CHARACTERISATION OF SUBSTRATE PREFERENCE IN *STAPHYLOCOCCUS* AUREUS SIDEROPHORE BIOSYNTHESIS

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Maxim Kolesnikov

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CHARACTERISATION OF SUBSTRATE PREFERENCE IN *STAPHYLOCOCCUS AUREUS* SIDEROPHORE BIOSYNTHESIS

submitted by	Maxim Kolesnikov	in partial fulfillment of the requirements for
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Examining Committee:

Dr. Michael Murphy, Microbiology and Immunology Supervisor

Dr. Lindsay Eltis, Microbiology and Immunology Supervisory Committee Member

Dr. Lawrence McIntosh, Biochemistry Additional Examiner

Additional Supervisory Committee Members:

Dr. Pauline Johnson, Microbiology and Immunology Supervisory Committee Member

ABSTRACT

Staphylococcus aureus is a common opportunistic pathogen and commensal resident of a majority proportion of the adult population. Emerging drug-resistant and hypervirulent strains such as methicillin-resistant *S. aureus* (MRSA) have reduced the efficacy of existing treatment options. Iron acquisition from the host is required for the establishment of infection. *S. aureus* possesses several mechanisms for iron acquisition, including the ferric-iron binding siderophores staphyloferrin A (SA) and staphyloferrin B (SB).

To explore the substrate preference of the SA and SB biosynthetic enzymes, crystal structures of biosynthetic enzymes were solved, and alternative substrates were tested. Crystal structures of the synthetases SfaD and SbnF were solved and compared to homologs from other species to define structural determinants of substrate preference. An analogue of SA, substituting D-lysine for D-ornithine during synthesis was produced *in vitro* and characterized using liquid chromatography and mass spectrometry. Furthermore, *S. aureus* was shown to be able to use this SA analogue for iron acquisition. Analogues of intermediates in the SB biosynthesis pathways were produced *in vitro*. The biosynthesis of a functional *S. aureus* siderophore analogue provided insights into the structures and substrate specificities of siderophore synthesis proteins. The modified siderophores may be of use to deliver antimicrobials into the cell or as a diagnostic for *S. aureus* infection.

LAY SUMMARY

Staphylococcus aureus is a common bacterial pathogen that causes severe diseases across the globe. Iron is an important metal that is needed by bacteria for growth. To infect the human body, *S. aureus* must steal iron from the host. To acquire iron *S. aureus* secretes small molecules called siderophores into the environment to find and bind iron. These are eventually reabsorbed by the bacterium. This work characterized the machinery that builds these siderophores in *S. aureus* and examined how it can be manipulated to produce modified products, including a new siderophore. Making modifications to these siderophores could be used to attach antibiotics as a new therapy or label to create diagnostic tools. If developed, this work may help design new compounds to fight *S. aureus* infections.

PREFACE

The work in this thesis involved contributions by several fellow scientists from the laboratories of Dr. Michael Murphy and Dr. Lindsay Eltis. Development and optimization of the HPLC detection assay and siderophore utilization bioassay was a collaborative effort between Dr. Jason Grigg, Mariko Ikehata and myself. Adam Crowe and Eugene Kuatsjah of the Eltis lab provided assistance with MS analysis of siderophore samples.

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LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CA-MRSA	Community-associated MRSA
Can	Collagen adhesin
C-DAE	Citryl-diaminoethane
ClfA	Clumping factor A
EHEC	Uropathogenic Escherichia coli
FnBPA	Fibronectin-binding protein A
Fur	Ferric uptake regulator
HPLC	High-pressure liquid chromatography
IPTG	Isopropyl β-D-thiogalactopyranoside
Isd	Iron-surface-determinant
K _d	Dissociation constant
LB	Luria-Bertani broth
L-DAP	L-2,3-diaminopropionic acid
MALDI-MS	Matrix-assisted laser desorption/ionization mass spectroscopy
MESG	2-amino-6-mercapto-7-methylpurine ribonucleoside
MRSA	Methicillin-resistant Staphylococcus aureus
MS	Mass spectrometry
NEAT	Near iron transporter
NIS	NRPS-independent siderophore
NRPS	Nonribosomal peptide synthetases

PDB	Protein data bank	
PLP	Pyridoxal 5'-phosphate	
PNP	purine nucleoside phosphorylase	
PP _i	Pyrophosphate	
PVL	Panton-Valentine Leucocidin	
SA	Staphyloferrin A	
SB	Staphyloferrin B	
SCCmec	Staphylococcal cassette chromosome mec	
SD	Standard deviation	
SeMet	Selenomethionine	
TMS	Tris minimal succinate	
TSST	Toxic shock syndrome toxin	
WT	Wild-type	

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Chapter 1: Introduction

1.1 Staphylococcus aureus pathogenesis

Staphylococcus aureus is a gram-positive opportunistic pathogen, frequently found as a human commensal in the general population [1]. *S. aureus* colonizes nearly a third of the adult population, residing commonly on the skin in locations such as the nasal cavity, underarms, groin, and forearm [2, 3]. The predominant site of a persistent *S. aureus* population is in the nasal cavity, with researchers linking nasal colonization to medical complications as early as the 1930s [4, 5]. Although often asymptomatic, *S. aureus* is currently known to cause a wide variety of infectious diseases including bacteremia, toxic shock syndrome, infective endocarditis as well as multiple skin and soft tissue infections [6]. Moreover, it is one of the five most commonly acquired nosocomial infections, imposing an annual economic cost on society that has been estimated to be as large as 13.8 billion dollars in the United States alone [7, 8].

Treatment of *S. aureus* has been exacerbated over the decades by the emergence of drugresistant strains [9]. Early clinical use of penicillin, although effective, was limited by the rapid spread of penicillin-resistance in *S. aureus*, first identified in the 1940s [10, 11]. This was eventually followed by methicillin and vancomycin resistance in 1961 and 2002, respectively [12, 13]. Vancomycin resistance, in particular, has raised a great degree of concern as vancomycin treatment is currently one of the last therapeutic options available for methicillinresistant *Staphylococcus aureus* (MRSA) infections [14]. Vaccine development has been unsuccessful with several trials failing at the phase II stage [15-17].

The global threat of *S. aureus* is most commonly associated with MRSA, the emergence of which was first described in 1961 shortly after the introduction of methicillin [11]. Globally, MRSA often represents more than half of infective *S. aureus* isolates, spread by existing clones

or horizontal transfer of the staphylococcal cassette chromosome *mec* (SCC*mec*) which confers resistance to methicillin and by extension most β -lactam antibiotics [18, 19]. Resistance to β lactams, in conjunction with increased resistance to other antibiotics, has been exacerbated by changes in MRSA epidemiology in the 1990s which lead to infections in communities of healthy individuals with no prior hospitalization such as athletes and enlisted personnel [20, 21]. This is in contrast to MRSA infections initially being relegated to hospitals [22]. Such strains with increased virulence have now been reclassified as community-associated MRSA (CA-MRSA) and their emergence has expanded the reach of *S. aureus* infections and underscored the need for new treatment options [23].

The relative success of *S. aureus* in causing severe infections can be attributed to its wide range of virulence factors that provide mechanisms for evading the immune response from the host as well as factors for nutrient acquisition [24]. The presence and specificity of virulence factors vary between strains as many virulence genes are found on mobile genetic elements [25]. In general, *S. aureus* gains access to sterile tissues via open wounds and uses adhesin proteins such as clumping factor A (ClfA), collagen adhesin (Can), and fibronectin-binding protein A (FnBPA) to anchor itself in the host [26-29]. Secreted virulence factors such as toxins are regulated by the global virulence regulator Agr, for example, alpha-toxin and Panton-Valentine Leucocidin (PVL) which lyse red blood cells, leukocytes and damage surrounding tissue [30-34]. Lysis of the surrounding tissue provides access to essential nutrients, in particular, transition metals, such as iron. Additional toxins, such as the toxic shock syndrome toxin (TSST), which stimulates the release of IL-1, IL-2, TNF- α , and other cytokines, act to evade and overwhelm the immune system [32, 35]. *S. aureus* resistance to oxidative stress is provided by the production of the enzymes catalase and superoxide dismutase, which counteract hydrogen peroxide and

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superoxide produced by leukocyte activity [36-42]. These factors contribute to the high persistence of *S. aureus*, with studies showing its ability to survive and proliferate within host macrophages and epithelial cells [43-45].

1.2 Iron acquisition by *Staphylococcus aureus*

1.2.1: Iron uptake strategies of S. aureus

Transition metals are essential nutrients to nearly all forms of life due to their use as enzymatic cofactors in key intracellular processes [46]. Iron, in particular, is a crucial component of numerous systems such as in respiration where iron-bound haem is incorporated into cytochromes for electron transport [47]. A strategy commonly used by humans and other vertebrates to prevent infection is the restriction of these nutrients from pathogens. The various mechanisms which sequester Fe, Mn, Zn, and other essential nutrients are collectively termed nutritional immunity and form a defence against microbial proliferation [48-51]. Within the human host, over 90% of iron is located intracellularly [52, 53]. The most abundant carrier of iron is the haemoprotein haemoglobin found within erythrocytes [53, 54]. Iron located in serum is bound by the proteins transferrin and lactoferrin, while additional mechanisms provide regulated circulation and recycling of iron within the host [51, 55-57]. *S. aureus* has evolved several distinct and redundant iron-uptake systems to overcome these limitations.

When presented with an iron-limited environment, *S. aureus* alters its protein expression profile via the ferric uptake regulator (Fur) [58, 59]. In the presence of iron, iron-bound Fur binds to the Fur box, a short segment of DNA with a defined consensus sequence, and represses downstream genes [58, 59]. When iron is limiting, apo-Fur dissociates from DNA, allowing gene

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expression [58, 59]. The Fur regulon contains several gene loci encoding for iron and heme uptake systems.

As the most abundant source of iron in the human body, haem is a target of the *S. aureus* iron-surface-determinant (Isd) system [60-65]. This system contains several cell-wall anchored receptors as well as a membrane-bound transporter and intracellular proteins. Haemoglobin and haemoglobin-haptoglobin complexes are bound by receptors containing <u>NEA</u>r iron <u>T</u>ransporter (NEAT) domains, which extract haem and transfer it via other proteins to the IsdEF ABC transporter [62, 66-68]. Intracellular haem is subsequently degraded, providing iron to the bacterium [62]. The acquisition of haem from haemoglobin is a crucial virulence factor that is utilized in concert with haemolytic toxins secreted by *S. aureus* during infection [69]. During colonization and stages of infection where haem is not available, such as survival within macrophage phagolysosomes, iron may be acquired instead via the use of siderophores, small molecular weight ferric-iron binding molecules secreted by many bacterial species [69].

1.2.2: Siderophore-mediated iron acquisition

Siderophores are a diverse family of ferric-iron chelators, produced and secreted into the environment by numerous microbial species [70-72]. The affinity of these small (100-1000 Da) molecules for iron has been estimated to be in the range of 10^{12} - 10^{52} M [71, 73]. To date, over 500 individual siderophores have been characterized in bacteria, fungi and certain plants [70-72, 74]. Consequently, siderophore-mediated iron acquisition is commonly a key virulence factor for many human pathogens, including *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* [75]. Siderophores are generally classified into three types based on the chemical groups that coordinate the Fe³⁺ atom: catecholates, hydroxamates, and carboxylates (Figure 1.1), additionally

mixed-type siderophores containing more than one type of chemical group have been characterized [76-78]. *S. aureus* produces two chemically distinct hydroxamate type siderophores: staphyloferrin A (SA) and staphyloferrin B (SB) [69].



Figure 1.1 Schematic of siderophores, highlighting different types of iron-coordinating groups.

Enterobactin (A), produced by *Escherichia coli* utilizes catecholate functional groups. Ferrioxamine (B) and pyochelin (C) represent examples of siderophores containing hydroxamate and mixed-type coordinating groups, respectively. Iron-coordinating groups are labelled in red.

SA is a carboxylate-type siderophore that is comprised of two citrate molecules joined by

a D-ornithine backbone (Figure 1.2A) and is produced by a variety of staphylococci, including S.

aureus and S. epidermidis [79, 80]. In S. aureus, SA biosynthesis and export is managed by

products of the sfaABCD operon which is Fur regulated via an intergenic Fur box [81-83].

Assembly of SA has been shown to require a functioning TCA cycle as a source of citrate and is

therefore linked to environmental factors that may disrupt TCA cycle function in *S. aureus*, such as high glucose concentrations [82, 84]. As glucose is the second most abundant metabolite in human serum, the role of SA during infection is limited to solid tissue. Recent studies have identified a role for this siderophore in specific environments such as in abscesses or epithelial cells [85]. Also, the full role of SA during infection is yet to be determined.

SB is also a carboxylate type siderophore, and is produced by a smaller number of staphylococcal species than SA [86]. SB is synthesized form L-2,3-diaminopropionic acid (L-DAP), citrate and α -ketoglutarate (Figure 1.2B) [87]. In *S. aureus*, SB is assembled and exported by products of the *sbn* operon [87]. Synthesis of SB is supported by additional enzymes for biosynthesis of the precursors citrate (SbnG), L-DAP and α -ketoglutarate (SbnA, SbnB and SbnI) [88-92]. Gene deletion mutants of *S. aureus* unable to produce SB show a significantly decreased ability to survive iron-restricted conditions and decreased virulence in mouse infection models [93]. These conclusions have been supported by gene expression data showing upregulation of the *sbn* operon in serum [58, 94].



Figure 1.2 SA (A) and SB (B) coordinate ferric iron in a hexadentate manner. Groups that participate in iron binding are coloured in red.

1.2.3: Transport of SA and SB

Ferric-iron bound SA and SB are taken up from the environment via the ABC transporters HtsABC and SirABC, respectively [83, 95]. Both receptors are specific for their respective siderophore with dissociation constants (K_d) in the sub-nanomolar range [96, 97]. However, both transporters require the function of an ATPase named *fhuC*, as neither operon encodes a dedicated ATPase to power transport across the membrane [98]. Crystal structures of HtsA in complex with SA and SirA in complex with SB show both siderophores bound in positively-charged pockets that undergo conformational changes upon binding (Figure 1.3) [96, 97].



Figure 1.3 Crystal structures of HtsA (A) and SirA (B) bound to SA and SB, respectively, show positively-charged substrate-binding pockets.

1.2.4: Transport of xenosiderophores

In addition to dedicated transport of SA and SB, *S. aureus* encodes a nonspecific receptor system targeting hydroxamate type siderophores produced by other bacteria for transport. This is mediated by the receptors FhuD1 and FhuD2, as well as the permease FhuBG [99, 100]. This complex is presumed to have a lower affinity for ligands, allowing it to transport a broad spectrum of xenosiderophores available in the immediate environment of the bacterium. Currently, four siderophores have been identified as transported via this system: aerobactin, coprogen, ferrichrome A and desferrioxamine B [86]. Similarly, the SstABCD system is able to transport catecholate-type siderophores such as bacillibactin and ferrated catecholamines [101]. The presence of these systems provides an advantage in complex communities and may play a role in infections with several bacterial pathogens present.

1.2.5: Siderophore synthesis

Despite the great diversity in their structures, most siderophores are assembled by one of two distinct methods: nonribosomal peptide synthetases (NRPS) and the NRPS-independent siderophore (NIS) synthetase pathway [102]. Most hydroxamate, catecholate, and mixed-type siderophores are assembled from amino and/or aryl acids by NRPSs, large multifunctional enzyme complexes [102, 103]. Homologous complexes synthesize peptide antibiotics in other species [104]. NRPSs consist of a single or multiple polypeptide chains that form an assembly line with different catalytic sites for siderophore production [70, 102]. Generally, an NRPS consists of an adenylation domain, peptidyl carrier protein and a condensation domain [70, 102]. Synthesis of the siderophore in these active sites is defined by the covalent attachment of intermediates to the individual domains [70, 102]. Several examples of this pathway have been characterized and the mechanisms involved described [70, 102].

The lesser studied NIS pathway synthesizes the majority of carboxylate type siderophores [102, 105, 106]. These include aerobactin and achromobactin found in various bacterial genera. This pathway consists primarily of so-called NIS-synthetases with other enzyme classes occasionally involved in siderophore assembly [102, 107]. Unlike NRPS-mediated synthesis, all NIS synthetases are free enzymes without covalent retention of intermediates.

Most of the NIS synthetases discovered to-date cluster together in a single superfamily based on sequence and structure and can be divided into categories based on their substrate specificities. Type A synthetases utilize citric acid, type B α -ketoglutarate, and type C monoamide/monoester derivatives of a citric acid molecule, often the product of a previous synthesis step [102]. A mechanism for the NIS family was proposed based on the aerobactin pathway and validated with the structure of the type A synthetase AcsD from *Pectobacterium*

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chrysanthemi [105-107]. The active site in AcsD consists of an ATP-binding site that is conserved across the known NIS synthetase family, as well as the catalytic arginine R305 and ATP-coordinating histidine H444, shown to play a key role in catalysis [107]. All three classes catalyse a Mg^{2+} -dependent condensation of a carboxylic acid with either an amine or alcohol. The carboxylate is initially adenylated by a single ATP molecule, followed by displacement of the AMP group by the nucleophilic amine or alcohol (Figure 1.4 B) [107]. Catalytic residues and the Mg^{2+} ion act to correctly position substrates in the active site and polarize bonds to facilitate nucleophilic attack [107].



Figure 1.4 Schematic of a generic condensation reaction catalysed by NIS synthetases. (A) Initial adenylation of a carboxylic acid displaces a pyrophosphate group and is followed (B) by a nucleophilic attack by either an amine or alcohol. R-COO is a citric acid, α -ketoglutarate or citrate-derived molecule.

1.2.6: SA and SB synthesis pathways

S. aureus synthesizes SA and SB via two distinct NIS pathways [81, 87]. SA is

assembled by products of the Fur-regulated sfa gene locus, which encodes the NIS synthetases

SfaD, SfaB, the putative ornithine racemase SbnC, and a membrane-embedded efflux protein, SfaA [81]. As described previously, SA consists of two citrate molecules bridged by a Dornithine backbone. Citrate is likely diverted from the TCA cycle, as SA production shuts down when the TCA cycle is inactive, and SbnC likely provides D-ornithine via conversion from Lornithine pools present in the cell [81, 82]. SA is synthesized by the sequential reactions of SfaD and SfaB (Figure 1.5) [81]. Both enzymes group by protein sequence with type-B NIS synthetases but are defined as type A due to their utilization of citrate [81]. SfaD activates citrate by adenylation to form a citrate-adenylate intermediate, which reacts with the δ -amine of Dornithine. The remaining α -amine present on the intermediate acts as the nucleophile on the citrate-adenylate intermediate produced by SfaB. SA can be synthesized *in vitro* using SfaD, SfaB and the necessary substrates [81].



Figure 1.5 Biosynthetic pathway of SA.

SA is synthesized by the sequential reactions of SfaD and SfaB from two molecules of citric acid and one molecule of D-Ornithine.

SB is synthesized in four steps by three NIS synthetases and a pyridoxal 5'-phosphate (PLP)-dependent decarboxylase encoded on the *sbn* locus (Figure 1.6) [87]. PLP-dependent enzymes exist as a Schiff base which is displaced by the substrate [108]. Synthesis begins with the type A synthetase SbnE condensing L-DAP with citrate via the β -amine, producing the first intermediate [87]. This is followed by PLP-dependent decarboxylation by SbnH of the L-DAP originating carboxyl group. Addition of another L-DAP molecule by the type C SbnF and α -ketoglutarate by the type B SbnC results in the final SB molecule. *In vitro* synthesis of SB has been shown using these four enzymes and the necessary substrates [87].



Figure 1.6 Biosynthetic pathway of SB.

SB is synthesized by the NIS synthetases SbnE, SbnF, and SbnC as well as the decarboxylase SbnH from two molecules of L-DAP and one molecule each of citric acid and α -ketoglutarate.

1.3 Applications of modified siderophores

Modified siderophores have been proposed as a tool for both scientific and clinical purposes with extensive research into siderophore conjugation to other effector molecules [109]. Several naturally occurring examples exist such as analogues of hydroxamate type siderophores called sideromycins, produced by several species from the *Streptomyces* and *Actinomyces* genera [110, 111]. These molecules consist of siderophores or siderophore mimics conjugated to antimicrobial molecules, such as albomycin where the siderophore is coupled to a nucleoside analogue via a peptide linker [109]. Efforts to produce siderophore conjugates for possible "trojan horse" applications began in earnest in the 1980s and have resulted in multiple proof-ofconcept studies with *in vitro* activity against target species [109]. In particular, catechol conjugates to β -lactam antibiotics, such as between pyoverdine from *Pseudomonas aeruginosa* and ampicillin, were shown to be effective [112]. Similarly, siderophores conjugated to cationized BSA to increase binding to immune cells were used to successfully vaccinate and prevent uropathogenic *Escherichia coli* (EHEC) infection in mice [113]. However, high rates of resistance have been reported for these approaches and although there is currently a conjugate siderophore undergoing clinical trials, no compounds have yet made it to the clinic [109, 114].

Aside from bactericidal applications, other uses for conjugated siderophores have been proposed. A vibrioferrin conjugate to a modified fluorophore was shown to selectively target several vibrios including *V. parahaemolyticus*, *V. cholerae and V. vulnificus* in a complex bacterial community containing *S. aureus* and *E. coli* [115]. These results suggest the possibility for applications in the scientific community for rapid analysis of bacterial diversity.

Synthesis of siderophore conjugates has been limited in some cases by the difficulty of purifying or synthesizing the siderophore. No protocol exists for high-yield purification of SA or SB and while both have been chemically synthesized, yields remain a significant challenge [116, 117]. While recent progress has been made with synthesizing some modified siderophores *in vitro*, very little work has been done with carboxylate type siderophores and enzymes of the NIS pathway.

1.4 Objectives

The emergence of S. aureus as a hypervirulent and multi-drug resistant pathogen has underlined the need for novel treatment methods. Acquisition of iron from the host plays an important role during infection and can be hijacked by siderophore conjugates as shown in several other bacterial species. Additionally, new ideas for alternative uses of conjugated products present an opportunity to provide tools to the field of S. aureus research. The relative lack of research into carboxylate type siderophore conjugation with useful molecules and low yields from chemical synthesis provide a rationale for determining a method for modified siderophore production *in vitro*. For the first part of my research, I characterized the substrate preferences of the NIS synthetases SfaD, SbnE, and SbnF as well as the decarboxylase SbnH. This was followed by the production of a modified SA, detected and validated by a combination of HPLC and MS. Utilization of the modified siderophore by S. aureus for iron-acquisition was demonstrated by bioassay. Structures of the NIS synthetases SfaD and SbnF provided insight into the similarity of these proteins to other members of the family and were used to generate several active site mutants to confirm catalytic residues. This study has provided insights into the structure and substrate preferences of the NIS synthetase family of enzymes and created an initial protocol for the production of a modified SA.

Chapter 2: Methods

2.1 Bacterial strains and growth conditions

DNA was propagated using *Escherichia coli* strain DH5 α . Proteins were expressed using *E. coli* strain BL-21 (DE3). Strains were grown in Luria-Bertani broth (LB). For selection and plasmid maintenance all media was supplemented with 25 µg ml⁻¹ of kanamycin.

Bacteria	Strain name	Description or genotype	Source or reference
Escherichia coli	BL-21	F-ompT gal dcm lon hsdSb(rb-mb-) λ(DE3 [lacI lacUV5-T7 gene ind1 sam7 nin5]) [malB+]κ-12(λs)	Novagen
Escherichia coli	DH5a	F-endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB 20 φ 80dlacZ Δ M15 Δ (lacZYAargF) U169, hsdR17(rk-mk+), λ -	Life Technologies
Staphylococcus aureus	JE-2	USA300, SCCmecIV, pvl+	University of Nebraska Medical Center

Table 2.1: Bacterial strains used in this study

2.2 Site-directed mutagenesis

SfaD and SbnF were predicted to contain several conserved catalytic residues. To validate these predictions, whole plasmid site-directed mutagenesis was used to generate point mutations in the target amino acid sequence. SfaD R351A, H496N, SbnF R292A, and H426N were generated using the same method. Using existing protein expression constructs on the pET28A vector, complementary mutagenesis primers were designed and purchased from Integrated DNA Technologies with a 5' phosphate tag. Amplification reactions contained Phusion polymerase (NEB), Ampligase (Epicentre) as well as sufficient nucleotides to amplify and ligate mutant plasmid. Amplicons were subsequently digested with DpnI for 3 hours at 37°C

to remove methylated or hemi-methylated DNA. Ten μl of digestion product was transformed into heatshocked *E. coli* DH5α cells. Mutations were confirmed by sequencing (Genewiz Inc).

Table 2.2:	List of	plasmids	used in	this study	
				•/	

Plasmid name Description		Antibiotic Resistance	Source or references
pET28a	Expression vectors carrying an N terminal His-tag/thrombin/T7 tag	Kanamycin in E. coli	EMD Biosciences
pET28a-SbnE	pET28a derivative for SbnE expression	Kanamycin in E. coli	[87]
pET28a-SbnH	pET28a derivative for SbnH expression	Kanamycin in E. coli	[87]
pET28a-SbnF	pET28a derivative for SbnF expression	Kanamycin in E. coli	[87]
pET28a-SbnC	pET28a derivative for SbnC expression	Kanamycin in E. coli	[87]

Table 2.3: List of primers used in this study

Description	Names of Primer(s)	Primer sequence (5'-3')
SfaD R351A	R351A 5'mutagenesis	CATATTACAGGTGAGATT <mark>GC</mark> TACATTA
		TCAGAACAAACG
SfaD H496N	H496N 5'mutagenesis	GCATTGCATTAGAGGCT <mark>A</mark> ACTTGCAAAA
		TGCTATTGC
SbnF R292A	R292A 5'mutagenesis	GAACACTT CAACGAAA <mark>GC</mark> AGTGTTGGCG
		CCTCATAC
SbnF H426N	H426N 5'mutagenesis	GGTATTGCCTTTGAATCG <mark>A</mark> ATGCACAAAAT
		ATGATGCC

2.3 **Protein expression and purification**

Proteins were purified using the same protocol unless otherwise noted. One liter of LB supplemented with 25 μ g/mL kanamycin was inoculated with 5 mL of an overnight culture of BL-21 cells with the desired protein encoded within a pET-28a vector. The culture was grown at 37°C for 4-6 h until an OD₆₀₀ of 0.6-1. The culture was then induced by addition of 0.3 mM isopropyl β-D-thiogalactopyranoside (IPTG) and incubated for 16-20 h at 22°C with constant

shaking at 200 rpm. Cell pellets were collected by centrifugation at 4000 rpm for 7 minutes, resuspended in 40 mM Tris (pH 8.0), 100 mM NaCl and lysed at 10,000-13,000 psi using an EmulsiFlex-C5 homogenizer (Avestin). Supernatants containing protein were isolated by centrifugation at 18,000 rmp for 1 h. Collected supernatant was loaded on a 5 mL HisTrap HP column (GE Healthcare) and eluted with a linear imidazole gradient (0-500 mM). Collected protein was dialyzed overnight into 40mM Tris (pH 8.0) at 4°C. Dialysed fractions were further purified by anion exchange chromatography using a Source 15Q column (GE Healthcare) and eluted with a NaCl gradient (0-500 mM). Collected fractions were dialyzed overnight into 40mM Tris, pH 8.0, containing 100 mM NaCl at 4°. All proteins were concentrated to >10mg/mL, flash frozen and stored at -80°C. Selenomethionine-incorporated (SeMet) SfaD was expressed by methods previously described using a defined minimal media with supplemented selenomethionine [118]. Purification was done using the same procedure as all other proteins used in this study.

2.4 NIS synthetase pyrophosphate release assay

NIS synthetases catalyse a condensation reaction utilizing one molecule of adenosine triphosphate (ATP) and releasing one molecule each of adenosine monophosphate (AMP) and pyrophosphate (PP_i). The *S. aureus* NIS synthetases SfaD, SbnE, and SbnF conform to this mechanism and were assayed for turnover with wild-type (WT) and alternative substrates using the EnzCheck pyrophosphate assay kit (Sigma). In the presence of inorganic phosphate, the substrate 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) is converted to ribose 1-phosphate and 2-amino-6mercapto-7-methylpurine by purine nucleoside phosphorylase (PNP). This results in a shift of the absorbance maximum from 330 nm to 360 nm. Phosphate is

generated by inorganic pyrophosphatase which converts PP_i to phosphate. The measured absorbance gain at 360 nm represents turnover of the enzyme.

UV-visible spectra were measured using a Varian Cary 50 UV-visible spectrophotometer. A 200 μ L reaction contained 1 mM ATP, 1 mM of any additional enzyme substrate, 200 μ M MESG, 10 μ L 20X reaction buffer (1.0 M Tris-HCl, 20 mM MgCl₂, pH 7.5, 2 mM sodium azide), 1 U PNP, 0.03 U inorganic pyrophosphatase and 10 μ M NIS synthetase. Reactions were started by addition of the NIS synthetase and followed for 5-10 minutes.

Absorption data were plotted as standard deviation (SD) + error. Initial substrate concentrations were kept consistent unless derived from a mixed isomer stock, in which case all calculated rates defined as apparent. To determine reaction rates, the slope of a linear portion of the progress curve was measured for a period of one to three minutes starting at 30 seconds after the addition of enzyme. The period measured depended on the enzyme studied: SfaD 3 min., SbnE 2 min., SbnF 1 min. All rates calculated for SbnF are defined as apparent due to substrate concentration being derived from an estimate.

2.5 CO₂ release assay of SbnH

SbnH catalyses the second step in the SB pathway and is a decarboxylase to produce CO_2 and citryl-diaminoethane. The rate of CO_2 formation by SbnH in the presence of either its WT substrate or modified products produced by SbnE was measured via a coupled enzymatic assay with phosphoenolpyruvate carboxylase (catalyses the addition of bicarbonate to phosphoenolpyruvate to form oxaloacetate) and malic dehydrogenase (catalyses the NADHdependent reduction of oxaloacetate to form malate) [119].

Oxidation of NADH by malic dehydrogenase was measured at 340 nm for three minutes. The reactions were performed in 75 mM Tris-HCl, 25 mM NaCl, 8 mM MgSO₄, pH 8.0 (Buffer A). To remove residual CO₂ from the reactions, Buffer A (200 mL) was purged with N₂ for 1 hour and then placed in an anaerobic glove box maintained under a positive N₂ atmosphere to equilibrate for 16 hours. The powdered forms of the substrates, malic dehydrogenase, and phosphoenolpyruvate carboxylase were placed into the glove box, allowed to equilibrate overnight and were dissolved in Buffer A. SbnH was also introduced into the glove box where all the following steps were undertaken at 25 °C. Reactions were performed in a total volume of 500 μ L and monitored at 340 nm for three minutes using a NanoDrop 2000c UV-visible spectrophotometer (ThermoFisher). Each reaction contained 0.2 mM NADH, 500 μ M phosphoenolpyruvate carboxylase and an estimated 200 μ M SbnH substrate. The reactions were initiated with the addition of 10 μ M SbnH. All reactions were run in triplicate.

For data analysis, SbnH enzyme activity was defined as an absorbance change after three minutes that was greater than the negative control.

2.6 Synthesis of SB intermediates, SA and D-Lys-SA

As SbnH and SbnF, the second and third enzymes in SB synthesis, respectively, use pathway intermediates which are not commercially available, a one-pot reaction mixture was adapted from *in vitro* synthesis of SA and SB, as described by other groups [81, 87]. This method was also used to generate SA and D-Lys-SA used for HPLC and MS analysis.

To generate substrate for SbnH, 1 mL reactions containing 1 mM citrate, 1 mM L-DAP, 3 mM ATP, 1mM MgCl₂ and 10 μ M SbnE were set up in water and incubated overnight at room temperature. Protein was filtered out by centrifugation using nanosep protein centrifugal filters (PALL Laboratory) at 10,000 g for 10 minutes. The resulting filtrate was collected and used for subsequent assays. Alternative products were generated by replacing either citrate or L-DAP in

the reaction mixture. SbnF product generation followed the same procedure but was supplemented with $10 \mu M$ SbnH.

Production of SA and testing of alternative SfaB products were set up as 1 mL reactions containing 2 mM citrate, 1 mM D-ornithine, 3 mM ATP, 1 mM MgCl₂ and 10 μ M of SfaD. For alternative SfaB products, D-ornithine or citrate was substituted out. The reactions were incubated overnight, and protein was removed by filtration. SfaB to 10 μ M was added to continue the reaction and removed following 6-8 hours of incubation. The reaction products were processed with the same procedure as described above.

To estimate the concentration of the final product in the reaction mixtures, samples were analysed by HPLC. Reaction mixtures were analysed using an Agilent 1260 Infinity HPLC equipped with a Waters Xbridge 3.5 µm Amide column (2.1 x 100 mm). Compounds were mixed 50:50 with acetonitrile prior to injection, run in 1.25 mM ammonium acetate (pH 5.1 by glacial acetic acid) and eluted with acetonitrile. Peaks at 210 nm corresponding to ATP and AMP were identified by comparison with standards and a decrease of the ATP peak was measured. The product was estimated to be at 90% of precursor concentration.

2.7 SbnF structure determination

Initial SbnF crystals were grown by setting up the JCSG Core Suites crystallization screen (QIAGEN) using the sitting-drop vapor diffusion method at 4 °C in 2-µl drops with a 1:1 mixture of ~16.7 mg/mL SbnF and reservoir condition. Hits were identified based on visual examination under a microscope and selected for further optimization. Crystals grown for structure determination were grown with reservoir conditions of 0.18 M tri-ammonium citrate and 20% w/v PEG 3350 at 4 °C. For cryoprotection crystals were soaked for ~30 seconds in reservoir buffer supplemented with 30% (v/v) glycerol. Crystals were then flash-frozen in liquid nitrogen and stored until data collection. Crystals were initially screened on a Rigaku MicroMax 007-HF generator, VariMax HR optics, and Saturn CCD 944+ detector. Diffraction data were collected at the Stanford Synchrotron Radiation Lightsource beamline 7-1. Diffraction data were processed and scaled using XDS [120]. Crystals grew in the space group $P2_12_12_1$ with two molecules in the asymmetric unit (Table 2.4). Phase determination by molecular replacement was conducted using the NIS synthetase AsbB from *Bacillus anthracis* (PDB entry code 3TO3), using the AutoSol program in Phenix [121]. Model building and refinement were performed using the Phenix suite and further visualized using COOT [121, 122]. Structure figures were made using Chimera [123].

Data collection			
Space group	$P2_{1}2_{1}2_{1}$		
Cell dimensions			
a, b, c (Å)	71.72, 137.96, 150.00		
α, β, γ (°)	90, 90, 90		
Resolution range (Å)	37.48 - 1.80 (1.86 - 1.80) *		
R-merge	0.050 (0.335)		
Ι/σΙ	13.5 (3.1)		
CC1/2	0.996		
Refinement			
Resolution (Å)	1.8		
No. reflections	136049		
R-work	0.1653 (0.2304)		
R-free	0.1946 (0.2818)		
No. atoms			
Macromolecules	9572		
Ligand	39		
Water	1313		
Average B-factor (Å ²)	28.50		
Protein	27.20		
Ligand/ion	43.70		
Water	37.90		
R.m.s deviations			
Bond lengths (Å)	0.017		
Bond angles (°)	1.52		
Ramachandran favored (%)	97		
Ramachandran outliers (%)	0.17		

 Table 2.4: Data collection and refinement statistics for SbnF

*Values in parentheses are for the highest-resolution shell.
2.8 SfaD structure determination

SfaD crystal conditions were identified and optimized in the same manner as for SbnF. Crystals were grown with a reservoir condition of 0.1 M MES (pH 5.0), 5% w/v PEG 6000 with a final pH of 6.0 at 4 °C. For cryoprotection crystals were soaked for ~30 seconds in reservoir buffer supplemented with 25% (v/v) glycerol. Data were collected at the Stanford Synchrotron Radiation Lightsource beamline 7-1. Diffraction data were processed and scaled using XDS. Selenomethionine (SeMet) crystals of SfaD were grown in the same reservoir conditions as native crystals. Data for SeMet crystals were collected at the Canadian Light Source beamline 08B1-1. Diffraction data were processed and scaled using XDS. Phasing of the SeMet dataset was done by PhaserMR and an initial structure was built by Autobuild from the phenix suite [121]. The structure was refined as for SbnF

Data collection	
Space group	$C222_1$
Cell dimensions	
a, b, c (Å)	77.74, 116.81, 165.45
α, β, γ (°)	90, 90, 90
Resolution range (Å)	38.9 - 2.0 (2.1 - 2.0)*
R-merge	0.0228 (0.125)
Ι/σΙ	15.03 (5.12)
CC1/2	0.999 (0.936)
Refinement	
Resolution (Å)	2.0
No. reflections	101622
R-work	0.1771 (0.2096)
R-free	0.2207 (0.2506)
No. atoms	5583
Macromolecules	5108
Water	475
Average B-factor ($Å^2$)	30.60
Protein	29.97
Water	39.16
R.m.s deviations	
Bond lengths (Å)	0.008
Bond angles (°)	1.01
Ramachandran favored (%)	97
Ramachandran outliers (%)	0

Table 2.5: Data collection and refinement statistics for SfaD

*Values in parentheses are for the highest-resolution shell.

2.9 HPLC analysis of SA and alternative SfaB products

As direct measurement of SfaB activity was not possible due to background signals from protein aggregation, reaction mixtures were set up with all successful substitute substrates identified for SfaD as described above and analysed by HPLC to detect final products. Reaction mixtures were analysed using an Agilent 1260 Infinity HPLC equipped with a Waters Xbridge 3.5 µm Amide column (2.1 x 100 mm). Compounds were mixed 50:50 with acetonitrile prior to injection, run in 1.25 mM ammonium acetate (pH 5.1 by glacial acetic acid) and eluted with acetonitrile.

Detection of iron-bound SA and alternative products of SfaB was done by continuous monitoring at 340 nm during the sample run. Additional validation of the detected peaks was carried out by supplementation of the sample by 0.8 mM FeCl₃ before injection and comparison of peak height to un-supplemented samples.

2.10 Mass spectrometry of SA and D-Lys-SA

SA and D-Lys-SA reactions prepared and processed as described above were filtered using a 0.2 μ m syringe filter and diluted to an estimated concentration of 100 μ M with acetonitrile-water (3:97 vol/vol) supplemented with 0.1% formic acid. Diluted sample was directly injected into an Agilent 6550 time of flight mass spectrophotometer. Analysis of the sample was performed with electrospray ionization in negative ion mode with a scanning range of 200-500 or 200-600 *m/z*. The peaks corresponding to SA and D-Lys-SA were determined based on the characteristic [M-H⁺] -479 and 493 *m/z* fragments.

2.11 Bioassay of SA and D-Lys-SA samples

To evaluate whether D-Lys-SA could be used by *S. aureus* for iron acquisition, a bioassay on iron-chelated media previously described was used [83]. In brief, *S. aureus* JE2 was

seeded into Tris Minimal Succinate (TMS) square agar plates [124]. D-Lys-SA samples were prepared by HPLC purification by collecting the 340 nm absorption peak corresponding to D-Lys-SA. The collected peak sample was concentrated and applied to sterile paper disks placed on the seeded agar plates. A control sample using SA collected and processed in an identical manner and a negative control sample of filtered, sterile H₂O were applied to filter discs as well. Agar plates containing samples were incubated at 37°C for 24-48 h and the growth radius was recorded.

Chapter 3: Results

3.1 Characterization of the structure and substrate preference of SA pathway NIS synthetases

3.1.1: Structure of SfaD

SA is synthesized by the sequential reaction of the NIS synthetases SfaD and SfaB from two molecules of citrate and one molecule of D-ornithine (Figure 1.5). SfaD is the first enzyme in the pathway, catalysing a condensation reaction between one molecule each of D-ornithine and citrate, utilizing one molecule of ATP in the process. Although the use of citrate defines SfaD as a type A NIS synthetase, its protein sequence is most similar to type B synthetases. Of the available structures in the protein data bank (PDB), the closest homolog based on protein sequence is the type C NIS synthetase AsbB from *Bacillus anthracis* (26% identity). The closest type A homolog is IucA from *Klebsiella pneumoniae* (24% identity).

To determine whether the structure of SfaD represented a novel type of NIS synthetase, the crystal structure of SeMet labelled SfaD was solved by anomalous dispersion and used to phase a native dataset of SfaD to a final resolution of 2 Å. The structure represents a nearly complete polypeptide, with 4 residues missing at the N-terminus, 2 at the C-terminus and no discernable density for three flexible loop regions of 13, 4 and 10 residues in length (Figure 3.1). The structure does not have substrates or other large molecules bound. Visually, the structure of SfaD conforms to the architecture of previously described NIS synthetases and comprises of three α -helix rich domains in a "cupped hand" topology. However, comparison of SfaD with the type A NIS synthetase IucA from *K. pneumoniae* and type A synthetase AcsD from *Pectobacterium chrysanthemi* shows limited structural similarity with RMSD values, based on alignment of the C α atoms, of 3.0 Å and 3.4 Å, respectively (Table 3.1). A structural similarity search of structures in the PDB, identified the type C synthetase IucC from *K. pneumoniae* as the most similar with an RMSD value of 3.0 Å (Table 3.1). Superimposition with an ATP-bound structure of AcsD shows that, although the overall structures are dissimilar the ATP-binding pocket is relatively well conserved (Figure 3.2). Repeated attempts to grow crystals in the presence of the native substrates, as well as to soak compounds into existing crystals, resulted in several crystals and datasets which neither improved resolution nor contained density for substrates or missing residues.

NIS synthetase	RMSD (Å)	Z-Score	Number of residues aligned	PDB entry
IucC	3.0	36.5	506	6CN7
AcsD	3.4	36.2	539	2W02
IucA	3.0	35.6	514	5JM8
AsbB	3.2	30.5	541	3TO3
DfoC	3.0	29.8	535	5070
AlcC	3.1	29.2	538	2XO2

Table 3.1: Structural similarity of SfaD to NIS synthetases

Superimposition of SfaD with structures deposited in the PDB using the DALI server, ranked by Z-score [125]. Hits with a Z-Score lower than 2 are considered spurious.



Figure 3.1 Structure of SfaD.

(A) The structure of SfaD conforms to the fold of previously described NIS synthetase structures but lacks density for several flexible loop regions. (B) A close-up view of the active site region

denoted by the red box in (A). The red line connects endpoints of a 13 amino acid segment absent from the structure.



Figure 3.2 Conserved ATP binding site of SfaD compared to AcsD. Superposition of SfaD (cyan) to an ATP-bound AcsD (tan) structure shows a conserved ATP binding site. Conserved residues within hydrogen bonding distance are displayed as sticks. Atoms are coloured by heteroatom, and ATP carbon atoms are coloured purple.

3.1.2: Active site mutants of SfaD.

As the structure of SfaD did not provide details regarding enzyme function due to missing residues and lack of bound substrates, a biochemical approach was taken to define the catalytically important residues. The function of key residues in homologous NIS synthetases has been defined by other groups and is best characterized in the type A synthethase AcsD form the achromobactin synthesis pathways in *P. chrysanthemi*. Sequence alignment of SfaD with AcsD and other type A synthetases identified a conserved predicted catalytic arginine and an ATP-coordinating histidine at positions 351 and 496 respectively. The R351A and H496N site-

directed mutants were generated by PCR, transformed into *E. coli* and confirmed by sequencing. Purified protein mass was confirmed by MALDI-MS.

Catalysis of ATP by SfaD and other NIS synthetases produces pyrophosphate (PP_i) as a by-product of the reaction. As the SfaD substrates are commercially available, the reaction can be monitored spectroscopically at 360 nm by coupling PP_i release to conversion of a fluorescent nucleotide (Section 2.4). Using a standard reaction containing defined concentrations of substrates, the specific activity of SfaD and active site mutants was determined (Section 2.4). Lack of enzyme turnover was defined as a specific activity at or below the negative control rate. Neither the R351A nor the H496N mutant showed detectable activity (Table 3.2 and Figure 3.2). Histidine 208 is another conserved residue in NIS synthetases and in the AcsD structure this residue interacts with the central carboxyl group of citric acid. A H208A variant was made and was found to retain wild-type activity (Table 3.1).





Activity of active site SfaD mutants was measured by a coupled pyrophosphate release assay monitored at 360 nm for 10 minutes. No absorbance change was detected for either the R351A (blue) or H496N (green) mutant. All measurements performed in triplicate. Data plotted as standard deviation and error (thin dotted line).

Table	3.2	Specific	activity	of SfaD	mutants
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SfaD mutant	Specific activity (nmole min ⁻¹ µM ⁻¹)
Physiological reaction	560 ± 40
Negative control (no D-ornithine)	60 ± 40
R351A	N.D.
H496N	N.D.
H208A	550 ± 40

^aThe specific activity was determined using a coupled pyrophosphate release assay. The reactions were initiated by adding 10 μ M SfaD. All measurements were performed in triplicate. N.D. (Not Detected) defined as activity below negative control sample.

3.1.3: Substrate preference of SfaD

To characterize its substrate preference, the specific activities of SfaD with analogues of the native substrates D-ornithine and citric acid were determined using the PP_i release assay described previously. Analogues were selected based on commercial availability and presence of the required amine and carboxyl groups necessary for the condensation reaction to occur as described (Figure 1.4). Successful catalysis by SfaD was defined as a significant specific activity above the negative control. SfaD turnover in reactions with a single substituted component was detected with 6 of out 7 D-ornithine and 3 out 8 citric acid substitutions tested (Table 3.3). Additionally, SfaD is able to utilize either enantiomer of ornithine and lysine (Figure 3.4 A and B). Replacement of citrate with alternate compounds showed turnover with DL-isocitric acid, hydroxycitrate and tricarballylic acid (Figure 3.5 B and D). The specific activities measured for DL-isocitric acid, diaminobutyric, diaminopropionic acids and 5-hydroxylysine are apparent due to the mixed isomer stocks used (Figure 3.4 C).



Figure 3.4 Activity of SfaD with D-ornithine and its analogues.

(A) Turnover as measured by absorbance at 360 nm for SfaD physiological reaction (red), Lornithine substitution (blue) and control reaction without D-ornithine present (black). (B) Absorbance of physiological reaction (red), negative control (black), DL-5 hydroxylysine (green), L-Lysine (orange) and D-Lysine (purple). (C) Absorbance of physiological reaction (red), negative control (black), DL-diaminobutyric acid (yellow), DL-diaminopropionic acid (blue) and putreschine (purple). Reactions were measured continuously for 10 minutes. All measurements performed in triplicate. Data plotted as standard deviation and error (thin dotted line).



Figure 3.5 Activity of SfaD with citrate and its analogues.

(A) SfaD turnover as measured by absorbance at 360 nm with WT substrates (red), glutaric acid (blue), 3OH3CH3 glutaric acid (blue) and a negative control (black). (B) Turnover of physiological reaction (red), negative control (black), tricarballylic acid (blue) and diglycolic

acid (brown). (C) SfaD turnover with WT components (red), negative control (black), DL-malic acid (orange) and malonic acid (green) with separate negative controls for either (yellow and cyan). (C) Turnover of WT SfaD reaction (red), negative control (black), hydroxycitrate (green) and DL-isocitric acid (brown). Reactions were monitored for 10 minutes. All measurements performed in triplicate. Data plotted as standard deviation and error (thin dotted line).

D-ornithine substitute	Specific activity (nmole min ⁻¹ µM ⁻¹)	Citrate substitute	Specific activity (nmole min ⁻¹ μM ⁻¹)
L-ornithine	420 ± 40	Glutaric acid	N.D.
D-lysine	610 ± 40	3-hydroxy-3- methylglutaric acid	N.D.
L-lysine	170 ± 40	DL-malic acid ^a	N.D.
DL-2,3-			
Diaminopropionic	580 ± 40	Malonic acid	N.D.
acid ^a			
DL-2,4-	620 ± 40	Diglycolic acid	N.D.
Diaminobutyric acid ^a	020 - 10		
DL-5-Hydroxylysine ^a	480 ± 40	DL-Isocitric acid ^b	350 ± 40
Putrescine	N.D.	Hydroxycitrate	380 ± 40
		Tricarballylic acid	550 ± 40
Physiological reaction (D-ornithine + citrate)	560 ± 40	Negative control (- D- ornithine)	60 ± 40

Table 3.3 Specific activity of SfaD^a

^aAll measurements were performed in triplicates. N.D. (Not Detected) is defined as activity below negative control sample.

^bReported specific activity is apparent.

3.1.4: Substrate preference of SfaB

The synthesis of SA is catalysed by the sequential reactions of SfaD and SfaB. SfaB is a type A synthetase that catalyses the condensation of citrate with the δ -citryl-D-ornithine product of SfaD (Figure 1.5). The identification of alternative substrates for SfaD allowed testing of both substrates of SfaB. Several attempts were made to adapt the previously described PP_i release assay for use with SfaB. However, high background signals due to aggregation of protein interfered with measurements. Instead, reaction mixtures containing SfaD and substrates were set up and incubated overnight. These reactions were filtered to remove SfaD, SfaB was added and

incubated for 6-8 hours. Following the second incubation, SfaB was removed and the sample was analysed by HPLC using an amide column. Control reactions were set up without SfaB to distinguish the final product from the SfaD-generated intermediate. Further controls included a physiological reaction mixture to generate SA. Iron-bound SA can be detected by HPLC by absorption at 340 nm and is distinct from intermediate signal (Figure 3.6). As no iron was supplemented into the reaction mixture, synthesized SA is believed to be a mix of apo and holo-SA. Analysis of reactions with citrate and each of the identified SfaD substrate analogues showed a single peak present for the reaction with D-lysine (Figure 3.7A). No peak was observed in the intermediate control (no SfaB present). Henceforth this product is referred to as D-Lys-SA. As the WT product of SfaB is a functional siderophore, approximately equimolar FeCl₃ was added to the reaction mixture prior to HPLC analysis to evaluate whether the product was capable of binding ferric iron. The peak in the HPLC trace of FeCl₃-supplemented sample eluted at the same time point but with a greater absorption at 340 nm than sample without supplementation (Figure 3.7B).



Figure 3.6 Detection of SA by HPLC.

HPLC trace of wild type synthesis reaction run through an amide column and monitored at 340 nm. SA t_R 17.4 min. Reaction control t_R 16.8 min.



Figure 3.7 D-Lys-SA detection by HPLC.

D-Lys-SA ($t_R = 17.4 \text{ min}$) was detected via HPLC as a single peak at 340 nm by analysing the reaction mixture using an amide column (A). D-Lys-SA peak signal was significantly increased by the addition of FeCl₃ to the reaction mixture prior to analysis ($t_R = 17.5 \text{ min}$) (B).

3.1.5: Validation of D-Lys-SA

The modified product detected by HPLC, D-Lys-SA, represents an extension of the Dornithine backbone by one methylene group and would increase the molecular weight from 480.123 Da to 494.137 (Figure 3.8). To confirm the identity of this molecule, reaction mixtures were tested by negative ion-mode MS. MS analysis confirmed the presence of a species with a weight of 493.157 that was absent in reactions lacking SfaB (Figure 3.9B). Analysis of reaction mixtures of citrate with D-ornithine to produce SA showed a peak corresponding to a weight of 479.120 that was absent in reactions without SfaB (Figure 3.9A).



Figure 3.8 Predicted structure and iron-coordination of D-Lys-SA.



Figure 3.9 MS analysis of SA and D-Lys-SA reaction mixtures. MS analysis of SA reaction mixtures showed an m/z peak at 479.120 (A) under negative-ion mode. The same conditions showed an m/z peak at 493.157 (B) for the D-Lys-SA reaction mixture.

To evaluate whether *S. aureus* was able to use D-Lys-SA for iron acquisition, the product was purified by HPLC and concentrated. A bioassay on iron-limiting media containing *S. aureus* showed halos of growth for both SA and D-Lys-SA with no growth for an H₂O control (Figure 3.10).



Figure 3.10 Bioassay analysis of D-Lys-SA samples.

Addition of 10 μ l to a filter disk (white circle) located on solid, iron-chelated, agar media of either purified D-Lys-SA (A), SA (B) or H₂O (C) produced halos of *S. aureus* growth for the D-Lys-SA and SA samples. Extent of the growth halo has been emphasized with a red circle for clarity.

3.2 Characterization of the structure and substrate preference of SB pathway enzymes

3.2.1: Structure of SbnF

SB is synthesized by a four-enzyme pathway encoded on the *sbn* locus (Figure 1.6). SbnF, is a type-C synthetase that catalyses the condensation of one molecule of L-DAP and citryl-diaminoethane (Figure 1.6). For insights into enzymes of the SB pathway the crystal structure of SbnF was solved by molecular replacement with the NIS synthetase AsbB (36% amino acid identity) and refined to a resolution of 1.8 Å. The structure consists of two molecules in the asymmetric unit and each protein is missing three residues in a single flexible loop region (Figure 3.11). SbnF retains the "cupped hand" domain architecture of NIS synthetases and SfaD. Comparing the structure directly to AsbB (used for molecular replacement) by superposition of the C α atoms generates an RMSD value of 1.8 Å (546 residues aligned). Alignment with the structure of AcsD gives an RMSD value of 3.2 Å (517 residues aligned). Interestingly, alignment with SfaD (RMSD 2.4 Å) (500 residues aligned) shows a high degree of similarity between both enzymes. Two molecules of citrate, present in the crystallization conditions, are bound within the protein (Figure 3.12). Alignment with an ATP-bound structure of AcsD (PDB entry 2W02) identifies the placement of one of the citrates in the triphosphate region of the ATP binding pocket. The other citrate molecule is bound in a putative citryl-diaminoethane (C-DAE) binding site (PDB entry 2X3J) (Figure 3.13). Attempts to crystallize the other NIS synthetases in the pathway (SbnE and SbnC) or obtain substrate-bound structures of SbnF were unsuccessful.



Figure 3.11 Structure of SbnF.

SbnF is found as a dimer in the asymmetric unit. The fold and domain organization are similar to other NIS synthetases.



Figure 3.12 The structure of SbnF contains two citrates bound in the predicted active site. Residues within hydrogen-bonding distance have been displayed as sticks. One citrate is located in a histidine-coordinated pocket, whereas the second citrate appears to mimic a triphosphate in the ATP binding site.



Figure 3.13 Citrate bound in a predicted substrate-binding site in SbnF.

A superposition of the structure of SbnF (green) and that of AcsD bound to substrate (tan, PDB entry 2X3J). The citryl-diaminoethane bound to AcsD (blue) and citrate bound to SbnF (grey) and residues within hydrogen bond distance are drawn as sticks. Oxygen and nitrogen atoms are coloured red and blue respectively.

3.2.2: Active site mutants of SbnF

SbnF shares a higher degree of sequence and structural similarity to other characterized NIS synthetases than SfaD. Sequence alignment of SbnF with other synthetases showed the presence of catalytic arginine and histidine residues at positions 292 and 426, respectively. To confirm their role in catalysis, site-directed mutants R292A and H426N were generated by PCR, transformed into *E. coli* and confirmed by sequencing. Mass of expressed protein was confirmed by MALDI-MS. To test the activity of both mutants, the specific activity of WT protein was first defined using the previously described PP_i release assay. Turnover was defined as specific activity above the negative control. WT substrate had a specific activity of 360 ± 40 nmole min⁻¹

 μ M⁻¹ and a negative control without L-DAP present gave a calculated specific activity of 200 ± 40 nmole min⁻¹ μ M⁻¹. No activity was detected for either mutant (Figure 3.14). Activity of the control reaction (no L-DAP) can be attributed to SbnF turnover with citrate only.





Activity of active site SfaD mutants was measured by a coupled pyrophosphate release assay monitored at 360 nm for 5 minutes. No turnover was detected for either the R292A (blue) or H426N (orange) mutant. All measurements performed in triplicate. Data plotted as standard deviation and error (thin dotted line).

3.2.3: Substrate preference of SbnE

To characterize the substrate preference of enzymes in the SB pathway, activity of the first three enzymes (SbnE, SbnH and SbnF) with substrate analogues was measured. The same group of analogues used for SfaD characterization was used to provide a comparison to the SA pathway. SbnE, the first enzyme in SB synthesis and a type A NIS synthetase, utilizes one molecule each of L-DAP, citric acid and ATP (Figure 1.6). Specific activity with substrate analogues was measured as described using a PP_i release assay. To validate detection of enzyme function, specific activity with known physiological substrates or control reactions lacking citrate or L-DAP were tested. Physiological substrates produced a specific rate of 650 ± 40 nmole min⁻¹ μ M⁻¹. Control reactions produced rates of 40 ± 40 (no citrate) and 70 ± 40 nmole min⁻¹ μ M⁻¹ (no L-DAP).

Testing of the citrate analogues showed turnover with tricarballylic acid, isocitric acid, and hydroxycitrate, mirroring the results recorded with SfaD (Table 3.4 and Figure 3.15B). In contrast with SfaD, no substitute displayed a specific activity higher than the natural substrate (Table 3.4). Furthermore, none of the L-DAP replacements displayed a specific activity higher than the negative control (Table 3.4 and Figure 3.16)



Figure 3.15 Activity of SbnE with the substrate citrate and analogues.

Testing of SbnE citrate substitutions showed no absorbance change for several tested compounds (A) when compared against a WT SbnE reaction (red). (B) Turnover was detected with DL-isocitric acid (green), hydroxycitrate (brown) and tricarballylic acid (blue). Reactions were monitored for 5 minutes. All measurements performed in triplicate. Data plotted as standard deviation and error (thin dotted line).



Figure 3.16 Activity of SbnE with the substrate L-DAP and analogues.

Reactions of SbnE in the presence of L-DAP replacements did not produce any absorbance increase. Reactions were monitored for 5 minutes. All measurements performed in triplicate. Data plotted as standard deviation and error (thin dotted line).

D-ornithine substitute	Specific activity (nmole min ⁻¹ µM ⁻¹)	Citrate substitute	Specific activity (nmole min ⁻¹ µM ⁻¹)
L-ornithine	N.D.	Glutaric acid	N.D.
D-lysine	N.D.	3-Hydroxy-3- methylglutaric acid	N.D.
L-lysine	N.D.	DL-Malic acid	N.D.
DL-2,4- Diaminobutyric acid	N.D.	Malonic acid	N.D.
DL-5-Hydroxylysine	N.D.	Diglycolic acid	N.D.
Putrescine	N.D.	DL-Isocitric acid	230 ± 40
		Hydroxycitrate	550 ± 40
		Tricarballylic acid	480 ± 40
Physiological reaction (L-DAP + citrate)	650 ± 40	Negative control (no L-DAP)	70 ± 40

Table 3.4 Specific activity of SbnE^a

^aAll measurements were performed in triplicate. N.D. is defined as activity below the negative control sample.

3.2.4: Substrate preference of SbnH

SbnH is the second enzyme in the SB synthesis pathway and decarboxylates the product of the first enzyme SbnE, releasing CO₂ (Figure 1.6). The identification of several functional substrate analogues for SbnE provided an opportunity to study the substrate preference of SbnH. As the orientation of hydroxycitrate and isocitric acid in the active site of SbnE is unknown, several potential products are possible (Figure 3.17). To track turnover of SbnH, a CO₂ release assay in a positive pressure nitrogen atmosphere glovebox was adapted from an existing protocol. This assay couples CO₂ release to utilization of NADH by malic dehydrogenase, resulting in a decrease in absorbance at 340 nm. The products of the one-pot SbnE reactions were tested as substrates. SbnE was filtered out and substrates were allowed to equilibrate in the glovebox overnight. To confirm that turnover of enzyme could be detected, physiological substrates and products of control reaction lacking either the enzyme SbnE or the substrate L-DAP were tested. The absorption changes observed were not linear with time. Enzyme activity was qualitatively assessed by the absorption change after 3 minutes. Physiological substrate produced an absorbance reading of -0.164 ± 0.012 , control reactions produced readings of -0.058 ± 0.008 (No-SbnE) and -0.057 ± 0.009 (No L-DAP). The background signal seen in the control reaction mixtures lacking SbnE or L-DAP can potentially be attributed to residual CO₂ present in the glovebox or assay buffers.

Using this metric, SbnH showed turnover with SbnE products derived from all three citrate analogues tested (tricarballylic acid, hydroxycitrate and isocitric acid substituted SbnE products) as well as the physiological substrate (Figure 3.18). Control reactions lacking either SbnE or the SbnE substrate L-DAP showed a significantly smaller decrease in absorbance (Figure 3.18).



Figure 3.17 Possible products of SbnE citrate substitutions.

(A) SbnE is capable of producing the WT product citryl-L2,3-diaminopropionic acid, a tricarballylic substitution product (D) and two possible products for isocitrate (B and C) and hydroxycitrate (E and F) substitutions.



Figure 3.18 Activity of SbnH with citryl-L2,3-diaminopropionate and analogues. Activity of SbnH with products of SbnE reactions containing L-DAP and either citrate or the indicated alternative substrate. SbnH showed catalysis with all three modified SbnE products. All measurements performed in triplicate. Data plotted as standard deviation and error (thin dotted line).

3.2.5: Substrate preference of SbnF

SbnF is a type C NIS synthetase catalysing condensation of a molecule of L-DAP to the product of SbnH, utilizing a molecule of ATP in the process. Due to the uncertain nature of hydroxylysine and isocitric acid placement in the SbnE active site, several different SbnH products are possible (Figure 3.19). Substrates derived from tricarballylate, hydroxycitrate and isocitrate instead of citrate were produced using SbnE and SbnH. Specific activity of SbnF was determined as described previously using a PP_i release assay. Turnover was defined as specific activity above the negative control. WT substrate produced a specific activity of 360 ± 40 nmole min⁻¹ μ M⁻¹ and a negative control without L-DAP present gave a calculated specific activity of 200 ± 40 nmole min⁻¹ μ M⁻¹. Assay of SbnF activity showed turnover the hydroxycitrate and tricarballylate derived substrates (Table 3.5, Figure 3.20 A and B). The specific activity with the

isocitrate-derived substrate was not statistically significantly different from the control (Table 3.5 and Figure 3.20 C).







Figure 3.20 Activity of SbnF with the substrate citryl-diaminoethane and analogues. Enzyme turnover as measured by absorbance at 360 nm was detected with (A) hydroxycitrate (blue) and (B) tricarballylic acid (green). (C) Specific activity with isocitrate was not significant from control (black). Reactions were monitored for 5 minutes. All measurements performed in triplicate. Data plotted as standard deviation and error (thin dotted line).

Table 3.5 Specific activity of Sbi

D-ornithine substitute	Specific activity (nmole min ⁻¹ μ M ⁻¹) ^{bc}
Physiological reaction	160 ± 40
DL-Isocitric acid	N.D.
Hydroxycitrate	310 ± 40
Tricarballylic acid	210 ± 40

^aAll measurements were performed in triplicate. N.D. is defined as activity below the negative control sample.

^bReported parameters are apparent.

^cValues normalised to negative control (no L-DAP).

Chapter 4: Discussion

4.1 Substrate preference of SA pathway enzymes

4.1.1: Substrate preference of SfaD

To date six NIS synthetases have been structurally characterized. Of those, two are type A NIS synthetases. SfaD is categorized as a type A synthetase due to it using citrate as a substrate, but it is more closely related to type B and C synthetases based on amino acid sequence identity [81]. To define the structural features of SfaD, a crystal structure of apo-SfaD was solved. Comparison of SfaD to other synthetases shows a similar domain architecture, but relatively high RMSD values when superimposed with other NIS synthetases indicates SfaD has structurally unique features. Conservation of the ATP binding site and key catalytic residues suggests that SfaD is conserved in the core active site region but is much more variable on the periphery. The significance for this variability is unknown. Comparison with type C synthetase SbnF showed that SfaD is structurally most similar to another NIS synthetase from *S. aureus*.

Unfortunately, the structural model of SfaD is missing residues 204 to 217, including several residues predicted to form the citrate-binding pocket of the active site. Attempts to crystallize SfaD and active site mutants of SfaD in complex with substrates to stabilize this loop were unsuccessful. The SfaD crystal structure leaves unanswered questions regarding the high degree of substrate promiscuity.

The general enzymatic mechanism of NIS synthetases has been described biochemically and supported by structural analysis of the active site [102, 107]. In addition, some of these synthetases were shown to turn over non-native substrates, with several reported cases of nucleophile substitution and one case of citrate substitution [126-128]. The high degree of structural similarity within the family and conservation of key amino acid residues suggested that *S. aureus* homologs may also be able to accommodate a variety of substrate analogues.

Using a PP_i release assay to measure specific activity, SfaD was found to process six analogues of D-ornithine and three analogues of citrate (Figures 4.1 and 4.2). All functional Dornithine replacements retained a similar chemical structure with a terminal carboxyl group and a pair of amine groups (Figure 4.3). Previously, only the D isomer of ornithine was shown to give rise to SA [81]. Here both the D and L enantiomers of ornithine and lysine are substrates of SfaD, consistent with more stringent substrate selection resting with SfaB, the second SA synthetase. Additionally, the lack of any enzyme activity with putrescine, suggests that the carboxylate group is required for substrate recognition by SfaD, as the condensation reaction occurs at the δ amine (Figure 4.4). Functional citrate substitutes retained the same overall length and carboxyl groups in the native substrate (Figure 4.5). No activity was detected with shorter substitutes such as malate and malonate. Similarly, removal of the central carboxyl group, such as in the case of glutaric acid was sufficient to prevent reaction. Successful analogues show the central hydroxyl group is not essential, as isocitrate or even hydroxycitrate retain activity (Figure 4.5). This degree of substrate promiscuity found in SfaD is thus far unique in NIS synthetases as previous examples of functional substitutions were limited to only a handful of compounds [107, 126-128].



Figure 4.1 Progression of citric acid substitutions through the SA synthesis pathway. No citrate substitute product was detected by HPLC, only the WT product citrate gives rise to WT SA.



Figure 4.2 Progression of D-ornithine substitutes through the SA pathway. D-Lysine substitution gives rise to a final product in the full SA pathway reaction.



Figure 4.3 Functional nucleophile substitutions in SfaD.

SfaD is capable of utilizing native D-ornithine (A), L-ornnithine (B), DL-diaminobutyric acid (C), DL-diaminopropionic acid (D), D and L enantiomers of lysine (E and F) as well as DL-5-hydroxylysine. All functional D-ornithine substitutions retain a terminal carboxylic acid group and a pair of amines.







Figure 4.5 Functional citrate substitutions in SfaD. SfaD is capable of catalysing reactions containing either the carboxylic acid citrate (A), tricarballylic acid (B), Hydroxycitrate (C) or Isocitrate (D). All functional citrate analogues retain the three carboxyl groups found on citrate.

4.1.2 Production of a SA analogue.

The second and final enzyme in SA synthesis pathway, SfaB, is a type C NIS synthetase that adds a second citric acid group to the intermediate produced by SfaD. Although the continuous assay was not suitable likely due to the relative instability of the protein in solution, analysis by HPLC of incubated reactions provided some evidence regarding the substrate preference of SfaB. Of the nine alternate SfaD products tested, only with D-lysine was a product formed detectable by HPLC (Figures 4.1 and 4.2). Selectivity for D isoform is consistent with enantiomer specificity in assembly of the final siderophore product [81]. Exclusion of the diaminobutyric and diaminopropionic acid derived products may be due to the amino group being poorly positioned for condensation with a second citrate. The use of absorption at 340 nm in the HPLC analysis may have failed to detect products unable to bind iron sufficiently. However, for the analogue to be useful as a siderophore it must retain the ability to bind ferric iron. Overall these findings suggest that SfaB is the major substrate selection point in the SA pathway, as it successfully excluded all but one of the alternate SfaD products.

The detection of an SfaB product and the signal increase detected by HPLC upon addition of FeCl₃ suggested that this molecule may bind ferric iron and act as a siderophore. Modified siderophores require uptake by native receptors to be useful tools in "trojan horse" and other applications. The ability of *S. aureus* to bind and take up modified siderophore would be an indication of the viability of these approaches in this species.

MS analysis of complete and control reaction mixtures detected the presence of peaks with masses within error of the predicted mass of both SA and D-Lys-SA. The predicted mass and structure of D-Lys-SA corresponds to the structure of corynebactin from *Corynebacterium diphtheria* [129]. However, the stereochemistry of corynebactin and D-Lys-SA are unknown and these siderophores may be stereoisomers. Furthermore, a bioassay confirmed that *S. aureus* was capable of utilizing the new siderophore to acquire iron from iron restricted media. It is not known whether *S. aureus* is capable of utilizing corynebactin for iron acquisition. The smaller size of the halo in the D-Lys-SA sample may be a consequence of a difference in concentration as we did not quantify the SA or D-Lys-SA purified by HPLC. The fact that *S. aureus* is capable of growth using a modified form of SA suggests that further modifications of SA and SB, potentially conjugated to other molecules, may be a viable approach.

4.2 The SB pathway

4.2.1: Substrate preference of SB pathway enzymes

The SB biosynthesis pathway in *S. aureus* is more complex than SA synthesis. Four enzymes are required to sequentially assemble SB: SbnE, SbnH, SbnF, and SbnC. Successful substitute selection requires processing by all four enzymes. An important difference with the *sfa* pathway is the presence of the PLP-dependent decarboxylase SbnH. To investigate whether the SB pathway could be manipulated to produce alternate products, the first three enzymes were assayed for their activity with substrate analogues.

The first enzyme, SbnE, catalyses the condensation of citrate with L-DAP, similar to the condensation of citrate with D-ornithine catalysed by SfaD. SbnE and SfaD accepted the same citric acid replacements (Figure 4.6). However, in contrast to SfaD, SbnE, in the presence of citric acid, was found to not accept any replacements for L-DAP. As a result, a much higher degree of substrate selectivity is present at the initial stage of SB synthesis when compared to SA.



Figure 4.6 Citric acid substitute progression through the SB pathway.

Two substitutes for citric acid were able to generate products that were used by all enzymes tested.

The SbnE product citryl-L2,3-diaminopropionate is decarboxylated by the PLP-

dependent decarboxylase SbnH. Interestingly, SbnH decarboxylated the alternative SbnE

products; however, since the SbnE products were not characterized and more than one product could be formed, whether all or only some of these products are processed by SbnH was not determined.

The presence of one citrate molecule in the conserved ATP-binding site of SbnF and another in the putative C-DAE substrate site suggests that other carboxylic acids may interact with the active site of SbnF. This is observed in the PP_i release assays where negative controls that contained citrate showed a change in absorbance, suggesting that SbnF is capable of turning over with citrate. Additionally, assaying for SbnF activity with alternative substrates produced by SbnH showed that tricarballylic acid and hydroxycitrate substitution products were utilized by SbnF. The exclusion of both forms of the isocitrate variant indicates that structural isomers are excluded in later stages of siderophore assembly, as both SbnE and SbnH utilized structural isomers of isocitrate. Attempts to obtain a native-substrate bound structure using WT and sitedirected mutants of SbnF were unsuccessful.

Comparison of SfaD and SbnF to AcsD, a type A synthetase used for structural characterization of NIS synthetases, shows that catalytic residues are conserved (Figure 4.7). Active site mutants of SfaD R351A and SbnF R292A showed no discernable activity. Furthermore, no activity was detected for mutants of a predicted ATP-coordinating residue; SfaD H496N and SbnF H426N. The histidine residues can be clearly identified in the predicted ATPbiding pocket of the SfaD and SbnF structures. Additionally, the SfaD H208A mutant was generated. This residue was predicted to coordinate citrate based on structures of other synthetases but has not previously been described as essential. Phosphate assay results showed that this mutant indeed retained functionality with its native substrates. These findings confirm that the *S. aureus* NIS synthetases SfaD and SbnF retain the same mechanism described in other NIS synthetases and show a high degree of structural conservation in the active site region.



B


Figure 4.7 Key catalytic residues in SfaD and SbnF.

Superimposition of (A) SfaD (cyan) with AcsD (tan) and (B) SbnF (green) with AcsD (tan). Residues required for catalysis are drawn in sticks and are labelled.

The final enzyme in the SB pathway, SbnC, was not assayed due to difficulties with protein purification and stability, however, several observations regarding substrate preferences of enzymes in the SB pathway were made. The stringency of SbnE for L-DAP ensures that subsequent enzymes in the pathway are provided with the proper backbone for further assembly of the siderophore. Based on the exclusion of citrate substitution products by SfaB it would be possible for SbnC to act as the final control step in SB assembly.

4.3 Conclusions

The ability of *S. aureus* to scavenge iron from the human host has been repeatedly shown to play a crucial role in establishing an infection. The siderophores SA and SB form a key part of the iron-acquisition repertoire used by this pathogen. In my study, I was able to obtain structures of *S. aureus* NIS synthetases SfaD and SbnF. Comparison to existing NIS synthetase structures showed conservation of the core active site region with variability at the periphery. Furthermore, I made mutations of a catalytically essential arginine (SfaD R351A and SbnF R292A), a histidine that binds ATP (SfaD H496N and SbnF H426N), as well as a non-essential histidine (SfaD H208A), to confirm that both synthetases function in accordance with a previously described mechanism. Using enzymes from both SA and SB pathways I was able to show catalytic activity with a number of substrate analogues which provided insight into substrate specificities of the NIS synthetases SfaD, SbnE, SbnF and the decarboxylase SbnH. Finally, a modified SA was produced, detected and validated, showing that *S. aureus* is able to use modified SA for iron-acquisition.

4.4 Future Directions

The specificity of *S. aureus* NIS synthetases could be further characterized by defining the kinetic parameters with the physiological and alternate substrates identified in this study. To this end, I have collected preliminary enzyme rate data for the physiological and some alternate substrates.

Although progress has been made in determining the substrate binding sites of SfaD and SbnF, structures of both enzymes in complex with native and alternative substrates would shed light on why certain NIS synthetases are more promiscuous than others. Currently, I have generated crystals of site-directed mutants for both SbnF and SfaD in the presence of several substrates but have not been able to refine them to sufficient diffraction quality. Alternatively, computational docking methods could be attempted to model substrates into the apo-structures.

Similarly, a structure of SbnH in complex with substrate would reveal details about substrate preference that would complement the initial assay results obtained in this study. Crystals of inactive site-directed mutants have been generated, but solved structures did not possess discernable density for even a partial substrate molecule. If a crystallographic approach proves unsuccessful, an existing apo structure of SbnH may be used in docking trials to predict key coordinating residues which may be validated by site-directed mutants.

Finally, the aspect of generating a modified siderophore can be greatly expanded by testing additional substrates and making mutations on enzymes in both pathways to reduce substrate selectivity. The ability of *S. aureus* to use a modified SA for iron-acquisition presents an opportunity to further develop variants with altered backbone structure and work towards generating an easily modifiable siderophore. This could then be used in a semi-synthetic process

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to produce conjugates with antibiotic or other functional groups and adapted for further studies of a clinical nature.

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