THE SUF IRON-SULFUR CLUSTER BIOSYNTHETIC SYSTEM IS ESSENTIAL FOR *STAPHYLOCOCCUS AUREUS* VIABILITY AND DECREASED SUF FUNCTION RESULTS IN GLOBAL METBOLIC DEFECTS AND DECREASED SURVIVAL IN HUMAN NEUTROPHILS

By

CHRISTINA A. ROBERTS

A thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Microbiology and Molecular Genetics

Written under the direction of

Dr. Jeffrey Boyd

And approved by

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New Brunswick New Jersey

October 2016

ABSTRACT OF THE THESIS

THE SUF IRON-SULFUR CLUSTER BIOSYNTHETIC SYSTEM IS ESSENTIAL FOR *STAPHYLOCOCCUS AUREUS* VIABILITY AND DECREASED SUF FUNCTION RESULTS IN GLOBAL METBOLIC DEFECTS AND DECREASED SURVIVAL IN HUMAN NEUTROPHILS

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Thesis Director:

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Staphylococcus aureus remains a causative agent for morbidity and mortality worldwide. This is in part a result of antimicrobial resistance highlighting the need to uncover novel antibiotic targets and discover new therapeutic agents. In this study we explored the possibility of iron-sulfur (FeS) cluster synthesis as a viable antimicrobial target. RNA interference studies verified that Suf-dependent FeS cluster synthesis was essential in *S. aureus*. Two *S. aureus* strains were characterized that contained transposon insertions between *suf* genes (*suf**) resulting in decreased transcription of genes downstream of the insertions. We found that the *sufCDSUB* genes were cotranscribed and *suf* transcription was positively influenced by general stress sigma factor B. The *suf** strains had decreased activities of FeS cluster-requiring enzymes and decreased growth in media lacking metabolites that require FeS proteins for synthesis. Decreased FeS cluster synthesis also resulted in sensitivity to reactive oxygen and reactive nitrogen species. Decreased Suf function resulted in increased DNA damage and defective DNA repair. It also resulted in decreased flux though the TCA cycle and decreased cellular respiration. The *suf** mutants had perturbed intracellular non-chelated Fe pools. Defective FeS cluster synthesis did not

alter exoprotein production or biofilm formation, but it did result in decreased survival upon challenge with human polymorphonuclear leukocytes. The results presented suggest that FeS cluster synthesis is a viable target for antimicrobial development. The strains and DNA constructs described provide a genetic toolbox for further examination of FeS cluster synthesis in *S. aureus*.

Acknowledgements

I would like to thank Hassan Al-Tameemi, Ameya Mashruwala, Zuelay Rosario-Cruz, William Sause, Victor Torres, William Belden, and most of all Jeffrey Boyd for all their help in experimentation, data acquisition, scientific guidance, and positive encouragement that led to the fruition of this thesis project. Also a special thanks goes to Gerben Zylstra and Lee Kerkhof for making the time to serve as committee members for the thesis defense.

The content of this thesis will be submitted to a peer reviewed journal for publication.

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Table 1. Strains and plasmids used in this study.

Strains used in this study		
Strain name	genotype	Source/reference
WT	USA300_LAC	A.R. Horswill [84, 85]
JMB1102	Δ*sigB* (SAUSA300_2022)	[62]
JMB1163	Δ*acnA*::*tetM* (SAUSA300_1246)	[86]
JMB1165	Δ*nfu* (SAUSA300_0839)	[15]
JMB2078	*kat*::Tn (*ermB*) (SAUSA300_1232)	V. Torres
JMB2745	*sigB*::Tn (*ermB*) (SAUSA300_2022)	NARSA [85]
JMB2763	*nth*::Tn (*ermB*) (SAUSA300_1343)	NARSA [85]
JMB2726	*mutY*::Tn (*ermB*) (SAUSA300_1849)	NARSA [85]
JMB2950	*agrA*::Tn (*ermB*) (SAUSA300_1992)	NARSA [85]
JMB2952	*hemB*::Tn (ermB) (SAUSA300_1615)	NARSA [80]
JMB2963	*sdhA*::Tn (*ermB*) (SAUSA300_1047)	NARSA [80]
JMB2475	*acnA*::Tn (*ermB*) (SAUSA300_1246)	NARSA [80]
JMB3298	*addB*::Tn (*ermB*) (SAUSA300_0869)	NARSA [85]
JMB5853	*sodA*::Tn (*ermB*) (SAUSA300_1513)	NARSA [85]
JMB6876	*sufD**::Tn (*ermB*) (see Figure 2A)	P. Fey
JMB6877	*sufS**::Tn (*ermB*) (see Figure 2A)	P. Fey
JMB7237	*lacB*::Tn (*ermB*) (SAUSA300_2154)	NARSA [85]
JMB7592	Δ*nfu addB*::Tn (*ermB*)	This study
RN4220	Restriction minus	[85]
Escherichia coli PX5	Used for gene cloning	Protein Express

Plasmids used in this study		
Plasmid name	Insert	Function / reference
pCM11_*sufC*p	*sufC* promoter	sufC transcriptional activity
pML100	None	Gene expression / [79]
p*suf*KD1	*sufC* DNA	Suf depletion
p*suf*KD2	*sufC* DNA	Suf depletion
pCR2.1_TOPO	None	cloning

(Referenced on page 19)

Table 2. Primers used in this study

Name	Sequence
sufDfwdRT	CAAGTTGATGATAATGCATCGAAAG
sufDrevRT	ATGGTTCATAAGAGCGTCTGCTAA
sufSfwdRT	AACCATTGCAGAAATAGCTCATCA
sufSrevRT	GCTTGCGCCCCATCAAC
sufUfwdRT	AATGGCAAGTGCATCGATGA
sufUrevRT	GCATTGCTTCTCCAAGTGAATG
sufBfwdRT	CTGTTGTGGAAATCATTGTGCAT
sufBrevRT	GTTCGCCCAGTTTTGAATCG
sufCRT5	GATGAAATCGATTCAGGGTTAGACA
sufCRT3	TTCCCCACGCATTTGGTTA
sufCup	TTATTCAGCTGAACCGAACTCTTC
sufCdwn	CTCGTTCCCATAGCAAAACCT
pML100rev	GCCTGCAGGTCGACTCTAGAGG
pML100for	GGCGTATCACGAGGCCCTTTCG
Sufinternal3	TCTAATATCGGAAAATCCTTGATTACTTCATTAACGTC
Sufinternal5	GACGTTAATGAAGTAATCAAGGATTTTCCGATATTAGA

(Referenced on pages xiii, 19, and 23)

Table 3. FeS biosynthesis systems in select bacterial strains

Bacterial species	FeS assembly machinery	FeS biogenesis system predicted to be essential?	Reference
Acinetobacter baumannii [1]	Isc	Yes	[88]
Bacillus subtilis	Suf	Yes	[89]
Bacteroides fragilis	Suf	Yes	[90]
Burkholderia pseudomallei	Suf and Isc	Yes (IscS only)	[91]
Campylobacter jejuni	Nif	Yes	[92]
Clostridium difficile	Suf	Yes	[93]
Enterococcus faecalis [1]	Suf	Yes[2]	[94]
Escherichia coli	Suf and Isc	No	[95]
Francisella novicida	Suf	Yes	[96]
Haemophilus influenzae	Isc	Yes	[97]
Helicobacter pylori	Nif	Yes	[98]
Klebsiella pneumoniae [1]	Suf, Nif and Isc	No	[99]
Mycobacterium tuberculosis	Suf	Yes	[100]
Porphyromonas gingivalis	Suf	Yes	[101]
Pseudomonas aeruginosa [1]	Isc	Yes	[102]
Salmonella enterica	Suf and Isc	No	[103]
Staphylococcus aureus [1]	Suf	Yes	[16]
Streptococcus pneumoniae	Suf	Yes	[104]
Streptococcus pyogenes	Suf	Yes	[105]
Vibrio cholerae	Isc	Yes	[106]

[1]ESKAPE pathogen; capable of “escaping the biocidal effects of antibiotics.”
[2]Limited data set.

(Referenced on pages 3 and 14)

Table 4. Predicted *Staphylococcus aureus* iron-sulfur cluster binding and assembly proteins.

Gene product	Protein Function	FPR3757 locus	NCTC 8325 locus	Essential[a]	Fitness Cost[b]
PflA	Pyruvate formate lyase activating enzyme	SAUSA300_0221	SAOUHSC_00188	No	No
GltB	Glutamate synthase	SAUSA300_0445	SAOUHSC_00435	No	No
SufC	Fe-S cluster biogenesis	SAUSA300_0818	SAOUHSC_00847	Yes	-
SufD	Fe-S cluster biogenesis	SAUSA300_0819	SAOUHSC_00848	Yes	-
SufS	Fe-S cluster biogenesis	SAUSA300_0820	SAOUHSC_00849	Yes	-
SufU	Fe-S cluster biogenesis	SAUSA300_0821	SAOUHSC_00850	Yes	-
SufB	Fe-S cluster biogenesis	SAUSA300_0822	SAOUHSC_00851	Yes	-
LipA	Lipoate synthase	SAUSA300_0829	SAOUHSC_00861	No	No
Nfu	Fe-S cluster carrier	SAUSA300_0839	SAOUHSC_00873	No	Yes
SufA	Fe-S cluster carrier	SAUSA300_0843	SAOUHSC_00877	No	Yes
AddB	DNA helicase-nuclease	SAUSA300_0869	SAOUHSC_00904	Yes/No	Yes
SufT	Fe-S cluster assembly	SAUSA300_0875	SAOUHSC_00910	No	Yes
SdhB	Succinate dehydrogenase	SAUSA300_1048	SAOUHSC_01105	No	No
MiaB	23S rRNA methyltransferase	SAUSA300_1111	SAOUHSC_01185	No	Yes
-	Radical SAM	SAUSA300_1185	SAOUHSC_01679	No	No
AcnA	Aconitate hydratase	SAUSA300_1246	SAOUHSC_01347	No	Yes
Nth	Endonuclease III	SAUSA300_1343	SAOUHSC_01469	No	Yes
Fdx	ferridoxin	SAUSA300_1373	SAOUHSC_01504	Yes/No	Yes
-	Adenosine tRNA methylthiotransferase	SAUSA300_1536	SAOUHSC_01269	No	Yes
HemN	Coproporphyrinogen III oxidase	SAUSA300_1543	SAOUHSC_01686	No	No
-	Radical SAM	SAUSA300_1706	SAOUHSC_01877	No	No
HemH	ferrochelatase	SAUSA300_1782	SAOUHSC_01961	Yes/No	Yes
AirS	Environmental sensing	SAUSA300_1799	SAOUHSC_01981	No	No
-	Epoxyqueuosine reductase	SAUSA300_1806	SAOUHSC_01989	No	No
MutY	Adenine glycosylase	SAUSA300_1849	SAOUHSC_02005	No	No
IlvD	Isoleucine biosynthesis	SAUSA300_2006	SAOUHSC_02281	No	No
LeuC	Leucine biosynthesis	SAUSA300_2012	SAOUHSC_02287	No	No
SdaA	L-serine dehydratase	SAUSA300_2469	SAOUHSC_02839	No	Yes
MoaA	Molybdenum cofactor biosynthesis	SAUSA300_2219	SAOUHSC_02536	No	No
FdhA	Formate dehydrogenase	SAUSA300_2258	SAOUHSC_02582	No	No
BioB	Biotin synthase	SAUSA300_2371	SAOUHSC_02714	No	No
Nre	Environmental sensing	SAUSA300_2338	SAOUHSC_02676	No	No
NarG	Nitrate reductase	SAUSA300_2343	SAOUHSC_02681	No	No
NirD	Nitrate reductase	SAUSA300_2345	SAOUHSC_02683	No	No
NirB	Nitrite reductase	SAUSA300_2346	SAOUHSC_02684	No	No
NrdG	Ribonucleotide reduction	SAUSA300_2550	SAOUHSC_02941	No	No
CysJ	sulfite reductase	SAUSA300_2554	SAOUHSC_02947	No	No
ArcR	Environmental sensing	SAUSA300_2566	SAOUHSC_02964	No	No

[a] Gene essentiality was determined as reported elsewhere [14, 16, 67, 68].
[b] fitness cost was determined by Valentino *et al*. [16].

(Referenced on pages 8 and 15)

List of Figures/Illustrations

Figure 1. The Depletion of *suf* and Viability

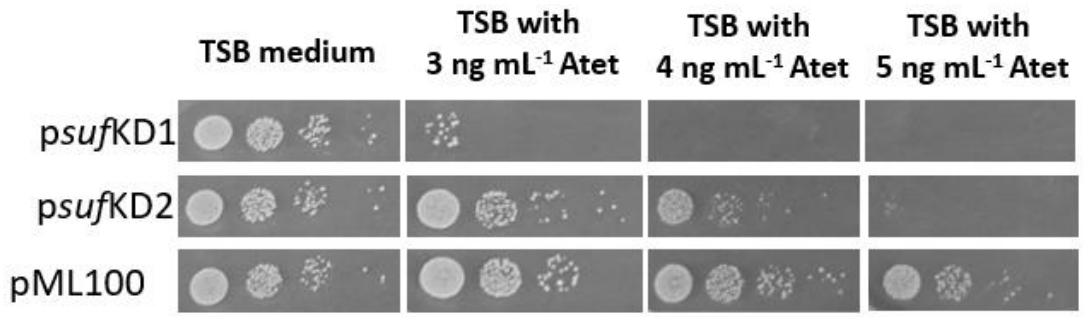

Figure 1. *suf* depletion decreases *S. aureus* viability. *S. aureus* strains containing p*suf*KD1, p*suf*KD2, or pML100 (empty vector) were serial diluted and spot-plated on solid TSB-Chloramphenicol media with and without anhydrotetracycline (Atet; inducer). Pictures from representative experiments are shown.

(Referenced on page 4)

Figure 2. Transposon Locations and Transcript Abundance

Figure 2. Transposon insertions decrease transcription of *sufU* and *sufB*. Panel A) Locations of the *sufS** and *sufD** transposon insertions. The *sufS** insertion is located between *sufD* and *sufS*, and the *sufD** insertion is located between *sufC* and *sufD*. Panel B) The *sufS** and *sufD** insertions decrease the transcription of *suf* genes downstream of the insertion sites. Total RNA was isolated from the WT, *sufS** (JMB6877), and *sufD** (JMB6876) strains and the transcription of the individual *sufCDSUB* genes were quantified. The data represent the average mRNA abundance from cells cultured in biological triplicates and cDNA libraries were analyzed in duplicate. Where indicated, Student t-tests (two tailed) were performed on the data and * denotes $p < 0.05$.

(Referenced on page 4)

Figure 2. Transposon Locations and Transcript Abundance

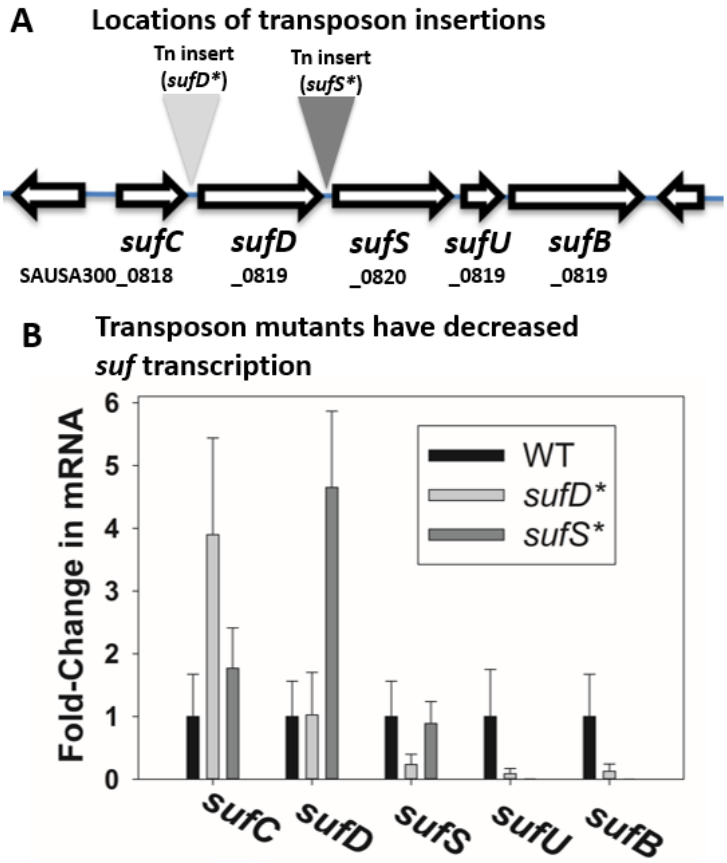

Figure 3. The Influence of Sigma Factor B and Cotranscription of *suf* Genes

Figure 3. The *sufCDSUB* genes are cotranscribed and transcription is positively influenced by sigma factor B. Panel A) Analysis of an RNA-seq data set indicate that *sufCDSUB* is co-transcribed. The RNA-seq dataset analyzed was previously published [36]. Panel B) The *suf* genes are co-transcribed. Top: a schematic of the *suf* operon, the locations of the amplicons are shown, and the predicted sizes of the amplicons generated using the following primer pairs: lanes 2 and 3, sufCRT5 and sufDrevRT; lanes 4 and 5, sufDfwdRT and sufinternal3; lanes 6 and 7, sufinternal5 and sufSrevRT; lanes 8 and 9 sufSfwdRT and sufBrevRT (Table 2) are displayed. Bottom: amplicons were generated from cDNA libraries using RNAs isolated from the WT and separated using agarose gel electrophoresis. The samples analyzed in lanes 3, 5, 7, and 9 were generated using a template that was not treated with reverse transcriptase. Panel C) The promoter of the *suf* operon contains potential sigma factor A (green) and sigma factor B (red) recognition sites. The predicted transcriptional start site is shown in blue and was determined by analyzing previously published RNA-seq data [36]. The annotated translational start site of *sufC* is in bold and underlined. Panel D) The transcriptional activity of the *sufC* promoter is modulated by sigma factor B (SigB). The transcriptional activity of *sufC* was monitored in the WT, Δ*sigB* (JMB1102), and Δ*nfu* (JMB1165) strains containing pCM11_*sufC*. The data shown in Panel D represent the average of biological triplicates with standard deviations shown. Student t-tests (two-tailed) were performed on these data and * denotes $p < 0.05$.

(Referenced on pages 5 and 6)

Figure 3. The Influence of Sigma Factor B and Cotranscription of *suf* Genes

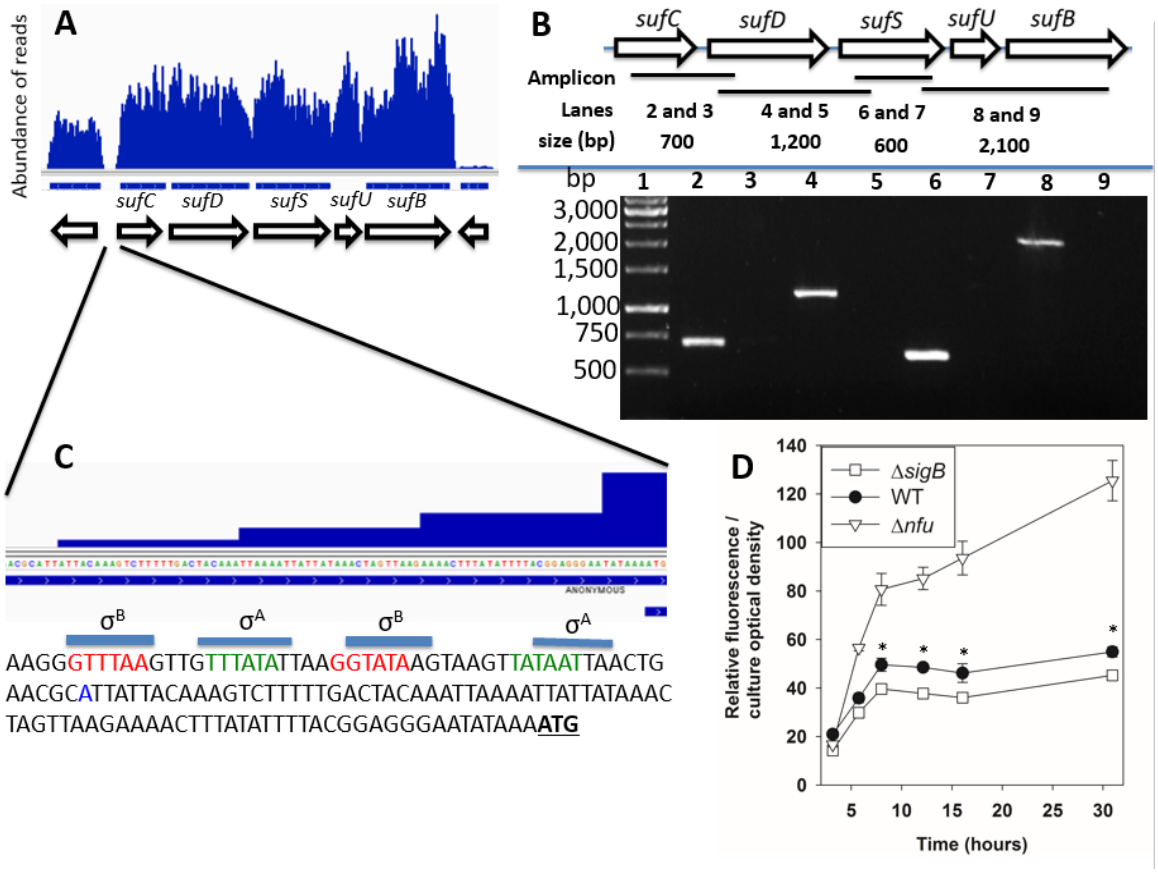

Figure 4. Activity of FeS-requiring Proteins

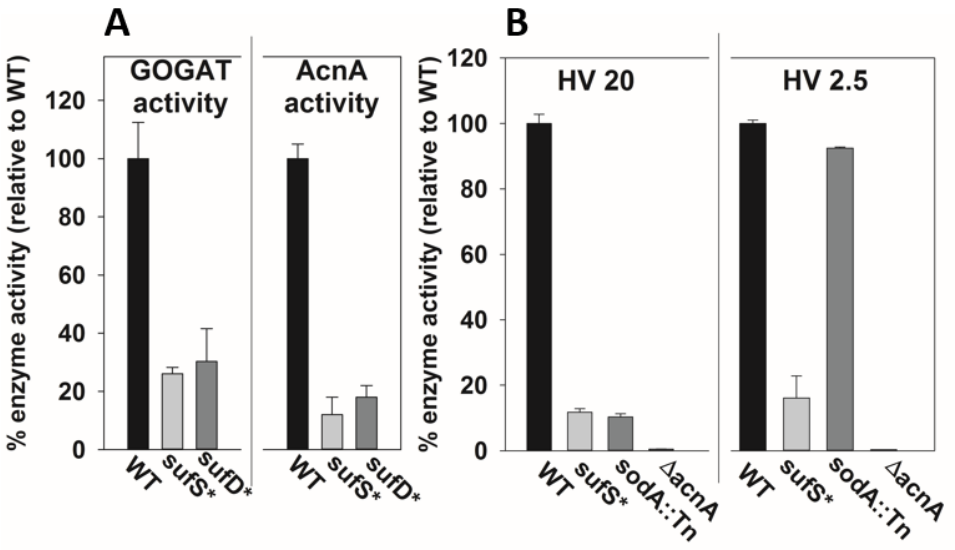

Figure 4. Iron-sulfur cluster requiring proteins have decreased activity in *S. aureus* strains with decreased *suf* transcription. Panel A) Aconitase (AcnA) and glutamate dehydrogenase activity was assessed in the WT, *sufD** (JMB6876), and *sufS** (JMB6877) strains. Panel B) AcnA activity is decreased in the *sufS** strain irrespective of culture aeration. The enzyme assays were conducted using the WT, *sufS** (JMB6877), *sodA*::Tn (JMB5853) and Δ*acnA* (JMB1163) strains cultured in TSB medium with altered gaseous headspace volume to liquid growth medium volume ratios (HV). Data represent the average of biological triplicates with standard deviations shown.

(Referenced on pages 6 and 22)

Figure 5. TCA Cycle Function

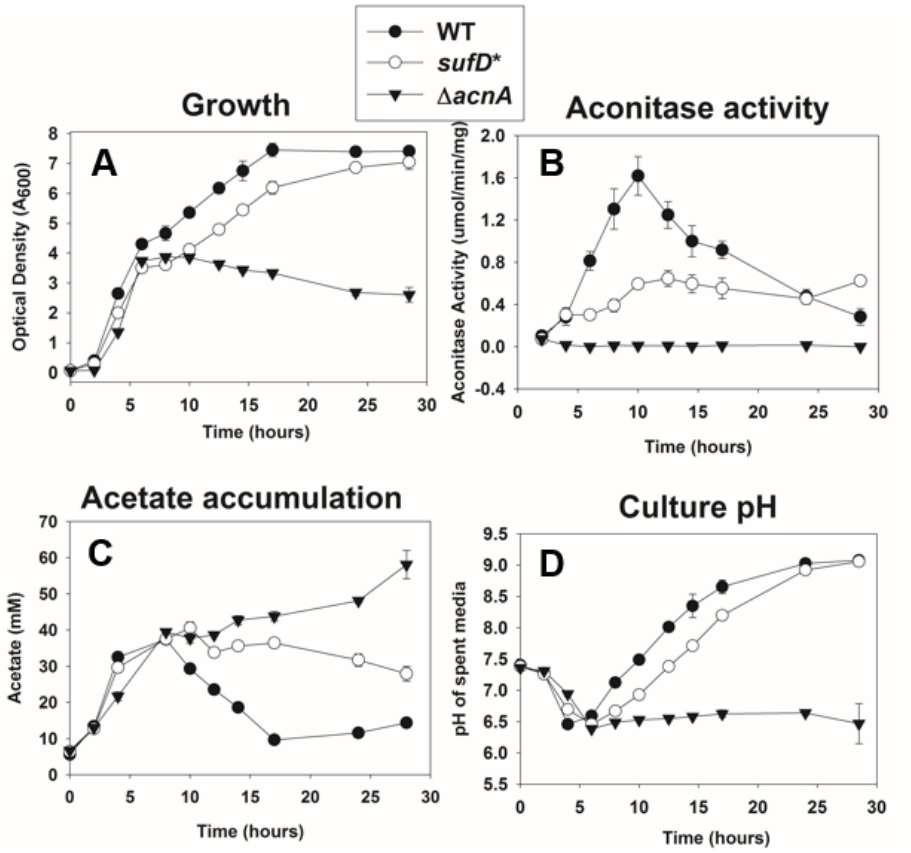

Figure 5. Decreased Suf function results in decreased TCA cycle function. Panel A) Growth profiles of the WT, *sufD** (JMB6876), and Δ*acnA* (JMB1163) strains. Panel B) Aconitase (AcnA) activity throughout growth. Panel C) Acetate accumulation in spent media throughout growth. Panel D) Spent media pH throughout growth. Data represent the average of biological triplicates with standard deviations shown.

(Referenced on pages 7 and 22)

Figure 6. Respiratory Flux and Proton Motive Force

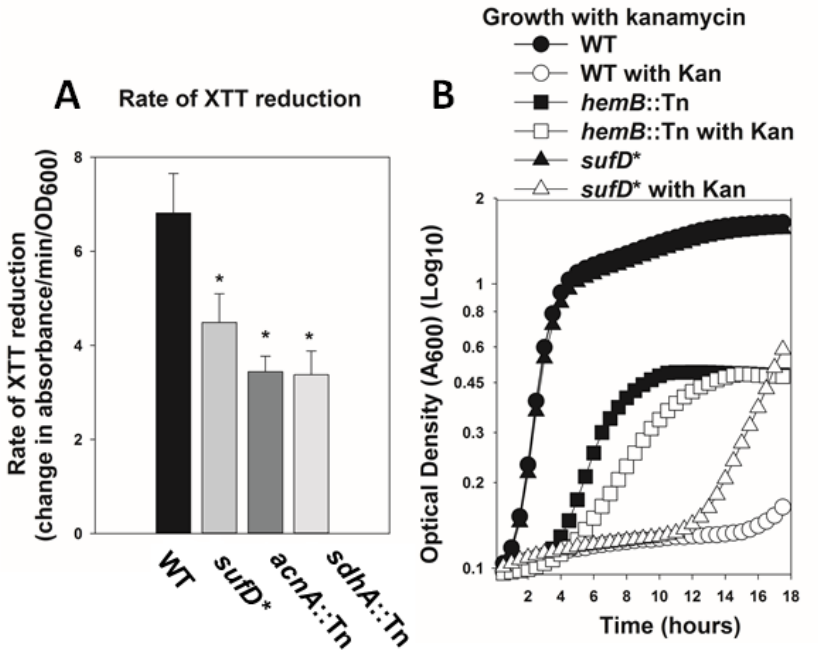

Figure 6. Decreased Suf function results in decreased cellular respiration and decreased proton motive force. Panel A) The rate of XTT reduction by WT, *sufD**(JMB6876), *acnA*::Tn (JMB2475), and *sdhA*::Tn (JMB2963) strains. Panel B) Representative growth profiles of the WT, *sufD** (JMB6876), *hemB*::Tn (JMB2952) strains with and without 4 ug mL^{-1} kanamycin. Data presented in panel A represent the average of biological triplicates with standard deviations shown. Student t-tests (two-tailed) were performed on these data and * denotes $p< 0.05$. Data shown in panel B are representative of at least three experimental trials.

(Referenced on page 8)

Figure 7. Auxotrophic Analysis on Solid Media

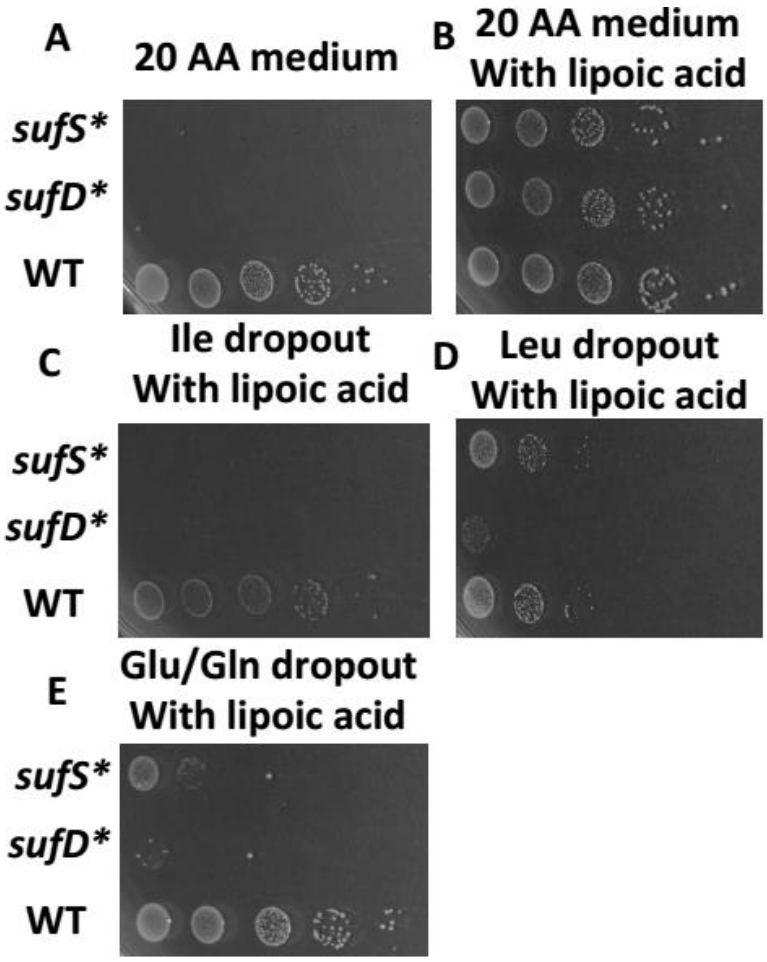

Figure 7. Auxotrophic analysis of strains with decreased *suf* transcription. An auxotrophic analysis was conducted in the WT, *sufS** (JMB6877), and *sufD** (JMB6876) strains. Strains were grown on solid chemically defined media containing: Panel A) the 20 canonical amino acids (AA); Panel B) 20 AA with lipoic acid; Panel C) 19 AA minus isoleucine with lipoic acid; Panel D) 19 AA minus leucine with lipoic acid; Panel E) 18 AA minus glutamate and glutamine with lipoic acid. Pictures from a representative experiment are shown.

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Figure 8. Effects on DNA Metabolism

Figure 8. Effect of decreased Suf function on DNA metabolism. Panel A) the frequency of spontaneous rifampicin resistance was measured in the WT, *sufS** (JMB6877), and *sufD** (JMB6876) strains. Panel B) The frequency of spontaneous rifampicin resistance was measured in the WT, *mutY*::Tn (JMB2726), *nth*::Tn (JMB2763), and *addB*::Tn (JMB3298) strains. Panel C) Sensitivity to methyl methanesulfonate (MMS) was monitored in the WT, *sufS** (JMB6877), and *sufD** (JMB6876) strains. Panel D) Sensitivity to diethyl sulfate (DES) was monitored in the WT, *sufS** (JMB6877), and *sufD** (JMB6876) strains. Panel E) Sensitivity to MMS was monitored in the WT, *mutY*::Tn (JMB2726), *nth*::Tn (JMB2763), *addB*::Tn (JMB3298), Δ*nfu* (JMB1165), and Δ*nfu addB*::Tn (JMB7592) strains. Panel F) Sensitivity to DES was monitored in the WT, *mutY*::Tn (JMB2726), *nth*::Tn (JMB2763), *addB*::Tn (JMB3298), Δ*nfu* (JMB1165), and Δ*nfu addB*::Tn (JMB7592) strains. The data presented in panels A and B represent the average of ten biological replicates with standard deviations shown. Where indicated, Student t-tests (two tailed) were performed on the data and * denotes $p < 0.05$. The data presented in panels C, D, E, and F represent the average of biological triplicates with standard deviations shown.

(Referenced on pages 9 and 11)

Figure 8. Effects on DNA Metabolism

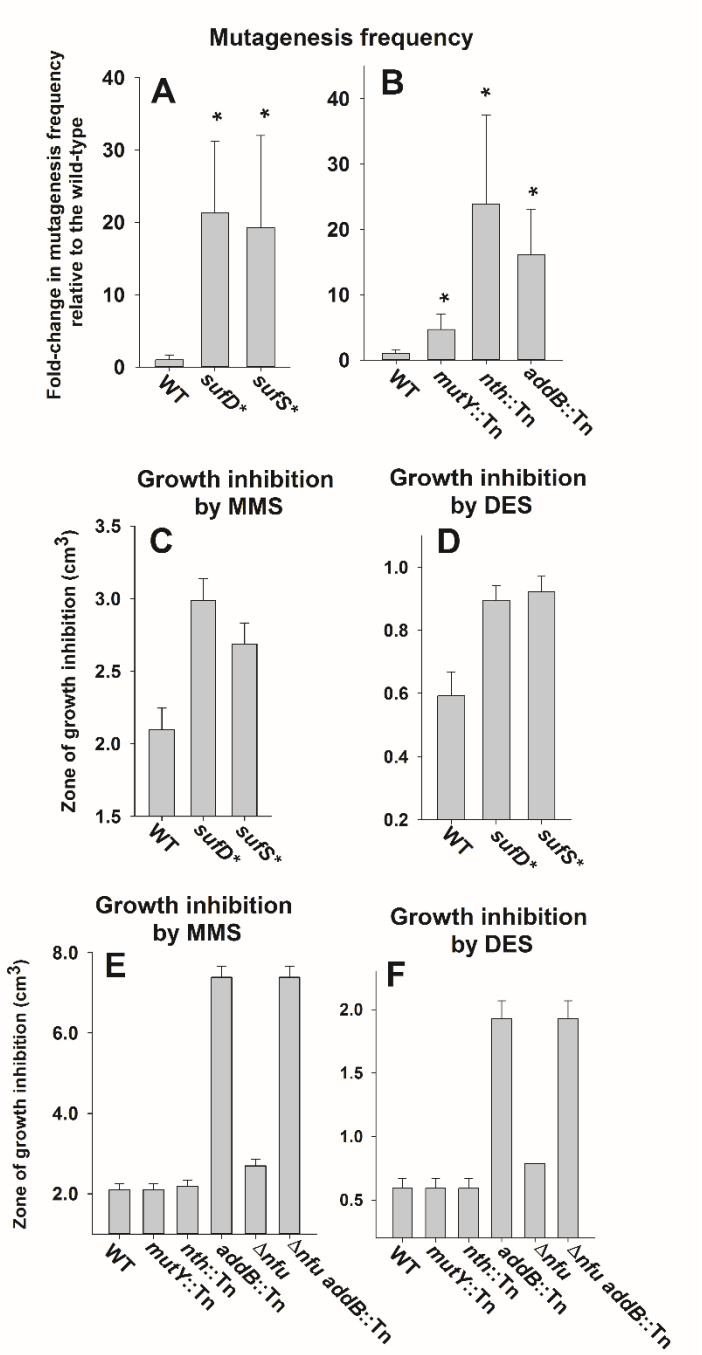

Figure 9. Sensitivity to ROS and RNS

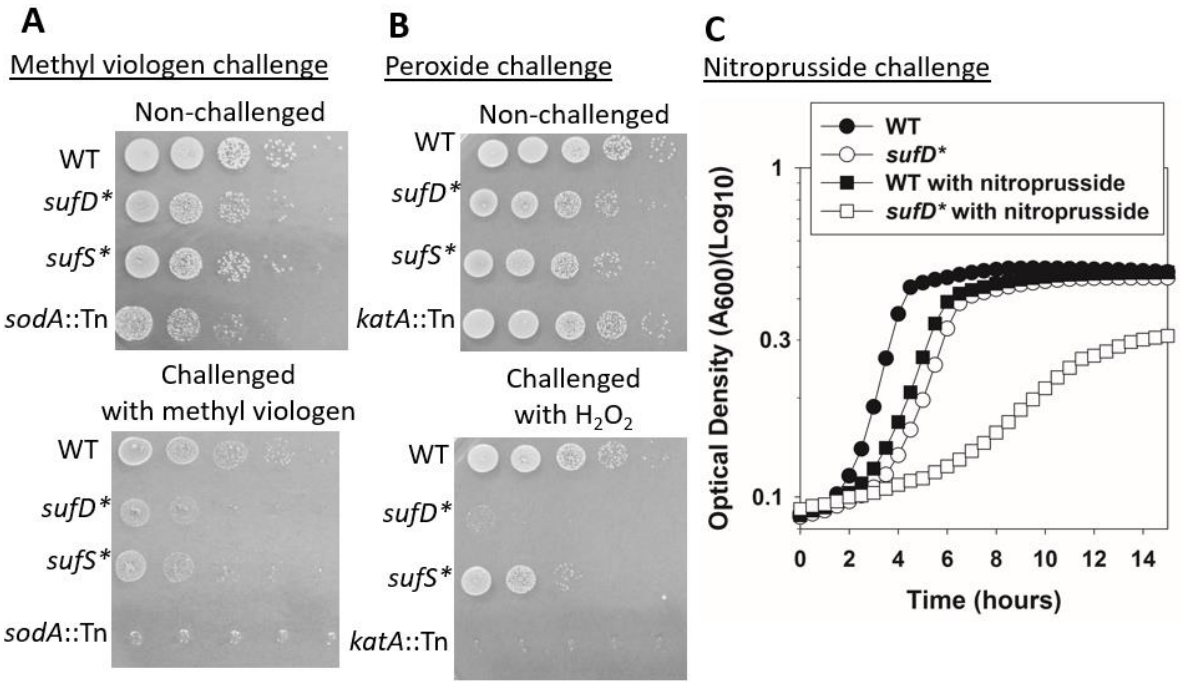

Figure 9. Decreased Suf function results in increased sensitivity to RNS and ROS toxicity. Panel A) Methyl viologen sensitivity was monitored in the WT, *sufS** (JMB6877), *sodA*::Tn (JMB5853), and *sufD** (JMB6876) strains. Cells were serial diluted and spot plated on TSB media with and without 30 mM methyl viologen. Panel B) Hydrogen peroxide (H_2O_2) sensitivity was monitored in the WT, *sufS** (JMB6877), *katA*::Tn (JMB2078), and *sufD** (JMB6876) strains. Strains were challenged with 500 mM H_2O_2 before the reaction was quenched, the cells were then serial diluted and spot plated on TSB medium. Panel C) Nitroprusside sensitivity was monitored in the WT, *sufS** (JMB6877), and *sufD** (JMB6876) strains. Growth profiles are shown in the presence and absence of 4 μM nitroprusside. Data from representative experiments are shown.

(Referenced on pages 10 and 11)

Figure 10. Intracellular Fe Pools

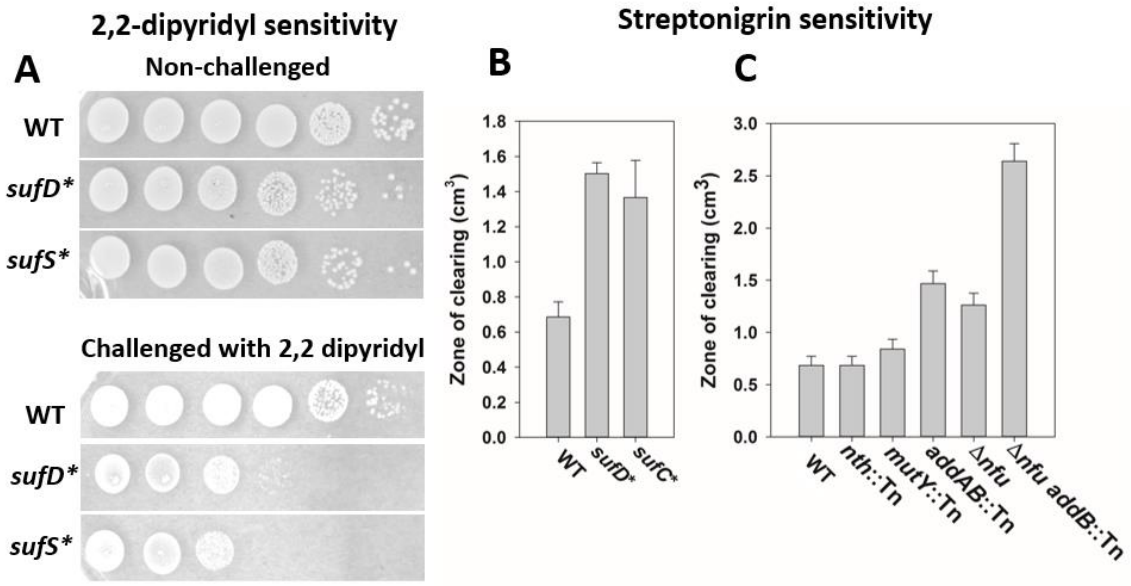

Figure 10. Decreased Suf function destabilizes intracellular Fe pools. Panel A) The WT, *sufS** (JMB6877), and *sufD** (JMB6876) strains were spot plated on solid TSB media with and without 250 mM 2,2-dipyridyl. Panel B) The WT, *sufS** (JMB6877), and *sufD** (JMB6876) strains were plated as top agar overlays on TSB solid media before the zones of inhibition as the result of streptonigrin toxicity were measured. Panel C) The WT, *nth*::Tn (JMB2763), *mutY*::Tn (JMB2726), *addB*::Tn (JMB3298), Δ*nfu* (JMB1165), Δ*nfu addB*::Tn (JMB7592) strains were plated as top agar overlays on TSB solid media before the zone of inhibition as the result of streptonigrin toxicity was measured. The data presented in panel B represent the average of biological triplicates with standard deviations shown.

(Referenced on page 11)

Figure 11. Exoprotein Production and Biofilm Formation

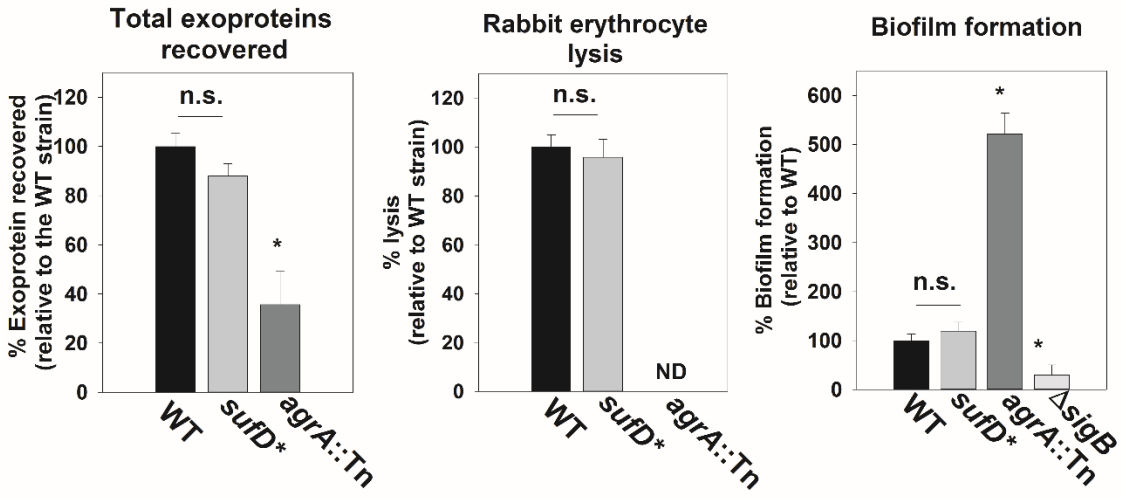

Figure 11. Necessity of proper Suf function for exoprotein production and biofilm formation. Total exoprotein production (Panel A), hemolysin activity (Panel B), and biofilm formation (Panel C) were assessed in the WT, *sufD** (JMB6876), *agrA*::Tn (JMB2950), and *sigB*::Tn (JMB2745) mutant strains. Student t-tests (two-tailed) were performed on the data presented and * denotes $p< 0.05$ and n.s. denotes $p> 0.05$. Standard deviations are shown.

(Referenced on page 12)

Figure 12. Survival in Human Neutrophils

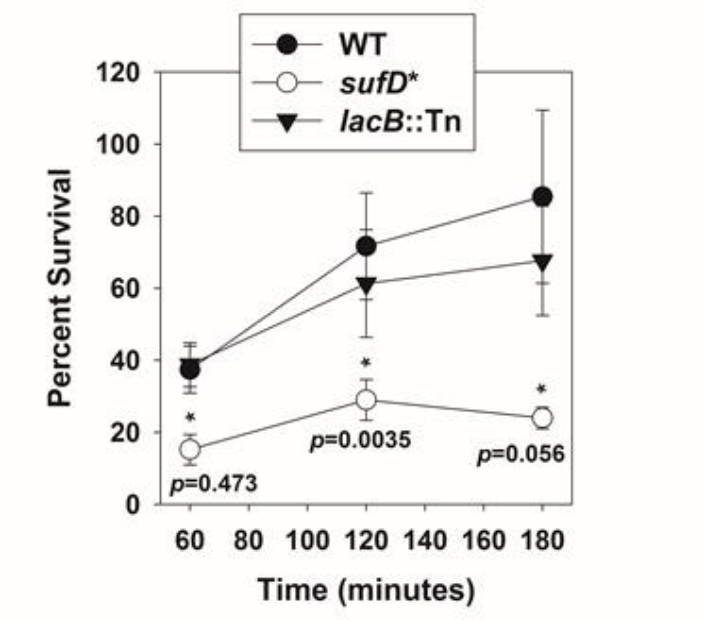

Figure 12. A strain with decreased Suf function has decreased survival in neutrophils. The WT, *sufD** (JMB6876), and *lacB*::Tn (JMB7327) strains were opsonized with 20% human serum, washed, then diluted to 2.5 x 10^7 CFU mL^{-1} and used to infect 250,000 polymorphonuclear neutrophils (PMNs) per well in a 96-well plate. Neutrophils were lysed upon addition of 1% saponin and CFU's were determined at various time points by plating. The data presented represent the average of biological triplicates with error bars representative of standard error of the mean. Student t-tests (two-tailed) were performed on the data presented and are shown for the *sufD** strain.

(Referenced on page 13)

Introduction

Staphylococcus aureus continues to cause morbidity and mortality worldwide. *S. aureus* is a human commensal that is frequently found colonizing the skin, nasal passages and throat [1]. While responsible for low morbidity maladies such as food poisoning and folliculitis, it is also capable of causing highly fatal afflictions such as endocarditis, bacteremia, and toxic shock syndrome [2, 3]. The high fatality rates are, in part, the result of resistance to clinically prescribed antimicrobials.

Upon entrance into the human host, *Staphylococcus aureus* encounters leukocytes that phagocytize the invading bacteria and bombard them with reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS have bactericidal and cytotoxic effects, which are, in part, the result of oxidation and eventual disassembly of protein–associated and solvent-exposed inorganic cofactors called iron-sulfur (FeS) clusters [4-8]. Proteins containing FeS clusters are necessary for diverse metabolic processes including environmental sensing [9], DNA repair [10], fermentation [11], cellular respiration [12], and central metabolism [13]. Because of the substantial reliance on FeS proteins for cellular processes, we and others have hypothesized that the disruption of FeS cluster assembly will result in metabolic standstill and eventually cell death. In *S. aureus*, this hypothesis is supported by results from high-density transposon mutant screens that suggest that the gene products required for FeS cluster synthesis are essential for survival [14-16].

Iron is a necessary nutrient and *S. aureus* strains defective in acquiring Fe have decreased virulence [17]. The importance of Fe during infection is highlighted by the fact that the human body goes to extraordinary lengths to prevent invading bacteria from acquiring the micronutrient, which has been termed “nutritional immunity [18].” *S. aureus*

produces two siderophores and has the capability to bring in Fe associated with siderophores produced by other organisms and heme [17, 19-21].

Upon acquisition, *S. aureus* uses Fe to metalate proteins, produce heme, or synthesize FeS clusters. The assembly of FeS clusters into proteins initiates with FeS cluster synthesis on the Suf machinery encoded by the *sufCDSUB* genes [15, 22]. Monoatomic Fe^{2+}, S^0, and electrons are combined to form an FeS cluster on the SufBCD molecular scaffold forming Fe_2S_2 linear clusters or Fe_4S_4 cubane clusters [23, 24]. Elemental sulfur is provided by the SufS cysteine desulfurase via the sulfur trafficking protein SufU [25]. The Fe and electron donors are unknown. SufC is an ATPase that has homology to membrane associated ATPases, SufD participates in Fe acquisition, and SufB is thought to be where FeS clusters are synthesized [23, 26]. In a previous study we were unable to create deletions of the *sufB* or *sufU* genes, which mirrored the findings from high-density transposon mutant libraries, suggesting that Suf function was essential for viability [14-16].

After synthesis, FeS clusters are either transferred directly to apo-proteins or to FeS cluster carriers that traffic the cofactors to apo-proteins [24, 27]. SufA and Nfu function as FeS cluster carriers in *S. aureus* [15, 28]. Genetic evidence suggests that SufT and bacillithiol also have roles in the maturation of FeS proteins [28, 38].

A majority of genetic and physiological studies on FeS cluster synthesis have been conducted in the bacterial model organisms, *Escherichia coli* and *Azotobacter vinelandii*. These organisms utilize more than one system to synthesize FeS clusters (ex. Suf and Isc). Many bacterial species, including *S. aureus*, utilize only one FeS cluster biosynthetic system and the Suf system is the most widely distributed [29].

Antibiotic resistance continues to plague healthcare settings worldwide. Infections caused by antibiotic resistant *S. aureus* result in a) increased mortality, b) increased stress on the healthcare system, and c) increased financial burden [30, 31]. Strains of *S. aureus* have recently been isolated that are resistant to last resort antimicrobials, including linezolid and vancomycin, prompting continued investigations into novel antimicrobial targets and discovery of new antimicrobials. Current FDA-approved antimicrobials target a limited number of metabolic processes [32] and recent discoveries prove that antimicrobials can still be isolated that are effective in inhibiting these processes [33]. Thus, expanding the list of antimicrobials that target alternate processes would provide more treatment possibilities and aid in multidrug therapy.

This study was initiated to determine if FeS cluster biogenesis is a viable antimicrobial target in *S. aureus*. RNA interference studies confirmed that Suf is essential for viability. Strains with decreased *suf* transcription had a decreased capability to assemble FeS clusters into apo-proteins. The decreased Suf function resulted in global metabolic defects including amino acid and vitamin auxotrophies and defective DNA repair. Defective FeS cluster synthesis also resulted in sensitivities to ROS and RNS, altered intracellular non-chelated Fe pools and decreased survival in human polymorphonuclear neutrophils. Importantly, decreased Suf function did not alter biofilm formation or toxin production, which are notable aspects of *S. aureus* pathogenesis. The synthesis of FeS clusters is hypothesized to be essential for a number of bacterial pathogens (Table 3). These observations, taken together with the findings within, lend strong support to the premise that the proteins catalyzing FeS cluster synthesis and assembly are strong targets for antimicrobial development.

Results

Expression of RNAs that are antisense to the *sufC* transcript decreases *S. aureus* viability. The conditional expression of an RNA that is antisense to a target mRNA has proven to be an effective means to deplete cells of the desired product [34]. We used mRNA depletion to further investigate the essentiality of Suf in *S. aureus*. DNA fragments corresponding to *sufC* were shotgun cloned into a plasmid vector under the transcriptional control of an anhydrotetracycline (Atet) inducible promoter. Two clones were identified that resulted in decreased growth in liquid TSB upon expression of the insert. The vectors contained fragments that expressed an RNA that was antisense to the 3' coding region of the *sufC* mRNA. The *sufC* gene is 762 nucleotides in length and the plasmids contained fragments corresponding to residues 515-762 (p*suf*KD1) and 572-750 (p*suf*KD2). *S. aureus* containing empty vector, p*suf*KD1, or p*suf*KD2 did not exhibit growth abnormalities when cultured on solid medium lacking inducer (Figure 1). As the concentration of Atet was increased, viability decreased in the cells containing p*suf*KD1 or p*suf*KD2, but not in cells containing the empty vector.

Transposon insertions between *suf* genes result in decreased *suf* transcription in *S. aureus*. Two strains were obtained that contained *bursa aurealis* mariner-based transposons between the coding regions of genes within the *suf* coding region [14, 35]. The transposons were located 62 and 63 base pairs upstream of *sufD* (*sufD**) or *sufS* (*sufS**), respectively (Figure 2A).

The effects of the *sufD** and *sufS** transposons on transcription of alternate *suf* genes was assessed. The abundances of mRNA transcripts corresponding to the genes upstream of the respective transposon were increased (Figure 2B). There was little effect on the accumulation of transcripts corresponding to the gene directly downstream of the

transposon, but mRNAs corresponding to *suf* genes further downstream, such as *sufU* and *sufB*, were decreased.

The *suf* genes are co-transcribed and transcription is modulated by sigma factor B. A previously published RNA-seq data set [36] was analyzed to further understand how the *sufD** and *sufS** transposons decreased transcription of *suf* genes downstream of the insertion sites. The reads that mapped to the *suf* genes were relatively evenly distributed (Figure 3A) leading to the hypothesis that the *suf* genes are transcribed as an operon using a common promoter.

We next analyzed whether the *sufCDSUB* genes are co-transcribed in *S. aureus*. Using a cDNA library generated from the WT as a template for PCR reactions, amplicons were generated that bridged various *suf* genes (Figure 3B), suggesting that the *sufCDSUB* genes were co-transcribed. To control for DNA contamination in the RNA preparation, PCR reactions were also attempted using a PCR template in which reverse transcriptase was left out of the reaction mixture, which is typically used to generate a cDNA library. Reactions using template not treated with reverse transcriptase did not generate amplicons showing that the amplicons were not the result of amplification from contaminating genomic DNA.

The reads from the RNA-seq data set [36] that mapped upstream of *sufC* were further analyzed. The first reads mapped to an adenine located 82 base-pairs upstream from the predicted translational start site (Figure 3C). Putative sigma factor A (σ^A) and sigma factor B (σ^B) recognition sequences were identified 14 and 28 base pairs upstream from the proposed transcriptional start site, respectively.

Sigma factor B is a general stress response transcriptional regulator in *S. aureus* [37]. The transcriptional activity of *sufC* was monitored in the WT and Δ*sigB* strains over

growth. The transcriptional activity of *sufC* is increased in a Δ*nfu* mutant, and therefore, a Δ*nfu* strain was included as a positive control [38]. The transcriptional activity of *sufC* was decreased in the Δ*sigB* strain (Figure 3D) confirming that sigma factor B positively influences *suf* transcription.

Decreased *suf* transcription results in lower activities of FeS cluster-requiring enzymes. The activities of enzymes that require FeS clusters for activity in the *sufS** and *sufD** strains was examined. Glutamate Synthase (GOGAT) is an FeS cluster-dependent protein that catalyzes the transfer of an amide group from L-glutamine to 2-oxoglutarate in order to produce L-glutamate [39]. The *sufS** and *sufD** strains had ~25% of the GOGAT activity of the WT (Figure 4A).

Aconitase (AcnA) is an FeS cluster-dependent enzyme that catalyzes the isomerization between citrate and isocitrate. The *sufS** and *sufD** strains had ~20% AcnA activity of the WT strain (Figure 4A).

S. aureus strains lacking accessory or non-essential factors utilized in the maturation of FeS proteins display more severe phenotypes when the dioxygen tension is increased ([15] and Mashruwala *et al*., in revision). The effect of dioxygen tension on AcnA activity in the WT, *acnA*, and *sufS** strains was assessed. A strain lacking *sodA*, which encodes for the major superoxide dismutase, was included as an experimental control. To modulate the concentration of dioxygen in the culture medium we varied the ratio of liquid media volume to culture vessel to gaseous headspace (HV ratio). The higher the HV ratio the higher the concentration of dissolved dioxygen [40]. A strain lacking SodA has low AcnA activity when cultured at a HV ratio of 20 and AcnA activity that is comparable to the WT when cultured at a HV ratio of 2.5 (Figure 4B), which confers that strains cultured at high HV ratios have increased ROS generation. AcnA activity was greatly decreased in the *sufS** strain and AcnA activity was not significantly altered as the

culture HV ratio was varied suggesting that the demand for the Suf FeS cluster biosynthetic system is not significantly influenced as the concentration of dioxygen is altered the culture medium.

Decreased Suf function results in a reduced rate of carbon flux through the TCA cycle. AcnA catalyzes the first committed step in the TCA cycle and therefore acts as a gatekeeper for flux though the TCA cycle. The finding that AcnA activity was decreased in the *sufS** and *sufD** strains led to the hypothesis that TCA cycle function would also be decreased in these strains.

The WT, Δ*acnA*, and *sufD** strains were cultured in TSB medium and growth was monitored over time. The initial growth rates of the WT, *sufD**, and Δ*acnA* strains were similar during exponential growth phase (<6 hours) (Figure 5A). During post-exponential growth phase (>6 hours) the *sufD** strain displayed a lag before growth commenced. The *acnA* mutant strain did not resume growth after this time, suggesting that growth beyond this inflection point requires TCA cycle function.

The activity of AcnA was also monitored over growth. The AcnA activity was decreased in the *sufD** strain throughout growth (Figure 5B). The largest difference in AcnA activity between the WT and *sufD** strains corresponded to the lag before (~8 hours) post-exponential outgrowth.

Acetate accumulated in culture media from all strains examined. Consistent with decreased TCA cycle function, acetate uptake was decreased and non-existent in the *sufD** and Δ*acnA* strains, respectively (Figure 5C). All the strains acidified the culture media at a similar rate. After ~6 hours the pH of medium from the WT and *sufD** cultures slowly increased, but the rate was lower in the *sufD** strain (Figure 5D). The pH of the spent medium from the Δ*acnA* strain did not increase after the initial acidification. Taken

together, these data were consistent with the hypothesis that AcnA activity was decreased in the *sufD** strain resulting in decreased flux through the TCA cycle.

Effective FeS cluster synthesis is necessary for cellular respiration. TCA cycle oxidation of fermentation byproducts results in electrons that can be used to respire dioxygen. The process of respiration requires FeS cluster synthesis. The respiratory enzyme succinate dehydrogenase (Sdh) requires an FeS cluster [12] and cytochrome oxidases require heme, which is synthesized using FeS cluster requiring proteins [41, 42].

The rate of respiratory flux in the WT, *sufD**, *sdhA*::Tn and *acnA*::Tn strains was determined using the tetrazolium dye XTT [43]. The *sufD**, *acnA*::Tn and *sdhA*::Tn strains exhibited significantly lower rates of XTT reduction, which is consistent with decreased electron flux through respiratory pathways (Figure 6A).

The resistance of *S. aureus* cells to aminoglycoside antibiotics is correlated with the electrical potential difference (Δψ) of the proton motive force (PMF) [44, 45]. Bacterial strains with decreased cellular respiration are more resistant to aminoglycoside antibiotics and prolonged treatment with aminoglycosides can select for respiration deficient strains [46, 47]. The effect of the aminoglycoside, kanamycin, on the growth of the WT and *sufD** strains was assessed. A *hemB* mutant, which is incapable of cellular respiration, was included as an experimental control. The *sufD** and *hemB*::Tn strains had more proficient growth in the presence of kanamycin than the WT (Figure 6B).

Decreased FeS cluster synthesis results in amino acid and vitamin auxotrophies. A number of *S. aureus* enzymes utilized to synthesize amino acids and vitamins require FeS clusters for function (Table 4). The growth of the WT, *sufD** and *sufS** strains was assessed on solid chemically defined media. The *sufS** and *sufD** strains did not grow on chemically defined media containing the 20 canonical amino acids. Supplementing the

growth medium with lipoic acid alleviated this growth defect. The *sufS** and *sufD** strains did not grow or grew poorly on chemically defined media containing lipoic acid, but lacking isoleucine, leucine, or glutamate and glutamine (Figure 7).

Decreased Suf function results in increased damaged DNA. The DNA repair enzymes MutY [48], Nth [49] and AddAB [10] each require an FeS cluster for function. Specific mutations in the *rpoB* gene, which encodes for RNA polymerase, provide bacterial cells with resistance to rifampicin [50]. The rate of spontaneous Rif resistance was determined for the WT, *sufD** and s*ufS** strains. Strains were plated onto TSB solid media containing rifampicin, as well as TSB media without rifampicin, allowing us to calculate the ratio of rifampicin resistant cells to total cells plated. The *sufD** and *sufS** strains both had a ~20-fold increase in rifampicin resistant cells when compared to the WT strain (Figure 8A).

We next examined if one or more of the described FeS cluster-requiring DNA repair enzymes had a role in preventing DNA mutagenesis when cultured under standard laboratory conditions. The rate of rifampicin resistance was determined using *mutY*::Tn, *nth*::Tn, and *addB*::Tn mutant strains. *S. aureus* cells lacking functional MutY, Nth, or AddAB gain resistance to rifampicin at a rate higher than the WT (Figure 8B).

The susceptibility of the WT, *sufD* and sufS** strains to chemical mutagens was investigated. The *sufD** and *sufS** mutants had increased sensitivity to growth in the presence of methyl methanesulfonate (MMS) (Figures 8C) or diethylsulfate (DES) (Figures 8D). The necessity for FeS cluster requiring DNA repair proteins for resistance to MMS or DES intoxication was examined. The *nth*::Tn and *mutY*::Tn mutant strains displayed sensitivities to MMS and DES that were similar to the WT (Figure 8E and 8F), but the *addB*::Tn mutant displayed increased sensitivity to both mutagens.

We sought genetic evidence to lend support to the hypothesis that decreased FeS cluster biogenesis was resulting in decreased AddAB activity and increased sensitivity to the DNA damaging agents. Unfortunately, we were incapable of creating *sufD* addB*::Tn or *sufS* addB*::Tn double mutant strains. However, we were able to create a Δ*nfu addB*::Tn double mutant. Like the *sufS** and *sufD** strains the Δ*nfu* mutant displayed increased sensitivity to MMS and DES (Figures 8E and 8F). The phenotypic effects of the *nfu* and *addB* mutations were not additive. Although not conclusive, these data suggested that strains defective in FeS cluster assembly have a decreased ability to repair damaged DNA because of decreased functionality of DNA repair proteins that require FeS clusters for function.

Decreased Suf function increases sensitivity to reactive oxygen and reactive nitrogen species. Oxidation of solvent accessible FeS clusters can result in cluster disintegration and impaired protein function (reviewed here [51]). Proteins requiring FeS cluster cofactors have been demonstrated to be targets for intoxication by reactive oxygen (ROS) and reactive nitrogen (RNS) species [4, 7].

The sensitivity of the *sufS** and *sufD** strains to growth in the presence of methyl viologen, which is a redox cycling agent that produces superoxide, was monitored. The *sufS** and *sufD** strains showed decreased growth when plated on solid media containing methyl viologen (Figure 9A). A strain lacking the major superoxide dismutase (SodA) also displayed decreased growth, verifying superoxide generation. The *sufS** and *sufD** strains also displayed decreased survival after challenge with a bolus of hydrogen peroxide (H_2O_2) (Figure 9B). A *kata*::Tn strain, which is unable to produce functional catalase, was included as a control.

Growth of the WT and *sufD** strains was monitored in chemically defined media in the presence and absence of nitroprusside, which interacts with intracellular thiols

resulting in the release of reactive nitrogen species [52]. The *sufD** mutant had a more severe growth defect than the WT when exposed to nitrosative stress. (Figure 9C).

***suf** mutant strains have altered intracellular Fe pools.* *S. aureus* strains lacking Nfu or bacillithiol have perturbed intracellular Fe pools [15, 28]. The hypothesis that the *suf** mutant strains have perturbed intracellular Fe pools was examined. Growth of the WT, *sufD** and *sufS** strains were monitored in the presence of 2,2-dipyridyl (DIP), which is a cell permeable divalent metal chelator with specificity for Fe [53]. The *sufS** and *sufD** strains displayed decreased growth when cultured in the presence of DIP (Figure 10A).

The antibiotic streptonigrin, in combination with Fe and an electron donor, causes DNA damage resulting in cell death [54]. Higher incidences of cell death are correlated with increased non-chelated intracellular Fe pools [55-57]. The *sufS** and *sufD** strains displayed an increased sensitivity to growth in the presence of streptonigrin (Figure 10B).

Streptonigrin can catalyze double stranded DNA breaks [58]. Strains defective in FeS cluster assembly were defective in repairing damaged DNA (Figure 8). We examined whether the increased streptonigrin sensitivity of strains defective in FeS protein maturation was the result of defective DNA repair. The streptonigrin sensitivities of the *nth*::Tn, *mutY*::Tn and *addB*::Tn strains were determined. The *nth*::Tn and *mutY*::Tn mutants had streptonigrin sensitivities similar to the WT, but the *addB*::Tn mutant displayed increased sensitivity to streptonigrin (Figure 10C). The streptonigrin sensitivity of the Δ*nfu* and Δ*nfu addB*::Tn mutants was also assessed. The streptonigrin sensitivity phenotypes of the *nfu* and *addB* mutations were additive suggesting that the increased streptonigrin sensitivity of strains defective in FeS cluster assembly was not exclusively caused by defective AddAB function in the Δ*nfu* strain.

Exoprotein production or biofilm formation is not significantly altered in *suf mutants.** *S. aureus* utilizes production and secretion of many virulence factors including, but are not limited to toxins, adhesins, proteases, and invasins, in order to increase success during pathogenesis [59]. The total abundances of exoproteins were quantified in the spent medium from the WT, *sufD** and *agrA*::Tn strains. An *agrA* mutant is deficient in exoprotein production, and therefore, was included as a control [60]. The *agrA*::Tn strain had decreased exoprotein production, but the apparent slight phenotype of the *sufD** strain did not reach statistical significance (p=0.049) (Figure 11A).

The amount of hemolytic toxins present in the spent media from WT, *sufD** and *agrA*::Tn strains was assessed by monitoring the ability of spent culture media to lyse rabbit erythrocytes. An *agrA*::Tn mutant has decreased production of hemolytic toxins and was included as a control. The WT and *sufD** strains showed similar hemolytic activity (Figure 11B), whereas exoproteins from the *agrA* mutant did not cause detectable lysis [60].

Biofilm formation was monitored aerobically using the WT, *sufD**, *agrA*::Tn and *sigB*::Tn strains. The *agrA* and *sigB* strains were included as experimental controls for increased and decreased biofilm formation, respectively [61, 62]. The WT and *sufD** strains formed similar amounts of biofilm whereas the *agrA*::Tn and *sigB*::Tn strains formed more and less biofilm than the WT, respectively (Figure 11C).

Effective FeS cluster biosynthesis is necessary for survival in human polymorphonucelar neutrophils. The findings that strains defective in FeS cluster synthesis had global metabolic defects, including increased intoxication by ROS and increased non-chelated Fe, led us to hypothesize that decreased FeS cluster synthesis would result in decreased survival in human polymorphonucelar neutrophils (PMNs).

We examined the ability of the WT, *sufD**, and *lacB*::Tn strains to survive challenge by human polymorphonucelar neutrophils (PMNs). The WT, *sufD**,* and *lacB*::Tn strains were individually combined with human PMNs and bacterial survival was monitored at various time points. The *lacB*::Tn strain was included to evaluate the contribution of the *bursa aurealis* transposon on bacterial survival. The *sufD** strain had decreased survival compared to the WT upon challenge with PMNs (Figure 12). The survival of the *lacB*::Tn strain was indistinguishable from the WT.

Discussion

Antimicrobial resistance is a problem for treating staphylococcal infections, as well as infections caused by alternate prokaryotic and eukaryotic pathogens including carbapenem-resistant *Enterobacteriaceae* [63] and artemisinin resistant *Plasmodium* spp. [64]. In this study we tested the hypothesis that the Suf FeS cluster biosynthetic system is a viable target for the development of antimicrobials. Suf-directed FeS cluster synthesis was essential for *S. aureus.* Iron acquisition and FeS cluster synthesis are predicted to be essential processes to nearly all organisms. We have assembled a list of human bacterial pathogens (Table 3) in which genetic screens have been used to predict essential genes. The genes that encode for FeS cluster biosynthesis factors are predicted to be essential (under standard laboratory growth conditions) for a majority of these organisms listed including *Mycobacterium tuberculosis*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *S. aureus*. The genomes of some organisms encode for multiple FeS cluster assembly machineries, which are, for the most part, functionally redundant. Lesions in genes encoding for individual FeS cluster synthesis factors are not lethal in these organisms (see *E. coli* and *Klebsiella pneumoniae*). Of the organisms that encode for an essential FeS cluster assembly system, Suf is the most widely distributed. These findings mirror the hypothesis that the Suf machinery is the most widely distributed FeS cluster biosynthetic system in bacteria [29]. In addition, mammals do not use the SufBCD system for FeS cluster biosynthesis [65].

Data presented suggest that *sufCDSUB* is transcribed as an operon and transposon insertions between *suf* genes caused decreased transcription of genes downstream in the operon resulting in decreased activities of FeS cluster dependent enzymes and global metabolic defects. Defective FeS cluster synthesis decreased growth on media lacking metabolites that require FeS cluster requiring proteins for synthesis.

Protein-associated and solvent-exposed FeS clusters are a primary target of ROS and RNS intoxication. The *suf** strains displayed increased sensitivity to H_2O_2, methyl viologen and nitroprusside. Decreased Suf function also resulted in a reduced flux through the TCA cycle, decreased cellular respiration, and destabilized non-chelated Fe pools. The *suf** strains had increased mutagenesis and a decreased ability to repair DNA, which was likely the result of decreased AddAB and Nth activities.

Decreased FeS cluster synthesis did not result in increased exoprotein excretion or α-toxin production. In addition, it did not result in increased biofilm formation, which is the etiological agent of chronic *S. aureus* infections [66]. However, the *sufD** strain displayed decreased survival in human PMNs. Similar findings were noted for a *S. aureus* strain lacking the FeS cluster carrier Nfu [15]. A *S. aureus* Δ*nfu* mutant also displayed decreased tissue colonization in a mouse model of infection [15]. A recent Tn-seq study identified *S. aureus* genes that are necessary for fitness in various models of infection. We created a list of predicted *S. aureus* FeS proteins (Table 4) and found that a number of processes that require FeS proteins are required for fitness including DNA repair (AddAB, Nth), RNA modification (MiaB), central metabolism (AcnA, Fdx, SdaA), and heme synthesis (HemN, HemH). The Nfu, SufA and SufT FeS cluster assembly factors were also required for fitness during infection.

We further analyzed the list of predicted *S. aureus* FeS proteins (Table 4) and the results from high-density transposon screens in hopes of determining why Suf may be essential [14, 16, 67, 68]. Besides *sufCDSUB*, the only genes encoding a FeS proteins predicted to be essential were *fdx* (ferredoxin) *hemH* (ferrochelatase) and *addB* (DNA helicase/exonuclease). Fdx and AddB were reported essential in one of the three studies whereas HemH was reported essential in two of the studies. The fact that we were able

to reconstruct the *addB::Tn* mutation in the USA300_LAC strain argues against AddB being essential in this background.

A couple of scenarios could explain the essentiality of Suf in *S. aureus*. There may be a currently unidentified FeS cluster requiring protein(s) or pathway that is essential for *S. aureus*. In support of this argument, replacing the native isoprenoid pathway, which requires FeS proteins (IspG and IspH), with the mevalonate pathway that does not rely on FeS proteins, allowed for *E. coli* viability in the absence of FeS cluster synthesis machinery [69]. In an alternate scenario, the wide variety of metabolic defects resulting from the lack of FeS cluster synthesis may result in metabolic standstill and cell death. The data presented herein support the latter argument. We found that decreased Suf function caused global metabolic defects in diverse processes including cofactor synthesis, DNA repair, intracellular Fe processing, cellular respiration, protection from ROS intoxication, and carbon processing. We should note that the genome of *E. coli* is predicted to encode 100+ FeS proteins, which are most likely in the apo-state in the Δ*isc* Δ*suf* strain and these strains are still viable if the need for IspG and IspH are bypassed [70].

Would Suf be a good drugable target? The core Suf biosynthetic system is large ($SufC_2BD$ 160 kDa) and requires assembly of three functional proteins. Suf also requires ATP hydrolysis for assembly of FeS proteins [23]. ATP hydrolysis drives large-scale conformational changes suggesting that the SufBCD is dynamic when building and transferring FeS clusters [71]. FeS biogenesis in *S. aureus* also requires sulfur mobilization from cysteine, catalyzed by SufS and the sulfide trafficking factor SufU. The Suf complex must interact with a number of alternate proteins including iron and electron donors, FeS cluster carriers, and/or target apo-FeS proteins. A number of additional FeS cluster assembly factors have been identified in *S. aureus* including SufT, Nfu and bacillithiol that may also may interact with Suf. Interactions with these factors, assuming

that they interact at alternate Suf surfaces, would require multiple interaction sites and potentially large surface areas. Cysteine desulfurases are PLP-dependent enzymes that can be divided into two groups based on sequence similarity. Group one cysteine desulfurases consist of SufS and CsdA, and group two consists of NifS and IscS. The human mitochondrial NFS1 is necessary for FeS cluster synthesis and belongs to group two which suggest that SufS could be an additional target for antimicrobial therapy in SufS dependent organisms [72-74]. The antimicrobial D-cycloserine irreversibly inactivates PLP-dependent enzymes and it was found to inhibit *Plasmodium falciparum* SufS and retard the infection lifecycle [75, 76]. We have not seen a Suf dependent effect of D-cycloserine in *S. arueus* (data not shown).

In summary, the data presented show that Suf-dependent FeS cluster biosynthesis is essential for *S. aureus*. We characterized two *S. aureus* strains that had decreased transcription of *suf* genes resulting in decreased activities of FeS cluster dependent enzymes. Defective FeS cluster synthesis resulted in broad metabolic defects and decreased survival in human PMNs. The data presented show that Suf is a viable antibacterial target in *S. aureus*. The *suf** mutants examined and plasmids constructed will provide a valuable genetic toolbox for the identification of Suf inhibitors and for further characterization of FeS cluster synthesis in *S. aureus*.

Future experimentation will involve the further characterization of *suf* genes. One gene, *sufT*, is hypothesized to be involved in FeS cluster assembly and maturation [28, 38]. Strains containing suppressor mutations were isolated on limiting methionine medium and the various mutations were mapped within the *rsbU* gene. These suppressor strains were able to bypass numerous defects exhibited by a Δ*sufT* mutant which include but are not limited to a Δ*sufT* mutant's lipoic acid auxotrophy and defects in the FeS cluster-requiring aconitase enzyme activity. Further suppressor analyses will give more insight

into the mechanism that SufT follows and the potential roles that other proteins like RsbU may play in facilitating FeS cluster assembly and maturation.

Materials and Methods

Materials. Phusion DNApolymerase, deoxynucleoside triphosphates, quick DNA ligase kit, and Restriction Enzymes were purchased from New England Biolabs. The plasmid mini-prep kit, gel extraction kit, and RNA protect were purchased from Qiagen. Trizol, High-Capacity cDNA Reverse Transcription Kits, and XTT (2,3-Bis-(2-Methoxy-4-Nitro5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) were purchased from Life Technologies. Oligonucleotides were obtained from Integrated DNA Technologies and are listed in Table 2. DNase I was purchased from Ambion. Lysostaphin was purchased from Ambi products. DL-Threo-3-isopropylmalic acid was purchased from Wako Pure Chemical Co. Tryptic Soy broth (TSB) was purchased from MP Biomedical. Difco BiTek agar was added (15 g L^{-1}) for solid medium. Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich and were of the highest purity obtainable.

Bacterial growth and media. Bacterial strains used in this study are listed in Table 1. Unless noted otherwise, these strains were built in the community-associated MRSA USA300 strain LAC that had been cured of the plasmid conferring resistance to erythromycin, pUSA03 [14]. The chemically defined minimal media was described previously [15] and where noted, was supplemented at 0.5 µg mL^{-1} with lipoic acid. *S. aureus* strains cultured in TSB were grown at 37°C with shaking at 200 rpm in 8 mL culture tubes containing 1 mL of liquid media unless otherwise stated. Top-agar overlays were made by diluting overnight cultures grown in TSB 1:100 in PBS and then adding 100 µL to 4 mL of 3.5% tryptic soy agar (TSA) before pouring atop of TSA plates. When noted, 1 µL of 2.5 mg mL^{-1} streptonigrin dissolved in DMSO, 4 µL of neat diethylsulfate or 6 µL of neat methyl methanesulfonate were spotted in the center of the plates. Antibiotics were added to TSB medium at the following concentrations: 3-5 ng mL^{-1} anhydrotetracycline (A-tet); 30 µg mL^{-1} chloramphenicol (Cm); 1.25 µg mL^{-1} rifampicin (Rif); 10 µg mL^{-1}

erythromycin (Erm). To maintain plasmids media, was supplemented with 15 μg mL^{-1} of chloramphenicol. Methyl viologen and 2,2-dipyridyl were added to solid media at 30 mM and 250mM, respectively.

Liquid phenotypic analysis was conducted in 96-well microtiter plates containing 200 μL of media using a BioTek 808E Visible absorption spectrophotometer and culture densities were read at 600 nm. Cells used for inoculation were cultured for 18 hours in TSB medium and cells were washed using PBS. The optical densities of the cell suspensions were adjusted to 2.5 (A_{600}) with PBS. Two μL of the washed cells were added to 198 μL of media. Where noted, sodium nitroprusside and kanamycin were added to liquid media at 4μm and at 4 μg mL^{-1}.

Genetic and recombinant DNA techniques. All transductions were conducted using phage 80α [77]. 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).

The Suf depletion plasmids were created as described elsewhere [78]. Briefly, the *sufC* gene and the 5' untranslated region was amplified using the sufCup and sufCdwn primers. The resulting amplicon was gel purified and treated with 0.03 units of DNase I (Ambion Carlesbad, CA) for 5 minutes. The digested DNAs were separated using agarose gel electrophoresis and DNAs of approximately 250 base pairs were purified. The purified fragments were treated with T4 DNA polymerase (NEB; Ipswich, MA) and subsequently treated with Taq DNA polymerase (NEB). The DNA fragments were cloned into pCR2.1_TOPO (Thermo-Fisher). After transformation and selection the colonies were pooled and plasmids purified. Plasmids were digested with EcoRI, insert fragments were gel purified and subsequently subcloned into pML100. After transformation and selection, colonies containing pML100 [79] were pooled and plasmids were purified and transformed

into *S. aureus* RN4220 and plated on TSA-Cm. Individual chloramphenicol resistant RN4220 colonies were inoculated into 200 µL of TSB-Cm medium in 96-well microtiter plates and cultured overnight. Cells were subcultured into liquid TSB media with and without A-tet and strains with decreased growth in the presence of A-tet were retained. Two positive clones were identified and the insert was confirmed by DNA sequencing using the purified plasmid as template.

RNA-seq analysis of suf operon. RNA-seq data was downloaded from the Gene Expression Omnibus (GEO accession number GSE48896) corresponding to NCTC8325-4 [36]. The downloaded SRA files were converted to fastq format using SRA toolkit then mapped to the *Staphylococcus aureus* genome using Tophat [107, 108]. The resulting bam files were sorted and indexed using SAMtools [109] then converted to tdf format using Integrative Genomics Viewer (IGV) tools [110]. The image of the *suf* operon was acquired using IGV [110].

Protein analysis. **Glutamate synthase (GltBD or GOGAT) assays**. GOGAT assays were conducted as previously described with slight modifications [28]. Briefly, strains were cultured overnight in TSB, cells were pelleted by centrifugation and resuspended in PBS (1:1). The resuspended cells were used to inoculate 5 mL (30 mL tube) of chemically defined media containing 20 AA and lipoic acid to an OD of 0.1 (A_{600}). Strains were cultured at 37ºC with shaking to an OD of 0.8 (A_{600}), cells were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.7). Cells were lysed anaerobically by the addition of 4 µg lysostaphin and 8 µg DNase. Cells were allowed to incubate at 37°C until full lysis observed (about 1 hour). Cell debris was removed by centrifugation. GOGAT was assayed by the addition of 60 µL of 50 mM glutamine (pH 7.7), 60 µL of 5 mM α-ketoglutarate (pH 7.7), 60 µL of cell-free extract and 60 µL of 0.75 mM nicotinamide adenine dinucleotide phosphate (NADPH) to 600 µL of lysis buffer. GOGAT activity was

determined by monitoring the rate of NADPH oxidation at 340 nm for 5 min [ε340 nm = 6.22 mM−1 cm−1 [111].

Aconitase assays. AcnA assays were conducted as previously described with slight modifications [38]. Strains were cultured overnight in TSB before diluting them into fresh TSB to an optical density of 0.1 (A_{600}). Cultures were diluted into 0.5 mL or 4 mL of TSB in 10 mL culture tubes. Cells were cultured for either 8 hours (Figure 4) or over growth (Figure 5) before they were harvested by centrifugation and cell pellets stored at -80°C. Cells were thawed and anaerobically resuspended with 200 µL of AcnA buffer (50 mM of Tris, 150 mM of NaCl, pH 7.4) and lysed by the addition of 4 µg lysostaphin and 8 µg DNase. Cells were allowed to incubate at 37°C until full lysis observed (~1 hour). Cell debris was removed by centrifugation and AcnA activity was assessed as previously described [112][15].

Protein concentration determination. Protein concentration was determined using a copper/bicinchoninic acid based colorimetric assay modified for a 96-well plate [82].

RNA isolation and quantification of mRNA transcripts. The WT, *sufD**, and *sufS** strains were cultured overnight in TSB (~18 hours) and diluted into 80 mL of fresh TSB to a final OD of 0.05 (A_{600}) in 300 mL flasks in order to mimic the growth conditions used for the growth and acetate accumulation experiments displayed in Figure 5. Cells were cultured for 8 hours before harvesting by centrifugation. Cells were treated with RNAProtect (Qiagen) for 10 min at room temperature, pelleted by centrifugation and cell pellets were stored at −80°C. Pellets were thawed and washed twice with 0.5 mL of lysis buffer (50 mM of RNAse-free Tris, pH 8). Cells were lysed by the addition of 20 µg of lysostaphin and incubated for 30 min at 37°C. RNA was isolated using TRIzol reagent (Ambion–Life Technologies) as per manufacturer protocol. DNA was digested with the

TURBO DNA-free kit (Ambion–Life Technologies). The cDNA libraries were constructed using isolated RNA as a template and a High Capacity RNA-to-cDNA Kit (Applied Biosysytems). An Applied Biosystems StepOnePlus thermocycler and *Power* SYBR Green PCR Master Mix (Applied Biosystems) was used to quantify DNA abundance. Primers for quantitative real-time PCR of the *sufC*, *sufD*, *sufS*, *sufU*, and *sufB* transcripts are listed in Table 2 and were designed using the Primer Express 3.0 software from Applied Biosystems.

***H_2O_2 killing assays*.** Bacterial strains were cultured with shaking to OD 10 (A_{600}) in TSB. Cells were pelleted by centrifugation and resuspended in an equal volume of PBS. The optical densities for all the strains were adjusted to an OD of 0.7 (A_{600}) in a total volume of 1 mL of 1X PBS. Cells were subsequently challenged with a bolus of H_2O_2 (500mM) and incubated for 1 hour at room temperature. Fifty µL of the reaction mixture was diluted 1:20 into PBS buffer containing catalase (1300 units mL^{-1}) and incubated for five minutes. Colony forming units (CFU) were determined by serially diluting cells and spot plating upon TSB agar plates.

Determination of pH profiles and acetic acid concentration in spent media. Strains cultured overnight in TSB (~18 hours) were diluted into 80 mL of fresh TSB to a final OD of 0.05 (A_{600}) in 300 mL flasks. At the indicated times, aliquots of the cultures were removed, culture OD (A_{600}) determined, and the cells and culture media were partitioned by centrifugation at 14,000 rpm for 1 minute. Two mL of either the culture supernatant or sterile TSB, which served to provide a pH reading for the point of inoculation, were combined with 8 mL of distilled and deionized water and the pH was determined using a Fisher Scientific Accumet AB15 pH mV Meter. The concentration of acetic acid in spent media was determined using the BioVision Acetate Colorimetric Assay kit following the manufacturer's suggested protocol.

***Static model of biofilm formation*.** Biofilm formation was examined as described elsewhere, with minor changes [62]. Briefly, overnight cultures were diluted into biofilm media to a final optical density of 0.05 (A_{590}) and added to the wells of a 96-well microtiter plate and incubated statically at 37 °C for 22 hours. Prior to harvesting the biofilms, the optical density at A_{590} of the cultures was determined. The plate was subsequently washed with water, the biofilms were heat fixed at 60 °C and the plates were allowed to cool to room temperature. The biofilms were stained with 0.1% crystal violet and washed to remove non-bound stain. The plates were dried and subsequently destained by the addition of 33% acetic acid and the absorbance of the resulting solution was recorded at 570 nm. The absorbance at A_{570} was standardized to an acetic acid blank and subsequently to the optical density of the cells upon harvest (A_{590} numbers). Finally the data were normalized with respect to the WT strain to obtain relative biofilm formation.

Total exoprotein analyses*.* Spent media supernatants were obtained from overnight cultures and filter sterilized with a 0.22-μm (pore-size) syringe filter and standardized with respect to culture optical densities (A_{600}). Subsequently, exoproteins were extracted from the spent media supernatant using standard trichloroacetic acid precipitation. The resultant protein pellets were resuspended and protein concentrations were determined using the biuret assay. The data were subsequently normalized with respect to the WT strain.

Hemolysis assays*.* The hemolytic activity of staphylococcal exoproteins was determined as previously described [83]. The data were subsequently normalized with respect to the WT strain.

XTT reduction assays*.* Measuring the reduction of the tetrazolium dye XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)- 2H-Tetrazolium-5-Carboxanilide) was used to assay respiratory activity. The intermediate electron carrier N-methyl dibenzopyrazine methyl

(PMS), was used to accelerate the reaction. Overnight cultures were grown in chemically defined 20 amino acid medium containing lipoic acid and then diluted 100-fold into fresh defined medium (~0.1 A_{600}). Strains were cultured with shaking to a final OD of 0.7 (A_{600}). Cells were washed with PBS and suspended in an equal volume of PBS. Five µL of culture was added to a 195 µL of reaction buffer containing 0.4 mg mL^{-1} PMS and 0.5 mg mL^{-1} of XTT. XTT reduction to its formazan derivative was monitored at 450 nm over time using a microtiter plate and BioTek 808E Visible absorption spectrophotometer. Data were analyzed as percent increase in absorbance relative to the initial absorbance reading.

Mutagenesis frequency. Overnight cultures (n=10) were grown in TSB medium were diluted 1:100 into fresh TSB (~0.1 A_{600}). The cells were cultured with shaking for 48 hours at 37°C. One-hundred µL of culture was spread plated on TSA plates supplemented with 1.25 µg mL^{-1} of rifampicin and colony forming units (CFUs) were determined after 36 hours incubation. Cultures were also serial diluted and spot-plated on TSA to determine total CFU. Mutagenesis frequency was calculated by dividing number of rifampicin resistant colonies by total CFUs.

Transcriptional reporter analysis. Strains containing the p*sufC*p transcriptional reporter plasmids were grown in TSB-Erm medium overnight. Cultures were then diluted (1:100) into 5 mL of fresh TSB-Erm media and allowed to grow for 30 hours, during which 200 µL aliquots were removed at varying time points and fluorescence and culture optical density (OD) (A_{600}) were measured with a Perkin Elmer HTS 7000 Bio Assay Reader. GFP was excited at 485 nm and emission was read at 535nm. Fluorescence was standardized with respect to culture OD.

***Opsonophagocytic killing assay*.** Strains were grown overnight in TSB and sub-cultured (1:100) the following day for 3 hours in fresh TSB. Human primary polymorphonuclear neutrophils (PMNs) were isolated by Dextran gradient as described previously [113]. Prior

to infection, 96-well plates were coated with 20% human serum in RPMI 1640 (10mM HEPES+0.1% human serum albumin (HSA)) for 30 minutes at 37°C. Following subculture of the bacteria, strains were opsonized with 20% human serum for 30 minutes at 37°C, washed and diluted to an approximate density of 2.5 X 10^7 CFU mL^{-1}. Approximately, 250,000 PMNs per well in a 96-well plate were infected with approximately 2.5 X 10^6 CFU to generate a multiplicity of infection (MOI) of 10. With the exception of time (T)=0, the infections were centrifuged at 1,500 RPM for 7 minutes to synchronize the bacteria with the PMNs. During centrifugation, 1% saponin was added to the T=0 infections to lyse the PMNS, CFUs were then determined by serial dilution plating on TSB. This procedure was followed for the remaining time points, up to T=180 minutes.

Blood samples were obtained from anonymous healthy donors as buffy coats (New York Blood Center). The New York City Blood Center obtained written informed consent from all participants involved in the study. This research was approved by the New York University School of Medicine institutional human subjects board.

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