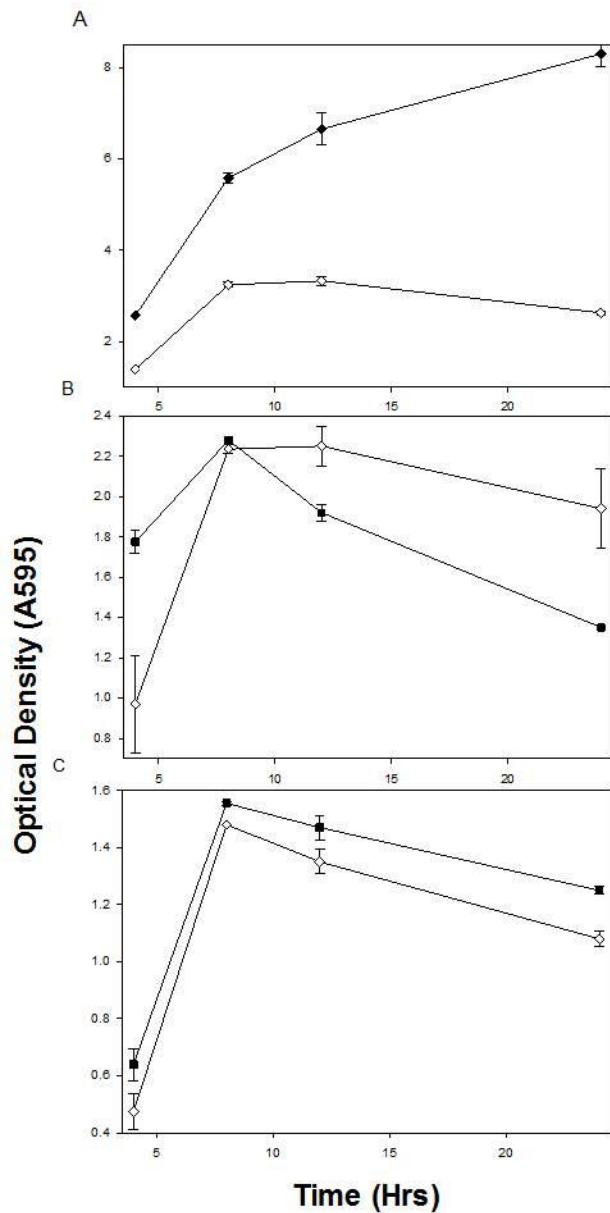


Panel A: The Δ *sufT*, *nfu::tetM* double mutant behaves similarly to an *acnA* mutant.

Strains WT JMB1100 (black squares); *nfu::tetM* JMB2316 (black triangles); Δ *sufT* JMB1146 (black circles); double mutant JMB2208 (black diamonds) and *acnA::tetM*

JMB1163 (black hexagons) were subcultured from overnight culture in fresh TSB to a final OD of 0.1 (A_{595}) and monitored for OD over the course of 14 hours. Additionally, samples were collected from the same growth cultures to monitor pH levels and are represented by the following; WT (white squares); *nfu::tetM* (white triangles); Δ *sufT* (white circle); Δ *sufT*, *nfu::tetM* double mutant (white diamonds), and *acnA::tetM* (white hexagons) were monitored for pH levels. **Panel B:** *The Δ sufT, nfu::tetM double mutant accumulates acetic acid similarly to an acnA mutant.* Strains WT (JMB1100) represented by black squares; Δ *sufT* JMB1146 (white circles); *nfu::tetM* JMB2316 (black triangles); double mutant JMB2208 (white diamonds); and *acnA::tetM* JMB1163 (black circles) were subcultured from overnight culture in fresh TSB to a final OD of 0.1 (A_{595}). Samples were collected at 2 hours increments and acetic acid concentrations were quantified as described. **Panel C:** *The aconitase promotor activity is extremely high in a Δ sufT, nfu::tetM double mutant.* Strains WT JMB2414 (Parent); mutant JMB2416 (*sufT*); mutant JMB2415 (*nfu*); double mutant JMB2420 (*sufT nfu*); mutant JMB2425 (*acnA*); mutant JMB2417 (*sufA*); double mutant JMB2418 (*sufT sufA*); and double mutant JMB2419 (*nfu sufA*) were subcultured from overnight culture in fresh TSB to a final OD of 0.1 (A_{595}) and allowed to grow for 24 hours. Luminescence units were determined as described and normalized for optical density.

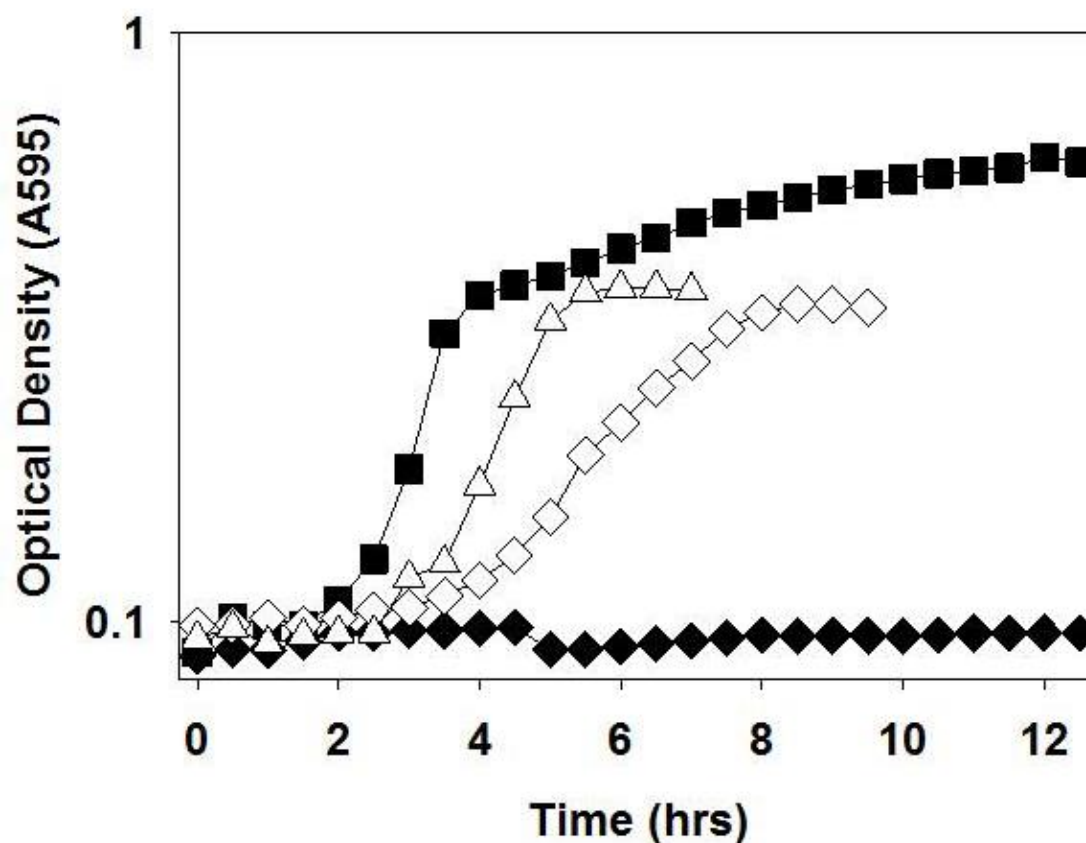
Figure 10: An *S. aureus* Δ *sufT* Δ *nfu* double mutant strain grows similar to wildtype in anoxic conditions



Panel A: An *S. aureus* *sufT* and *nfu* double mutant has severe growth defect in aerobic medium. Strains WT (JMB1100) represented by the black squares and Δ *sufT*, *nfu*::*tetM* (JMB2208) represented by the white diamonds were subcultured from overnight cultures in fresh TSB to a final OD of 0.1 (A₅₉₅) and allowed to be grown aerobically. Assay time

points displayed were collected and averaged in biological triplicate. Errors represent one standard deviation from the average. **Panel B:** *Growth analyses of an S. aureus sufT and nfu double mutant in anoxic medium with nitrate.* Strains JMB1100 (black square) and JMB2208 (white diamond) were subcultured from overnight cultures in fresh TSB greatly reduced or devoid of oxygen to a final OD of 0.1 (A_{595}) in the presence of 2mM nitrate as an electron acceptor. Assay time points displayed were collected and averaged in biological triplicate. Errors represent one standard deviation from the average. **Panel C:** *Growth analyses of an S. aureus sufT and nfu double mutant in anoxic medium without a terminal electron acceptor.* Strains JMB1100 (black square) and JMB2208 (white diamond) subcultured from overnight cultures in fresh TSB greatly reduced or devoid of oxygen to a final OD of 0.1 (A_{595}), as described, without the presence of an exogenous terminal electron acceptor.

Figure 11: An *S. aureus* Δ *sufT* Δ *nfu* double mutant strain can be partially corrected by culture supplementation with lipoic acid and SCFAs.



The WT (JMB1100) represented by black squares, and double mutant JMB2208 (black diamonds), were grown in *Staphylococcal* chemically defined media with 20 essential amino acids. Optical Density (A_{595}) was collected over the listed time in a Biotek 808E visible absorption spectrophotometer kept at a constant 37°C set at a medium shake speed. JMB2208 is also depicted in similar chemically defined medium supplemented with either 100 mM SCFAs (white diamonds) or 100 μ M lipoic acid (white triangles). All conditions, aside from the supplementation, were kept constant.

VI. Tables

Table 1: Staphylococcal Strains

Strain	Genotype/Description	Background	Source
RN4220	Restriction-negative <i>S. aureus</i>	RN1	[36]
JMB1100	<i>S. aureus</i> Parent Strain USA300	LAC	[75]
JMB1144	Δ <i>sufA</i> (USA300_0843)	LAC	[26]
JMB1146	Δ <i>sufT</i> (USA300_0875)	LAC	
JMB1163	<i>acnA::tetM</i> (USA300_1246)	LAC	[26]
JMB1165	Δ <i>nfu</i> (USA300_0839)	LAC	[26]
JMB1580	<i>nfu::kanR</i>	LAC	[26]
JMB1886	<i>attP::pLL39</i>	LAC	[26]
JMB2208	Δ <i>sufT</i> , <i>nfu::teM</i>	LAC	
JMB2223	<i>sufA::tetM</i>	LAC	[26]
JMB2224	<i>sufA::tetM</i> , Δ <i>sufT</i>	LAC	
JMB2316	<i>nfu::tetM</i>	LAC	[26]
JMB2414	WT, <i>pCM11_acnA</i>	LAC	
JMB2415	<i>nfu::tetM</i> , <i>pCM11_acnA</i>	LAC	
JMB2416	Δ <i>sufT</i> , <i>pCM11_acnA</i>	LAC	
JMB2417	<i>sufA::tetM</i> , <i>pCM11_acnA</i>	LAC	
JMB2418	Δ <i>sufT</i> , <i>sufA::tetM</i> , <i>pCM11_acnA</i>	LAC	
JMB2419	<i>nfu::kanR</i> , <i>sufA::tetM</i> , <i>pCM11_acnA</i>	LAC	
JMB2420	Δ <i>sufT</i> , <i>nfu::tetM</i> , <i>pCM11_acnA</i>	LAC	
JMB2425	<i>acnA::tetM</i> , <i>pCM11_acnA</i>	LAC	
JMB2501	<i>nfu::kanR</i> , <i>sufA::tetM</i>	LAC	[26]
JMB3055	WT, <i>acnA::ermB</i> , <i>attP:pLL39_acnA_FLAG</i>		
JMB3949	Δ <i>nfu</i> , <i>attP::pLL39</i> , <i>pXEN_isdB</i>	LAC	
JMB3965	WT, <i>attP::pLL39</i> , <i>leuCD::erm</i> ,	LAC	[26]
JMB3966	<i>attP::pLL39</i> , <i>ilvD::ermB</i> , <i>pEPSA5_ilvD_FLAG</i>	LAC	[26]
JMB4201	Δ <i>sufT</i> , <i>acnA::ermB</i> , <i>attP:pLL39_acnA_FLAG</i> , <i>pCM28_sufT</i>	LAC	
JMB4202	Δ <i>sufT</i> , <i>acnA::ermB</i> , <i>attP:pLL39_acnA_FLAG</i> , <i>pCM28</i>	LAC	
JMB4258	Δ <i>sufT</i> , <i>attP::pLL39</i>	LAC	
JMB4265	Δ <i>sufT</i> , <i>attP::pLL39_sufT</i>	LAC	
JMB4375	Δ <i>sufT</i> , <i>attP::pLL39_sufT</i> , <i>ilvD::ermB</i> , <i>pEPSA5_ilvD_FLAG</i>	LAC	
JMB4376	Δ <i>sufT</i> , <i>attP::pLL39</i> , <i>ilvD::ermB</i> , <i>pEPSA5_ilvD_FLAG</i>	LAC	
JMB4382	Δ <i>sufT</i> , <i>attP::pLL39_sufT</i> , <i>leuCD::erm</i> ,	LAC	
JMB4383	Δ <i>sufT</i> , <i>attP::pLL39</i> , <i>leuCD::erm</i> ,	LAC	

JMB4457	WT, attP::pLL39, pXEN-1_isdBp	LAC	
JMB4458	Δ sufT, attP::pLL39, pXEN-1_isdBp	LAC	
JMB4459	Δ sufT, attP::pLL39_sufT, pXEN-1_isdBp	LAC	
JMB3522	WT, pEPSA5_acnA_FLAG	LAC	[26]
JMB3505	Δ nfu, pEPSA5_acnA_FLAG	LAC	
JMB3507	Δ sufT, pEPSA5_acnA_FLAG	LAC	
JMB4261	sufA::tetM, pEPSA5_acnA_FLAG	LAC	
JMB3634	sufA::tetM, Δ sufT, pEPSA5_acnA_FLAG	LAC	
JMB3636	sufA::tetM, nfu::kanR, pEPSA5_acnA_FLAG	LAC	
JMB3509	nfu::tetM, Δ sufT, pEPSA5_acnA_FLAG	LAC	[26]
JMB3521	WT, pEPSA5_FLAG_leuCD	LAC	[26]
JMB3506	Δ nfu, pEPSA5_FLAG_leuCD	LAC	
JMB3508	Δ sufT, pEPSA5_FLAG_leuCD	LAC	
JMB3597	sufA::tetM, pEPSA_FLAG_leuCD	LAC	
JMB3599	sufA::tetM, Δ sufT, pEPSA5_FLAG_leuCD	LAC	
JMB3601	sufA::tetM, nfu::kanR, pEPSA5_FLAG_leuCD	LAC	
JMB3510	nfu::tet, Δ sufT, pEPSA5_FLAG_leuCD	LAC	

Table 2: Plasmids

Plasmid Name	Locus/Function	Source
<i>pAC</i>	Bacterial-2-Hybrid	[47]
<i>pBR</i>	Bacterial-2-Hybrid	[47]
<i>pJB38</i>	Mutant Construct	[76]
<i>pJB38_0875Δ</i>	For Chromosomal Deletion, (<i>sufT</i>)	
<i>pCM11</i>	Vector with promoterless gfp transcriptional reporter	[40]
<i>pCM11_acnA</i>	SAUSA300_1246, transcriptional reporter	
<i>pCM28</i>	Genetic complementation vector	[77]
<i>pEPSA5_acnA_Flag</i>	SAUSA300_1246 with C-terminal FLAG affinity tag	
<i>pEPSA5_ilvD_Flag</i>	SAUSA300_2006	
<i>pEPSA5_leuCD_Flag</i>	SAUSA300_2012-13 with N-terminal FLAG affinity tag	
<i>pLL39</i>	Chromosomal genetic complementation	[78]
<i>pLL39_0875</i>	SAUSA300_0875(<i>sufT</i>)	
<i>pXEN-1_isdB</i>	Transcriptional fusion of SAUSA300_1028 to <i>luxABCDE</i>	[53]

Table 3: Other Organisms Used

Organism	Description	Source
<i>Escherichia coli</i> – DH5 α	Cloning Strain	Protein Express

Table 4: Primers

Number	Primer	Sequence	Plasmid
1	08755BamHI.42873001K	CCCGGATCCGCGCCATTAAAATTAAC	pLL39
2	08753Sall.42910029K	CCCGTCGACGCGGGCCCAAACACAG	pLL39
3	875gfpKpnI	GGGGGTACCGCACCTAAGATACTATC	pCM11
4	875gfpHindIII	GGGAAGCTTCGATACTTGATATTGTT	pCM11
5	SAUSA300_0875do42567	CCCGGATCCCGAAATCCAATTTGTAA	Verify
6	SAUSA300_0875up42567	GGGGAATTCCTTCTGCTATAATATTC	Verify
7	875upBamHI	GGGGGATCCTTAGGAGGATGATTATT	
8	08753Sall.42873016K	CCCCAGCTGGCGGGCCCAAACACAG	
9	875upNdeI.43184010K	CCCCATATGGTAATTGACCCTGAATT	
10	875dwnXhoI.43184009K	GGGCTCGAGGCTCACACCAAGTGAA	
11	0875up3_NheI.42591004K	ACGCGTGGTACCGCTAGCGCTAGCG	

VII. References

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