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Serum iron uptake and virulence in Staphylococcus aureus

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

SERUM IRON UPTAKE AND VIRULENCE IN *STAPHYLOCOCCUS AUREUS*

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Abstract

The high affinity iron scavenging glycoprotein transferrin sequesters trace amounts of serum Fe^{3+} to concentrations below what is required to sustain microbial life. Iron may be liberated from this important innate immune factor after interaction with molecules that chelate or reduce Fe^{3+}. Organisms with cognate transport systems for these iron coordinating molecules can survive in the bloodstream using transferrin iron.

Staphylococcus aureus is an opportunistic bacterial pathogen. *S. aureus* executes numerous strategies for overcoming the innate immune barrier of iron deprivation in the bloodstream. In addition to specialized mechanisms for hemoglobin iron extraction, *S. aureus* can proliferate on serum iron, but factors enabling this growth are not described. Production of at least two siderophores (microbial iron chelators) has been documented on numerous occasions, and their contribution to growth on transferrin is documented here. Genomic inactivation of genes involved in production of molecules subsequently termed staphyloferrin A (SA; the *sfa* locus) and staphyloferrin B (SB; the *sbn* operon) resulted in a mutant severely incapacitated for growth in serum as well as on rarefied human transferrin as sole iron sources. Transport of staphyloferrins was correlated to adjacently encoded cognate ABC type transporter operons, *hts* (SA) and *sir* (SB), using previously constructed transport mutant strains. Mass spectrometry confirmed the molecular structure of SB as being the same as the previously described *S. aureus* metabolite, staphylobactin.

Alternate siderophores were not detectable for the double biosynthetic

mutant. Growth in the presence of transferrin could be rescued by addition of saturating concentrations of iron, and restored by molecules that bind Fe^{3+} through catechol-iron coordination, including mammalian catecholamine stress hormones. *In silico* analysis and mutational inactivation confirmed this transport function to be encoded by the *sst* operon. Biochemical assays revealed that the Sst transporter lipoprotein has a high affinity for ferrated catecholate iron ligands.

Siderophore biosynthesis and transport mutants displayed reduced virulence during systemic mouse infection. Decreased bacterial loads were documented in mouse hearts, an important finding as *S. aureus* is a leading cause of endocarditis. The data collected in this study show that acquisition of serum iron is an important part of staphylococcal pathogenesis, and suggest that therapeutics targeting the numerous facets of this process may be effective in combating invasive infection.

Keywords: *Staphylococcus aureus*, siderophores, staphyloferrin A, staphyloferrin B, serum, transferrin, ABC transporters, catecholamine hormones, catechol siderophores, mouse infection model

Coauthorship

The greater part of this thesis has appeared in published manuscripts. Work performed not exclusively by F.C. Beasley is denoted below.

Portions of chapters 2 and 3 have been published in a manuscript in the journal *Infection and Immunity*:

Beasley, F.C., C.L. Marolda, J. Cheung, S. Buac, and D.E. Heinrichs, 2011. *Staphylococcus aureus* transporters Hts, Sir and Sst capture iron liberated from human transferrin by staphyloferrin A, staphyloferrin B and catecholamine stress hormones, respectively, and contribute to virulence. Infect Immun submitted.

Writing of this manuscript and generation of figures was performed by F.C.B. with assistance from D.E.H. The Δ*sstABCD* mutant allele and complementing vector were generated by S.B. Bacillibactin purification was performed by C.L.M. SstD protein purification was done by C.L.M and J.C. SstD/ligand affinity determination experiments and analysis of data were performed by C.L.M. and J.C.

Significant portions of chapter 2 have also been published in a manuscript in the journal *Molecular Microbiology*:

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Murphy, and D.E. Heinrichs. 2009. Characterization of staphyloferrin A biosynthetic and transport mutants in *Staphylococcus aureus*. Mol Microbiol 72:947-963.

Writing of this manuscript and generation of figures was performed by F.C.B., D.E.H., and E.D.V. E.D.V. generated the Δ*htsABC* allele and *sfaABCsfaD* complementation vectors. HtsA protein purification for antibody generation and crystallization was performed by E.D.V. HtsA crystallization and structure determination were performed by J.C.G., Q.Z, and M.E.P.M. Mass spectrometry and analysis were performed at the University of Western Ontario Biological Mass Spectrometry Laboratory of G.A.L. by S.L. Radioactive transport assays were performed by E.D.V.

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Mass spectrometry was performed by S.L. in the laboratory of G.A.L. with samples provided by F.C.B. Writing of this manuscript was performed by D.E.H. and J.C.

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List of Abbreviations

^{o}C	degrees Celsius
ϕ	phage
A	absorbance
ABC	ATP-binding cassette
ADP	adenosine diphosphate
AMP	adenosine monophosphate
Am^{R}	ampicillin resistant
apo Tf	apotransferrin
ATP	adenosine triphosphate
bp	base pairs
CA	community acquired
CAA-RPMI	Casamino acids-RPMI
CAS	chrome azurol-S
C-Dae	citryl-diaminoethane
CFU	colony forming units
CID	collision-induced dissociation
Cm^{R}	chloramphenicol resistant
CNS	coagulase-negative staphylococci
CPS	coagulase-positive staphylococci
C-TMS	Chelex-treated Tris-buffered minimal succinate
D	dopamine
Da	Dalton

Dap	diaminopropionic acid
DCytB	cytochrome B
DHBA	dihydroxybenzoic acid
DMT1	proton-coupled solute transporter
DNA	deoxyribonucleic acid
E	epinephrine
ECF	extracytoplasmic function
EDDHA	ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid)
EDTA	ethylenediaminetetracetic acid
Em	erythromycin resistance cassette
Em^R	erythromycin/lincomycin resistant
epi	epinephrine
g	gram
g	gravitational force
GST	glutathione-S-transferase
GTP	guanosine triphosphate
h	hour
HFE	human hemachromatosis factor
holo Tf	holotransferrin
HoS-TMS	horse serum-Tris-buffered minimal succinate
Hts	heme transport system
HuS-TMS	human serum-Tris-buffered minimal succinate
IgG	immunoglobulin G
K_d	dissociation constant

kDa	kiloDalton
kg	kilogram
Km	kanamycin resistance cassette
Km^R	kanamycin/neomycin resistant
krpm	kilo-rotations per minute
L	litre
LC-ESI-MS	liquid chromatography electrospray ionization mass spectrometry
LC-MS	liquid chromatography mass spectrometry
LD	L-3,4-dihydroxyphenylalanine
L-DOPA	L-3,4-dihydroxyphenylalanine
LP	lipoprotein
M	molar
min	minute
µg	microgram
µL	microlitre
µM	micromolar
Met	methionine
mg	milligram
mL	millilitre
mm	millimetre
mM	millimolar
MRSA	methicillin resistant *Staphylococcus aureus*
MS	mass spectrometry

m/*z*	mass-to-charge ratio
n	number of replicates
NE	norepinephrine
NEAT	Near-iron transport
NIS	nonribosomal peptide synthesis-independent siderophore
nm	nanometre
nM	nanomolar
NRPS	nonribosomal peptide synthesis
NWMN	*Staphylococcus aureus* Newman
OD	optical density
OMR	outer membrane receptor
ORF	open reading frame
P	probability
PBP	penicillin-binding protein
PBP	periplasmic binding protein
PBS	phosphate buffered saline
PCP	peptidyl carrier protein
PCR	polymerase chain reaction
PPi	pyrophosphate
Rep	replicase
RNA	ribonucleic acid
SA	staphyloferrin A
SB	staphyloferrin B
Sbn	siderophore/staphyloferrin B biosynthesis

SCC	staphylococcal cassette chromosome
S.D.	Shine-Dalgarno sequence
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERP	*Staphylococcus epidermidis* RP62A
Sfa	staphyloferrin A biosynthesis
Sfna	staphyloferrin A biosynthesis
Sir	staphylococcal iron regulated
sp.	species
spp.	species (plural)
SSP	*Staphylococcus saprophyticus* ATCC15305
Sst	staphylococcal siderophore transport
Tc	tetracycline resistance cassette
TCA	tricarboxylic acid
Tc^R	tetracycline resistant
TE	transesterification
Tet	tetracycline
TMS	Tris-buffered minimal succinate
Tn*917*	transposon 917
TSB	tryptic soy broth
UPEC	uropathogenic *Escherichia coli*
V	volts
VISA	vancomycin intermediate *Staphylococcus aureus*
VRSA	vancomycin resistant *Staphylococcus aureus*

v/v	volume per volume
w/v	weight per volume

Chapter 1

Introduction

1.1 Iron

1.1.1 Iron chemistry

With the exception of some lactobacilli and spirochaetes (8, 204), iron is an essential nutrient for bacteria. Iron is most commonly found in Fe(II) (ferrous) and Fe(III) (ferric) valence states; however, it is able to achieve valence ranging from Fe(-II) to Fe(IV), and is amenable to ligand-mediated transition through a redox range of -750 to +1150 mV at neutral pH (198), making it an exceptionally versatile catalyst. The heme centre of cytochromes is crucial to respiratory and photosynthetic ATP biosynthesis; Fe–S clusters are central to redox reactions mediated by proteins such as nitrogenase and hydrogenase; and iron is a cofactor for numerous other metalloproteins. In aerobic, oxidizing environments, iron predominates in the ferric form (Fe^{3+}), forming poorly soluble hydroxides. A widely cited calculation describes the primary Fe^{3+} species in pH neutral solution to be $Fe(OH)_3$, restricting solubility to 10^{-18} M (93, 225). A revised solubility calculation posits $Fe(OH)_2^+$ to be the primary species, improving solubility to almost 10^{-9} M (41); nevertheless, this is below the threshold required to sustain microbial life in the absence of specialized acquisition mechanisms.

1.1.2 Iron toxicity

In spite of low solubility, residually soluble iron is a hazardous catalyst. Fe^{2+} readily reacts with hydrogen peroxide, generating hydroxyl radical, as shown in equation 1 (105). Soluble Fe^{3+} is renewed to Fe^{2+} by reaction with superoxide, shown in equation 2 (13).

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-$ (equation 1)

$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$ (equation 2)

Hydroxyl radical generated through equation 1 is extremely cytotoxic, being reactive with cell membranes (19), and inducing single and double strand breaks in DNA (30, 252). To mitigate toxicity of catalytic iron, most biological systems control iron levels tightly using high affinity storage and transport proteins, described below.

1.2 Iron absorption and storage in mammalian systems

1.2.1 Iron absorption

The average human body contains three to four grams of iron (26). The process of absorption from dietary sources, reviewed here (5, 75), occurs principally in the duodenum. Briefly, Fe^{3+} is reduced to readily soluble Fe^{2+} by the low pH of the intestinal lumen, and by the duodenal cytochrome B (DCytB), found on the apical membrane of the duodenal enterocyte. Fe^{2+} is transported into enterocytes through a proton-coupled solute transporter, DMT1. Release of Fe^{3+} from erythrocytes into plasma occurs through a solute carrier, ferroportin. In plasma, Fe^{3+} is bound by the carrier protein transferrin, which is described in detail below.

A surplus of bodily iron stimulates release of the peptide hormone hepcidin from the liver. Hepcidin binds to ferroportin on enterocytes, triggering its degradation and internalization. Rather than being passaged into plasma, subsequently absorbed dietary iron is instead compartmentalized within the

protein ferritin (described next), and excess iron can be shed through sloughing of enterocytes into the feces.

1.2.2 Bodily iron reservoirs: ferritin

Ferritin is a stable, hollow, 450 kDa proteinaceous sphere composed of 24 “heavy” and “light” subunits, capable of harbouring up to 4500 Fe^{3+} atoms (35). Ferritin is an important component of iron homeostasis in the body, specifically in regards to storage of surplus iron in a soluble and nontoxic form, and is found with high conservation in eukaryotes and prokaryotes alike (7). In animals, ferritin is most abundant in liver hepatocytes, which act as the body’s primary iron reserve (25). Release of ferritin into serum may aid in iron delivery from the liver to other tissues (73, 86). More importantly, elevated serum ferritin serves to reduce iron overload caused by hemochromatosis or some cancers (262).

1.2.3 Bodily iron reservoirs: heme and heme carrier proteins

The majority of iron in humans is found within heme prosthetic groups of metalloproteins, including peroxidases, cytochromes, and most abundantly, in the hemoglobin of erythrocytes, which contain around two-thirds of the total bodily reservoir (69). Variations on heme structures exist. The primary mammalian form is heme-*b*, a planar molecule composed of a large heterocyclic ring (protoporphyrin IX) with four central nitrogen atoms coordinating one atom of ferrous iron. The two remaining uncoordinated positions of heme iron permit interactions with axial ligands, including histidine/cysteine/methionine/tyrosine

residues of heme carrier proteins, water, or small molecule aqueous solutes, the most noteworthy being oxygen.

The largest reservoir of bodily heme is the circulatory hemoglobin pool. Hemoglobin is a globular heterotetramer found within erythrocytes whose primary function is to bind O_2 to heme prosthetic groups and deliver it from the lungs to all other tissues. Human blood contains approximately 1.3×10^{18} hemoglobin tetramers per mL (adapted from reference range provided by the Department of Pathology at the University of Texas Southwestern Medical Centre). Erythrocyte lysis and oxidative hemoglobin degradation leads to heme liberation into serum. Owing to its hydrophobicity, heme can intercalate into lipid bilayers, and is reactive with H_2O_2, producing hydroxyl radicals that lead to tissue damage (219). Capture of extracellular heme is a vital maintenance function for bodily well being and mechanisms exist for its removal.

Serum hemoglobin is scavenged by the glycoprotein haptoglobin (88). Haptoglobin irreversibly forms a soluble complex with hemoglobin. The complex is recognized by a specific receptor expressed on the surface of macrophages, internalized, then degraded (138). Iron captured in this fashion is released back to the serum in hemosiderin, a molecular aggregate comprised mostly of ferritin and denatured ferritin (71, 119, 270). Serum heme is readily bound by low and high density lipoprotein (175). This binding is transient, as the heme is then gradually removed by the glycoprotein hemopexin (120). Hemopexin delivers heme back to the liver, where it is internalized by receptor-mediated endocytosis; hemopexin is a true iron carrier protein, as it is released back into circulation undegraded (121).

1.2.4 Bodily iron reservoirs: transferrin and lactoferrin

Less than 5 mg of bodily iron (<0.1%) is found in the labile pool circulating within serum and lymph (adapted from (256)); this fraction, however, undergoes the highest turnover. After ferroportin-mediated export of dietary Fe^{3+} occurs from intestinal enterocytes, the binding and redistribution of iron in serum is performed chiefly by transferrin, an 80 kDa glycoprotein synthesized by the liver. The transferrin reference range for healthy adult serum is roughly 25 to 30 µM (adapted from (12)). Transferrin has two high affinity Fe^{3+} binding sites, with equilibrium constants of 4.7×10^{20} M^{-1} and 2.4×10^{19} M^{-1} at physiological pH; both have negligible affinity for Fe^{2+} (3). Binding of Fe^{3+} at either site is random and noncooperative (1). Serum transferrin is about 30% iron saturated in healthy individuals; saturation below 20% is a clinical indicator of iron deficiency (269). Transferrin is the primary agent of circulatory iron distribution, and its mode of action is summarized here (122). Briefly, upon contacting recipient tissue cells, ferrated (holo) transferrin binds a ubiquitously expressed cell membrane receptor. The amount of receptor found on a cell membrane is directly proportional to the cell's iron demand. Binding triggers endocytosis and internalization within a vesicular compartment, which is acidified to promote dissociation of iron. Fusion of the vesicle with the cell membrane returns deferrated (apo) transferrin, to the cell surface, where it is released back into circulation. Percent saturation of serum transferrin is sensed through the transferrin receptor and an interacting protein, human hemochromatosis factor (HFE protein), with increased saturation being a key stimulus for upregulation of hepcidin production, decreasing absorption of dietary iron (83).

The glycoprotein lactoferrin is highly related to transferrin in sequence (61.4% identical) and structure (260). This iron scavenging protein is found primarily in secretions such as mucus, milk, and tears, and releases iron at a much lower pH than transferrin (pH 3.0 vs 5.5) (11). It is also released by polymorphonuclear leukocytes as an antimicrobial agent during degranulation (47). Lactoferrin has no demonstrated role in iron transport, but is an important contributor to bacteriostasis in tissues and bodily fluids, by reducing the amount of iron available to pathogens (206, 210); this aspect of bodily iron homeostasis is discussed next.

1.2.5 Iron sequestration and innate immunity

Restricting iron availability is crucial for limiting the spread of infection in blood. Conversely, iron overload associated with diseases such as hemochromatosis increases susceptibility to bacteremic infection (130). The inflammatory response involves suppressing uptake of dietary iron. This renders serum anemic, a phenomenon known as hyposideremia. In this fashion, uptake of dietary iron can be further reduced even if transferrin saturation is already low. Hepcidin production by the liver and spleen is increased during inflammation (187, 199), and this is mediated at least in part by cytokines interleukin-6 (185) and interleukin-1α and 1β (142). Hepcidin binding to ferroportin on intestinal enterocytes triggers degradation of this transport protein and reduces absorption of dietary Fe^{3+} (186).

Hyposideremia also involves increasing levels of circulating iron binding proteins. Serum ferritin levels increase due to upregulated secretion by hepatic

cells in response to interleukin-1 (217, 254). Serum lactoferrin increases during septicemia and viral infection, as degranulating neutrophils deliver lactoferrin to sites of infection, leading to localized sequestration of iron (103, 163, 208). Hepatocyte secretion of the hemoglobin/heme removal proteins haptoglobin and hemopexin is also upregulated during the acute phase response (158, 263). Only one serum iron transport protein is maintained at a stable concentration during inflammation: transferrin (191). Its contribution to innate immunity should not be understated, however. The iron binding capacity of the transferrin pool is routinely high, as average saturation rarely exceeds 50% (214). When dietary absorption is repressed by acute phase increases in circulating hepcidin, the labile transferrin-bound iron supply is more rapidly depleted by erythropoiesis (205), effectively increasing the iron binding capacity of serum.

1.3 Bacterial strategies for acquisition of host iron

1.3.1 Active transport of iron ligands: an overview

As outlined above, animal serum maintains a consistently low level of freely dissolved iron through complexation to an abundant pool of transferrin. The labile iron pool may be further depleted through enhanced production of other serum iron scavenging proteins during the acute phase inflammatory response to disease. This effectively limits the amount of free iron below the threshold required to support microbial growth, meaning invasive pathogens must respond by expressing specialized, high affinity iron transport systems.

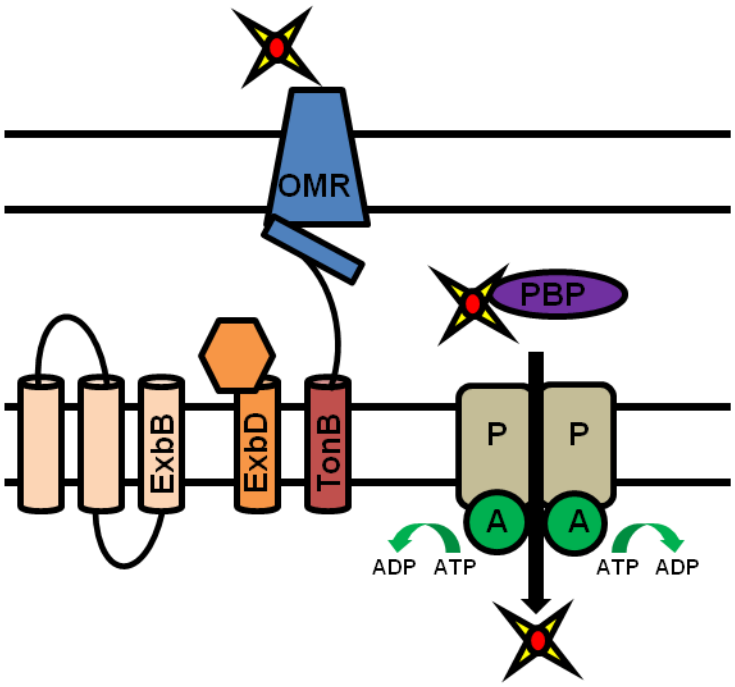
Bacteria import iron from the host milieu primarily as a liganded substrate. This can include heme extracted from host carrier proteins, or Fe^{3+} coordinated

by bacterial or host-synthesized molecules in a stable and kinetically labile arrangement. At the bacterial cytoplasmic membrane, most examples of iron transport are active processes involving ATP-binding cassette (ABC) transporters (Figure 1-1). These systems comprise three functional domains. The first is a high affinity receptor for recognition of the transported substrate; in Gram-positive bacteria this is covalently linked to the cytoplasmic membrane at a N-terminal lipidation motif, while in Gram-negative bacteria it is usually freely soluble in the periplasmic space. The second domain comprises the permease, a homodimeric or heterodimeric transmembrane domain which associates with the receptor and permits solute transport through the lipid bilayer. The final domain is an ATPase, a cytosolically-located, permease-associating, dimeric, ATP-hydrolyzing unit. The different components of the ABC transporter are usually distinctly translated proteins, but examples of translational domain fusions exist. (For a more comprehensive review on bacterial ABC transporters, see (52).)

In Gram-negative bacteria, the presence of an outer membrane and a periplasm necessitates additional components for substrate capture (Figure 1-1). An outer membrane receptor protein is involved in substrate recognition. A cytoplasmic membrane complex, comprising proteins TonB, ExbB, and ExbD, captures energy generated by proton motive force across the cytoplasmic membrane to drive substrate transport through the outer membrane receptor into the periplasm. The outer membrane receptor is a pore-forming β-barrel protein occluded by a N-terminal β-sheet “plug” domain. Conformational changes induced by ligand binding and interaction with TonB

Figure 1-1: Model of active ligand transport in Gram-negative and Gram-positive bacteria. (A) Model of transport in Gram-negative bacteria. Substrate (yellow star with red centre) is recognized at the outer membrane by the outer membrane receptor (OMR) and transported into the periplasm with energy provided by the Ton complex (TonB/ExbD/ExbB). A periplasmic binding protein (PBP) captures the substrate and delivers it to an ABC transporter comprising a homo- or heterodimeric permease (P) and dimeric ATPase (A), the latter hydrolyzing ATP to drive translocation of the substrate into the cytoplasm. **(B)** Model of transport in Gram-positive bacteria. Substrate at the outer face of the cytosolic membrane binds to a receptor lipoprotein (LP) and is translocated through a permease (P) with energy coming from ATPase-mediated (A) ATP hydrolysis.

A


OMR
PBP
ExbB
ExbD
TonB
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A
ADP
ATP
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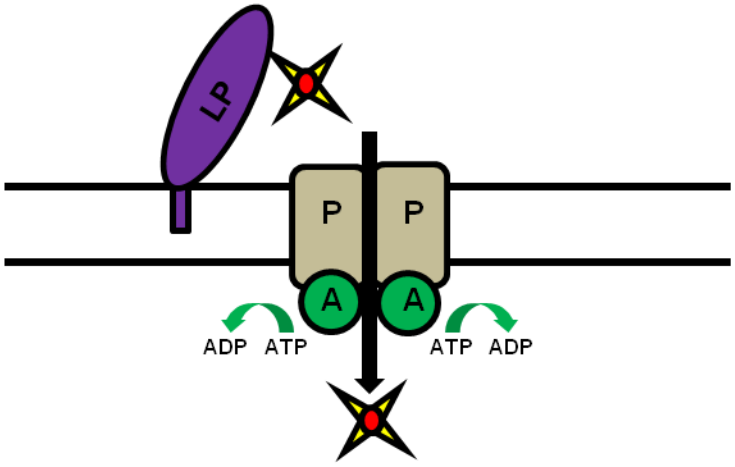
ADP

B


LP
P
P
A
A
ADP
ATP
ATP
ADP

move the plug to relieve occlusion, permitting translocation of the substrate to the periplasm, where it is recognized by the periplasmic binding protein. (For excellent, comprehensive reviews of Gram-negative transport across the outer membrane and periplasm, consult (66, 137).)

1.3.2 Heme uptake: capture from heme carrier proteins

The majority (~65%) of bodily iron is found in heme groups of hemoglobin, sequestered in red blood cells (48). The overwhelming abundance of hemoglobin in the mammalian host has selected for pathogens capable of exploiting this iron pool using specialized uptake mechanisms. In Gram-negative bacteria, heme uptake is initiated by binding of heme or hemoproteins to a bacterial outer membrane protein. In the case of *Neisseria* spp., two independent outer membrane receptors are involved. HmbR is a β-barrel/plug protein which recognizes heme and hemoglobin (242). HpuAB is a bipartite receptor, involving a β-barrel outer membrane protein (HpuB) working in concert with an outward facing lipoprotein (HpuA); this complex binds hemoglobin as well as hemoglobin-haptoglobin (148). Some bacterial outer membrane receptors recognize a variety of host hemoproteins; PhuR from *Pseudomonas aeruginosa* can bind heme, hemoglobin, haptoglobin-hemoglobin, and hemopexin (192). The mechanism(s) by which receptor proteins disengage heme from the host protein are not well described. Following extraction, passage of heme through the periplasm may require a periplasmic binding protein, as exemplified by PhuT of *P. aeruginosa*; this also

serves as the receptor component of the ABC-type transporter at the cytoplasmic membrane (248).

Heme capture by Gram-positive bacteria has received less scrutiny. Recognition of hemoproteins by pathogens including *Staphylococcus aureus*, *Bacillus*, and Group A *Streptococcus* occurs at the outer face of the cell wall and requires a complement of cell wall-anchored proteins that bind hemoproteins, extract heme, and serially passage it through the peptidoglycan to cytoplasmic membrane ABC transporters (84, 152, 168, 277). The iron-regulated surface determinant (Isd) system of *S. aureus* is fairly well understood and will be described in more detail in a later section.

1.3.3 Heme uptake: hemophores

Hemophores are secreted proteins that extract heme from solution or from heme carrier proteins and deliver it to the bacterial cell. The HasA hemophores form a unique, highly conserved family of proteins produced by pathogenic bacteria including *Serratia marcescens* (144), *P. aeruginosa* (146), and *Yersinia pestis* (218). HasA binds a large number of heme carrier proteins, including hemoglobin and hemopexin (261). Due to its binding affinity for heme exceeding that of host carrier proteins, transfer of heme is passive (145). An active, TonB-dependent transfer occurs at the bacterial surface, where outer membrane receptor protein HasR initiates passage of the heme molecule into the periplasm and toward the cytoplasmic membrane (143). Hemophore production by Gram-positive bacteria has been documented only for *Bacillus*

anthracis, which secretes soluble proteins with heme-binding motifs homologous to those found in its Isd system (157).

1.3.4 Direct uptake of Fe^{3+} from transferrin

Some bacteria secure transferrin and lactoferrin iron through direct binding and manipulation of these serum proteins using cell surface receptors. The best documented transferrin receptor system is found in *Neisseria* spp. This involves a bipartite outer membrane receptor complex, TbpAB, that binds transferrin and conformationally manipulates it to force release of Fe^{3+} (44, 150). TbpB, the outer membrane β-barrel protein, delivers Fe^{3+} to periplasmic chaperone FbpA; surprisingly, this transfer is not energetically dependent on TonB (194). FbpABC form an ABC-type transporter for active uptake of Fe^{3+} into the bacterial cytoplasm (131). Sporadic reports of transferrin binding by cell wall proteins of Gram-positive bacteria exist (111, 177, 247), but mechanistic data are lacking; this phenomenon may merely reflect the promiscuity cell wall adhesion factors demonstrate in interacting with a range of host molecules.

1.3.5 Indirect uptake of Fe^{3+} from transferrin: siderophores

Siderophores are small, high affinity Fe^{3+} chelators secreted by numerous bacteria, fungi, and plants in response to the stress of iron limitation. They scrounge soluble ferric iron or compete for it with other high affinity iron chelators. As will be discussed in greater detail in ensuing sections, siderophores come in a wide variety of molecular structures (also reviewed here (113)). Cellular uptake is preceded by ligand recognition through cell surface

receptors that are highly specific for particular molecular features of the substrate, rendering siderophore iron a privilege of organisms equipped with cognate transporters. Many siderophores are competitive with transferrin and lactoferrin for iron binding, and siderophore production has been shown to contribute to growth of numerous bacteria in serum (Table 1-1).

1.3.6 Indirect uptake of Fe^{3+} from transferrin: catecholamine stress hormones

The low affinity of transferrin and lactoferrin for Fe^{2+} means their function may be compromised by interaction with agents that reduce Fe^{3+}. Recent studies have demonstrated that the catecholamine stress hormones, including adrenaline (epinephrine), noradrenaline (norepinephrine), dopamine, and L-3,4-dihydroxyphenylalanine (L-DOPA), are capable of forming direct complexes with holotransferrin and hololactoferrin, and reductively liberating iron as Fe^{2+} (80, 220). Although Fe^{2+} is readily reoxidized in biological fluids, catecholamines have been proposed to provide the hexadentate coordination required to stabilize Fe^{3+} in solution via their catechol moieties, in a 3:1 molar ratio (220); thus these hormones may also act as biological chelators that compete with transferrin.

Elevated levels of catecholamines have been correlated to higher rates of postoperative sepsis (99) and experimental elevation of systemic norepinephrine has been shown to promote translocation of enteric bacteria in mice (156). Addition of catecholamines stimulates growth on transferrin or serum for pathogenic bacteria including *Bordetella bronchiseptica* (6), *Escherichia coli*

Table 1-1: Siderophores contributing to growth of bacteria in the presence of transferrin or in serum

Species	Siderophore	Ref.
Aeromonas spp.	Amonabactin	(161)
Bordetella bronchiseptica	Alcaligin	(78)
Burkholderia cepacia	Salicylic acid	(233)
Escherichia coli	Aerobactin, enterobactin	(135)
Mycobacterium spp.	Exochelins	(59, 90)
Pseudomonas aeruginosa	Pyoverdin, pyochelin	(238)
Pseudomonas pseudomallei	Malleobactin	(276)
Salmonella enterica	Salmochelin, enterobactin	(265)
Staphylococcus aureus	Staphyloferrins A and B	This study
Vibrio parahaemolyticus	Vibrioferrin	(274)
Vibrio vulnificus	Vulnibactin	(151)
Yersinia pestis	Yersiniabactin	(67)

(220), and *Staphylococcus epidermidis* (184).

1.3.7 Uptake of Fe^{2+}

Under aerobic (oxidizing) conditions, iron predominates in solution as Fe^{3+}; under anaerobic or microaerophilic conditions, however, transport of Fe^{2+} is a relevant iron uptake strategy for many bacteria, involving the Feo (ferrous iron) transport system. First characterized in *Escherichia coli* (107, 127), this comprises two or three proteins: 1) FeoB, a large protein with an integral membrane domain, acting as the permease component (127), and a N-terminal G-protein domain, acting as a GTPase driving active transport of Fe^{2+} (159); 2) FeoA, a small, cytosolic protein that activates the GTP-hydrolyzing activity of FeoB; 3) FeoC, a small hydrophilic protein associated only with γ-proteobacterial Feo systems, that may be a transcriptional repressor (32). FeoB-mediated iron uptake from the gastrointestinal tract contributes to colonization by and pathogenesis of *Campylobacter jejuni* (183) and *Helicobacter pylori* (258); FeoB also contributes to intracellular growth of *Legionella pneumophila* (215).

FeoB-mediated uptake of Fe^{2+} could theoretically work in concert with mechanisms that reductively convert Fe^{3+} on or around the bacterial surface. Extracellular ferric reductase activity has been reported for several bacteria (224, 257), but few reports empirically demonstrate a role for this phenomenon in microbial iron acquisition. Insofar as extracellular reduction contributes to the iron uptake strategy of pathogenic bacteria, this has only been shown for capture of transferrin and lactoferrin iron by *Listeria monocytogenes* under anaerobic conditions (14).

1.4 Siderophores

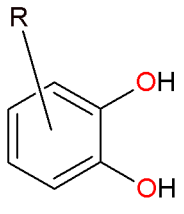
1.4.1 Siderophore chemistry and iron coordinating groups

Numerous plants, fungi, saprotrophic bacteria, and bacterial pathogens secrete small molecules called siderophores to serve as high affinity Fe^{3+} scavengers. Siderophores are assembled from amino acids, organic acids, and other small organic metabolites. Huge diversity has been documented in terms of the structure and iron-binding affinity of these compounds, although some broadly shared themes describe their physicochemical properties (for more comprehensive reviews, see (49, 171, 261)). Siderophores are small, being typically less than 1 kDa in mass. They are highly electronegative and surround Fe^{3+} to form a hexacoordinated complex involving three bidentate coordinating sites, although the stoichiometry of iron atoms to siderophore coordinating groups may vary if surrogate oxygen, nitrogen, or sulfur donors from solution compensate for vacancies. The stoichiometry of siderophore molecules to iron atoms may also deviate from 1:1 if the siderophore has more or less than three coordination moieties; rhodotorulic acid, for instance, has four, and coordinates iron in a 2:3 (Fe^{3+}:siderophore) molecular ratio.

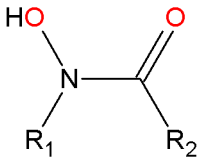
In general, three types of functional groups can act as siderophore iron coordination motifs (Figure 1-2A). First are the adjacent hydroxyl groups of catechol moieties (ortho-dihydroxybenzene), exemplified in the enterobactin structure (Figure 1-2B) (123). This siderophore group forms extremely stable ligand-Fe^{3+} complexes and includes some of the strongest known iron chelators. Most functional groups of catecholate siderophores are derived from a common

Figure 1-2: Siderophore Fe^{3+} coordination group types. (A) Siderophores coordinate Fe^{3+} in solution using catecholate, hydroxamate, or carboxylate moieties (Fe^{3+}-coordinating atoms in red). **(B)** Examples of siderophores include enterobactin (catecholate coordination), coprogen B (hydroxamate coordination), rhizoferrin (carboxylate coordination), and petrobactin (mixed catecholate and carboxylate coordination) (Fe^{3+}-coordinating atoms in red).

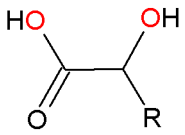
A



catecholate

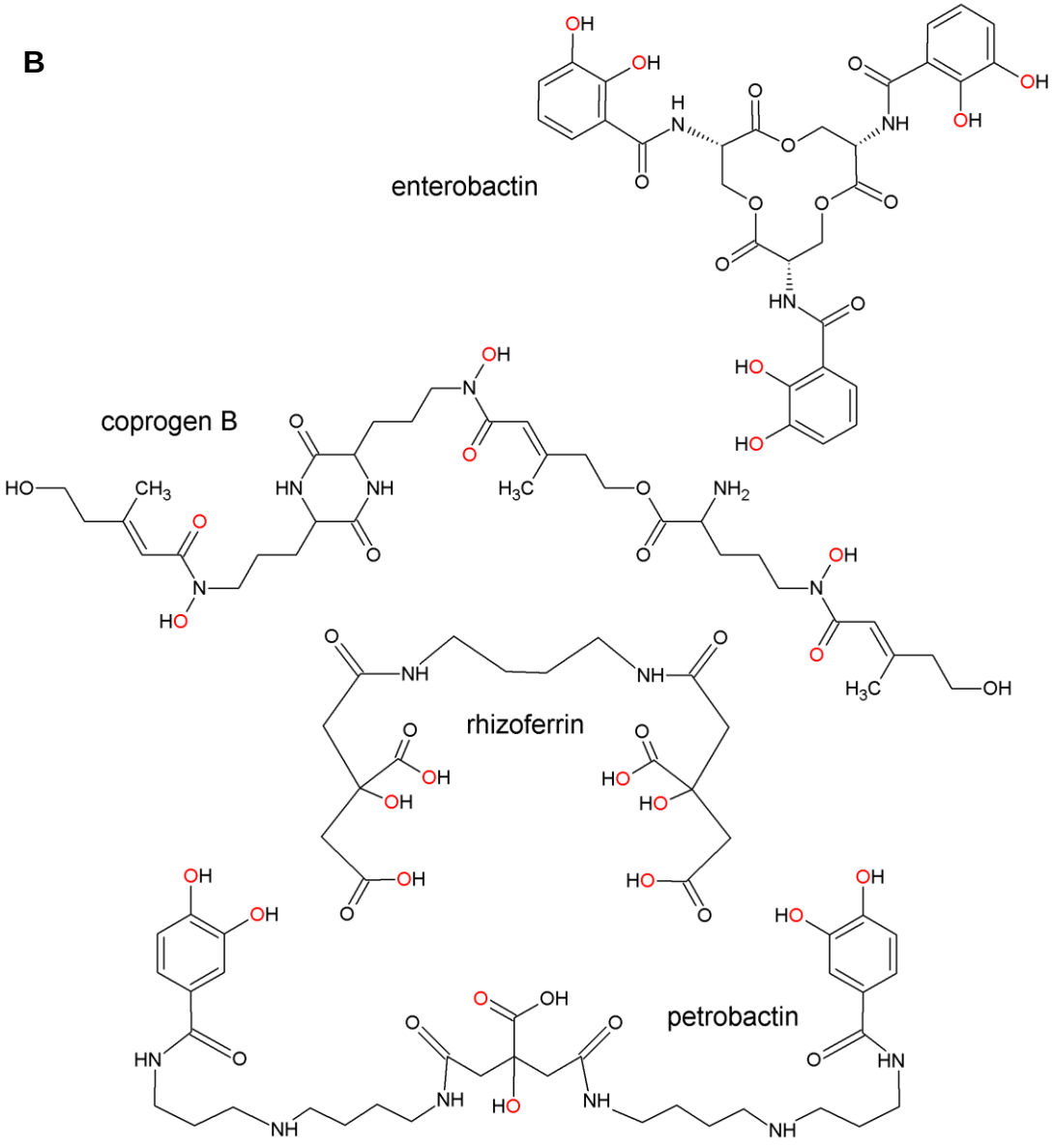


hydroxamate



carboxylate

B



precursor, 2,3-dihydroxybenzoic acid (Figure 1-3) (49). This molecule is itself capable of coordinating Fe^{3+} in a 3:1 molecular ratio, and is secreted as a siderophore by bacteria including *Brucella abortus* (154), *Azospirillum lipoferum* (222), and *Rhizobium leguminosarum* (195). Structurally similar biological molecules, including mammalian catecholamine stress hormones (Figure 1-3), have also been proposed to coordinate iron in solution; invasive bacteria with cognate transport systems could theoretically capitalize on these to meet nutritional iron demands (220).

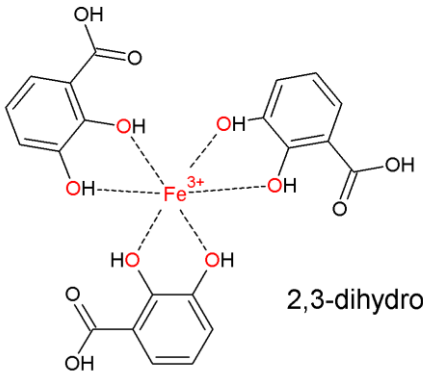
The second type of siderophore iron coordination involves hydroxamate motifs (Figure 1-2A), where adjacent carbonyl and aminohydroxyl groups provide an iron coordination site, as in coprogen B (Figure 1-2B) (271). This chemistry is frequent among fungal siderophores. The third type of coordination involves carboxylate motifs (Figure 1-2A). These may involve two sites provided by α-hydroxycarboxylates derived from citrate, or from carboxylic acid moieties, as in rhizoferrin (Figure 1-2B). Numerous siderophore molecules, such as petrobactin (Figure 1-2B) and aerobactin use combinations of the three iron coordinating groups (110, 141).

1.4.2 Nonribosomal peptide synthetase assembly

Siderophore assembly pathways fall into two broad classes: nonribosomal peptide synthesis (NRPS) and NRPS-independent siderophore (NIS) synthesis (34). NRPS siderophores involve polypeptidic scaffolds, often incorporating nonproteinogenic amino acids and their derivatives, assembled without the benefit of a ribosomal template (49). Numerous NRPS siderophores have been

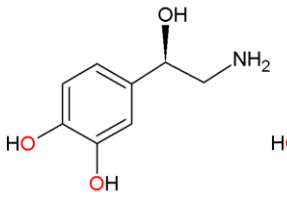
Figure 1-3: 3:1 coordination of Fe^{3+} in solution by small catecholate molecules. (A) Coordination of Fe^{3+} by three molecules of 2,3-dihydroxybenzoic acid (DHBA). **(B)** Catecholamine stress hormones, which have been proposed to coordinate Fe^{3+} in an arrangement mimicking DHBA.

A

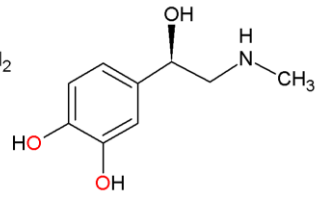


2,3-dihydroxybenzoic acid

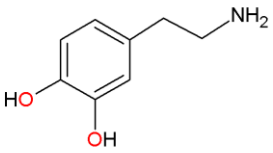
B



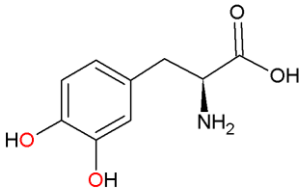
norepinephrine



epinephrine



dopamine



L-DOPA

described, including enterobactin (207), yersiniabactin (173), and vibriobactin (129). Important antibiotics such as penicillin (223) and vancomycin (244) are also NRPS metabolites. Independently of a ribosome, NRPS siderophores are condensed and modified stepwise on a multimodular enzyme, the NRP synthetase, for which the roles of functional domains can be summarized as follows (243): 1) Activation of an amino acid or growing intermediate aminoacyl-AMP by adenylation domains; 2) Transfer to peptidyl carrier protein (PCP) domains; 3) Condensation of PCP-bound amino acids by condensation domains; 4) Amino acid modifications, for example by epimerization domains; 5) Transesterification (TE) of the peptide chain from the terminal PCP onto the TE domain, with subsequent liberation of the mature metabolite through hydrolysis or macrocyclization. The modular arrangement of the NRPS synthetase essentially creates a protein assembly line, allowing metabolite assembly and modification to occur in a progressive, stepwise fashion.

1.4.3 Nonribosomal peptide synthetase independent assembly

Less well characterized are the mechanisms for assembly of NIS siderophores. These are created from the condensation of alternating subunits of dicarboxylic acids (usually succinate, citrate, and α-ketoglutarate) with diamines, amino alcohols, and alcohols (34). NIS synthetases are not homologous to their modular counterparts in NRPS pathways. They primarily serve to catalyze formation of amide and ester bonds between an organic acid and a substrate bearing an amino or a hydroxyl group (34). Individual NIS reactions are performed by individual synthetases. The proposed assembly of

aerobactin represents the archetypal pathway of NIS biosynthesis (54, 55), even though the biochemical roles of synthetases IucA and IucC have not been empirically characterized. Elucidation of the molecular structure of other siderophores and annotation of the genetic loci encoding their biosynthesis suggest rhizobactin (155), achromobactin (79), vibrioferrin (246), and alcaligin (128) are also assembled in an NRPS-independent fashion. Recent efforts have described the pathway involved in production of petrobactin (141, 197), which is a rare example of a siderophore assembled via a hybrid NRPS/NIS strategy.

A model has been proposed to classify NIS synthetases based on specificity for a carboxylic acid (34, 126). Type A enzymes, represented by IucA, catalyze condensation of a prochiral carboxyl group of citrate to an amine or alcohol functional group of a second substrate, resulting in an amide or ester bond, respectively. Type B enzymes catalyze a similar reaction substituting the C5 carboxyl of α-ketoglutarate for the citrate carboxyl. Type C enzymes, represented by IucC, catalyze condensation of monoamide/monoester derivatives of citrate or succinate to carboxyl groups on molecules with an amine or an alcohol. For the most part, predictions of NIS synthetase type based on phylogenetic analysis have accurately described their role in functional enzyme assays, but this is based on limited data, and at least one exception is known and will be discussed in a later section. Additional modifications to precursors, such as decarboxylation, isomerization, or oxidation, are performed by separate enzymes typically encoded by genes clustering near to those encoding the synthetases.

1.4.4 Siderophore uptake and iron liberation

In Gram-negative bacteria, siderophore import to the periplasm is mediated by outer membrane receptors and the Ton system, as previously described. Siderophore transport across the bacterial cytoplasmic membrane is typically performed by ABC-type transporters. Genetic loci for ABC transporters often reside in close genomic proximity to loci involved in biosynthesis of the cognate siderophore. Siderophore binding proteins sometimes exhibit plasticity in their substrate affinity, recognizing multiple siderophores within the same iron coordination chemistry. Examples include *E. coli* FhuA and FhuD, the outer membrane receptor and periplasmic binding proteins, respectively, for aerobactin and a broad host of exogenously derived hydroxamate type siderophores (65, 136). Homologous FhuD lipoproteins in *S. aureus* also recognize a variety of actively transported hydroxamate molecules (226).

Upon cellular internalization, two mechanisms may be used for liberation of siderophore-bound iron. Extraction from exceptionally high affinity siderophores often requires destruction of the molecule, while those with lower affinity may be deferrated through a reductive mechanism. Both scenarios occur in *E. coli*. Enterobactin, the strongest known siderophore (K_d = 10^{52} M^{-1}), is cleaved by esterase FesA at ester groups in the trilactone backbone, yielding subunits of 2,3-dihydroxybenzoyl-L-serine (43); concomitant reduction of Fe^{3+} occurs through an unknown mechanism (29). On the other hand, the hydroxamate siderophore aerobactin, with a more modest affinity for Fe^{3+}, is acted upon by cytoplasmic ferrisiderophore reductase FhuF, yielding Fe^{2+} for cellular processes, and reusable aposiderophore (166).

1.4.5 Contributions to virulence

Numerous studies have demonstrated the impact of siderophore-mediated iron uptake on virulence, even among organisms capable of extracting host heme iron; key reports are summarized in Table 1-2. Pathogenic bacteria may also produce siderophores for survival outside of a host niche, and rely on other iron uptake strategies *in vivo*; for instance, production of a catecholate siderophore is important for survival of the veterinary pathogen *Rhodococcus equi* as an environmental saprotroph, while having no significant impact on virulence in a murine infection model (176).

1.4.6 Siderocalin and stealth siderophores

The lipocalins are a family of roughly 20 small, soluble proteins, produced by prokaryotes and eukaryotes. Their three dimensional structure comprises an eight-stranded anti-parallel β-barrel that forms a cavity capable of enclosing low molecular weight solutes (77). The mammalian protein lipocalin-2, alternately named neutrophil gelatinase-associated lipocalin or, pertinently to this study, siderocalin, is secreted by macrophages and neutrophils during the inflammatory response to bacterial antigens such as lipopolysaccharide, leading to rapid rises in serum titres (76). Expression of recombinant siderocalin in *E. coli* serendipitously revealed it binds the catecholate siderophore enterobactin (91), and it is now known to sequester a variety of bacterial siderophores, primarily those with catecholic or phenolic moieties (91, 116). Siderocalin-deficient mice are more susceptible to sepsis and pneumonia caused by *E. coli* strains that rely on enterobactin for siderophore-mediated iron

Table 1-2: Siderophores contributing to the virulence of bacteria in animal models of colonization or infection.

Species	Siderophore	Animal model	Ref.
Bacillus anthracis	Petrobactin	Mouse inhalation anthrax	(31)
Bordetella bronchiseptica	Alcaligin	Neonatal pig intranasal colonization	(209)
Bordetella pertussis	Alcaligin	Mixed competition mouse respiratory infection	(28)
Burkholderia cenocepacia	Ornibactin	Rat agar bead lung infection	(234)
Escherichia coli	Aerobactin	Mouse intraperitoneal lethal challenge	(266)
Escherichia coli	Salmochelin	Mouse intraperitoneal lethal challenge	(72)
Mycobacterium tuberculosis	Carboxymycobactin	Mouse aerosol inhalation infection	(216)
Neisseria gonorrhoeae	Gonobactin	Chicken embryo bacteremia	(70)
Pseudomonas aeruginosa	Pyoverdin	Burned mouse subcutaneous infection	(170)
Salmonella enterica	Salmochelin, enterobactin	Mouse cecal colonization	(265)
Salmonella typhimurium	Enterobactin	Mouse intraperitoneal infection	(275)
Staphylococcus aureus	Staphyloferrins A and B	Mouse sepsis	This study
Vibrio cholerae	Vibriobactin	Mouse suckling intragastric infection	(112)
Vibrio vulnificus	Vulnibactin	Mouse suckling intragastric infection	(151)
Yersinia pestis	Yersiniabactin	Mouse pneumonic plague, bubonic plague	(67)

uptake (76, 272). Siderocalin is also capable of binding catecholamine stress hormones (172), further enhancing bacteriostasis through iron sequestration.

The binding of iron chelators by siderocalin is not indiscriminate, and many bacteria can subvert this immune factor by producing alternate, unrecognizable siderophores, or by incorporating structural modifications or substitutions within preexisting siderophore molecular templates. Siderocalin-evading siderophores are frequently described using the adjective “stealth”. The salmochelin family, for instance, includes enterobactin glucosylated at the C5 position of one or more of the molecule’s catecholic arms (22), and 2,3-dihydroxybenzoylserine subunits bridged by glucose residues (109). These enzymatic modifications to siderocalin-cognate siderophores are mediated by genes encoded in the *iroA* cluster, a virulence locus typically situated within pathogenicity islands or plasmids of exceptionally virulent strains of *E. coli* and species of *Salmonella* (17, 60, 236). Salmochelins are not bound by siderocalin, and *iroA*-expressing *E. coli* strains are insensitive to its growth inhibitory effects (72).

The anthrax pathogen also employs a similar strategy for bypassing siderocalin. *B. anthracis* produces two siderophores: petrobactin, which coordinates iron using an unusual combination of one carboxylate and two 3,4-catecholate moieties (Figure 1-2B) (20), and the more conventional bacillibactin, with three 2,3-catecholate-type coordination sites (167). Petrobactin is the only siderophore known incorporating 3,4-catechols. Owing to this unusual chemistry, petrobactin is not recognized by siderocalin, unlike bacillibactin, for which siderocalin has exquisite affinity (2). Petrobactin but not bacillibactin was found to contribute to the intracellular survival of *B. anthracis* in

macrophages and in a murine subcutaneous anthrax model of infection (33). This has been subsequently interpreted as proving a correlation between siderocalin evasion and pathogenesis, although other observations reveal bacillibactin to be dispensible in a range of host-independent conditions despite a high affinity for Fe^{3+}, convoluting the narrative that acquisition of petrobactin biosynthesis is necessarily an evolutionary countermeasure to siderocalin. Petrobactin but not bacillibactin is sufficient to sustain vegetative growth of *B. anthracis* in chelated culture medium, and furthermore to induce vegetative growth from dormant spores (33); the chronology of siderophore production at the onset of vegetative growth proceeds as petrobactin first, bacillibactin second (267). While petrobactin clearly contributes to the enhanced virulence of *B. anthracis*, it may be inadequate to describe its evolutionary significance based solely on its contribution toward virulence.

1.5 Bacterial regulation of iron transport mechanisms

1.5.1 The ferric uptake regulator is a conserved mechanism governing bacterial transcriptional responses to iron limitation

A decrease in intracellular iron stores is a key sensory trigger for the genetic expression of iron uptake mechanisms and virulence factors that play roles in liberating host iron stores or circumventing host immune strategies. The Fur protein (ferric uptake regulation) was first proposed for *Salmonella typhimurium* (63) and first described for *E. coli* (108); it is now recognized as the canonical global iron responsive regulator in bacteria. Fur is a homodimeric transcriptional repressor whose mode of action has been reviewed extensively

(64, 106). In brief, when cellular iron exceeds the level required for proper function of essential iron-containing metalloenzymes, association of one Fe^{2+} ion to each Fur monomer causes protein to bind a roughly 19-bp DNA motif, called the Fur box, within the operator region of target genes. The *E. coli*-deduced consensus Fur box reads as GATAATGATAATCATTATC (56), and similar sequences are found in the operator regions of iron responsive genes in other organisms. Typically, the Fur/DNA association blocks RNA polymerase, repressing gene transcription. As intracellular iron levels become depleted, Fur/Fe^{2+} dissociation occurs, alleviating DNA binding and derepressing iron responsive genes. Fur is known to indirectly activate some genes, via repression of small RNA silencing molecules (162), and direct upregulation of a target gene has also been reported (57).

1.5.2 Alternate examples of regulatory mechanisms contributing to iron homeostasis

Many bacteria use additional regulatory systems to respond to specific iron ligands. In Gram-negative bacteria, the most common mechanism involves the use of extracytoplasmic function σ factors (ECF factors), with the archetypal example being FecI in *E. coli* (for a review, see (27)). This factor enables *E. coli* to express genes specific for uptake of citrate-coordinated iron, genes that are Fur repressible but require additional positive regulation for expression that occurs only in the presence of extracellular ferric citrate. Briefly, under nonactivating conditions (in the absence of ferric citrate), ECF factor FecI is sequestered at the inner leaflet of the cytoplasmic membrane by

membrane-spanning anti-σ factor FecR. When ferric citrate is imported to the periplasm through TonB-dependent outer membrane protein FecA, with simultaneous interaction between FecA and FecR, FecI is released into the cytosol. FecI associates with RNA polymerase and recruits it to the operator region of the *fec* operon, which encodes the proteins required for reduction of iron in periplasmic ferric citrate and transport of Fe^{2+} through the cytoplasmic membrane.

While Fur functions to repress genes required for acquisition of iron when iron is abundant, additional regulatory measures may be necessary to help bacteria respond to toxic intracellular accumulation of iron or iron ligands. Recently, a heme responsive efflux system has been described for *S. aureus*, and bioinformatic analysis suggests this mechanism is conserved across a range of Gram-positive bacterial pathogens (240, 251). This involves a two component regulatory system, HssSR, which senses intracellular heme accumulation (HssS) and activates a transcriptional regulator (HssR) for the expression of an ABC-type heme efflux transporter (encoded by *hrtAB*). In this fashion, *S. aureus* can mitigate toxicity of dangerously rapid heme accumulation following derepression of Fur regulated heme uptake genes.

1.6 *Staphylococcus aureus*

1.6.1 The genus *Staphylococcus*

The Gram-positive bacterial genus *Staphylococcus* comprises 49 member species. Most are mammalian commensalists or opportunistic pathogens, occupying niches including skin, nares, and mucosal membranes. Four species

are of particular medical and veterinary relevance. *S. saprophyticus* is the second leading cause of uncomplicated urinary tract infection (117). *S. intermedius* is a leading cause of pyoderma in dogs and has become a significant reservoir of antimicrobial resistance factors for the genus (101, 102). *S. epidermidis* is a normal component of the epidermal flora of most mammals. Its ubiquity and ability to produce a viscous, hydrophobic, extracellular polysaccharide slime make it a leading cause of biofilm contamination of prostheses and indwelling medical devices (68).

The most nefarious staphylococcal species is *S. aureus*, the causal agent of a staggering variety of disease symptoms, ranging from cosmetic to lethal. Following traditional diagnostics, *S. aureus* is distinguished from the rest of its genus by its abundant production of secreted coagulase, an enzyme which converts serum fibrinogen to fibrin and promotes clotting. However, some strains of *S. lugdunensis* produce coagulase, as a bound form (165); and a recent emergence of veterinary strains of coagulase-secreting non-*aureus* species (213, 221) is further reducing the usefulness of this marker as a taxonomic tool. Accuracy notwithstanding, *Staphylococcus* species are for the time being classified as coagulase positive staphylococci (CPS, *i.e. S. aureus*) or coagulase negative (CNS, *i.e.* all other staphylococci).

1.6.2 Pathogenesis

S. aureus is a frequent commensal; 30% of the American population is asymptomatically colonized in the nostrils (92). Progression to disease is often opportunistic on immunocompromise, injury, or other medical complications of

the host; however, environmental strains exist that can severely infect healthy individuals. *S. aureus* causes suppurative skin and subcutaneous infections, including furunculosis, impetigo, cellulitis, mastitis, and flesh eating disease (10, 15, 42, 160, 174); respiratory infections, including nonsevere and necrotizing forms of pneumonia (89, 212); and sepsis, which can lead to osteomyelitis, septic arthritis, and endocarditis (62, 147, 181). Staphylococcal infection can also result in toxic shock if the invading strain produces superantigens (169). Severe, necrotizing disease manifestations are frequently caused by community-acquired strains, which are often capable of infecting individuals with no predisposing medical conditions; for recent reviews, consult (16, 53).

Systemically disseminated *S. aureus* can become persistent as the result of abscession (36). Colonies seeded in organ tissues induce inflammatory responses and attract macrophages, neutrophils, and other phagocytes. Recruited immune cells mediate liquefaction necrosis as the host seeks to prevent bacterial spread. Lesions containing necrotic tissue and leukocytes form around *S. aureus* communities enclosed by pseudocapsules, protecting them from the action of phagocytic immune cells. Abscesses that grow and rupture contribute to secondary waves of sepsis.

Invasion and persistence of *S. aureus* in a host is dependent on a battery of virulence factors. These include, but are not limited to, proteins and molecules involved in: attachment (fibrinogen/fibronectin/collagen binding proteins, polysaccharide capsule), resistance to immune cells (protein A, complement inhibitory protein, staphylokinases, proteases), destruction of immune cells (Panton Valentine leukocidins, phenol soluble modulins),

subversion of immune cells (superantigens, chemotaxis inhibitory protein), intracellular survival (catalase, carotenoid pigments), and lysis of erythrocytes (hemolysins); for recent reviews, consult (188, 189, 255).

S. aureus coordinates expression of virulence genes such that surface-associated factors are preferentially produced during exponential growth phase, promoting adherence, while secreted factors are preferentially produced in postexponential phase. This coordination is largely mediated by two regulatory mechanisms, whose genes are encoded within the *agr* locus (accessory gene regulator) and gene *sarA* (staphylococcal accessory regulator) (37-39, 253). Additional mechanisms also fine tune expression of virulence factors. The two component regulator SaeRS, for instance, is responsive to stimuli associated with phagocytic immune cells, such as hydrogen peroxide (85), leading to expression of secreted toxins and capsule (190, 259). As will be discussed further below, iron limitation is also a key sensory trigger for the expression of staphylococcal virulence factors.

1.6.3 Antibiotic resistance

Antibiotics are crucial to treatment of *S. aureus* disease. Alexander Fleming originally documented staphylococcal sensitivity to what is now known as penicillin in his hallmark 1929 report on the antibacterial properties of the fungus *Penicillium notatum* (74). By the 1940's, penicillin was being hailed as the miracle cure to a wide range of infections. The β-lactam ring of the penicillin molecule irreversibly binds cell wall transpeptidases (264), also known as penicillin binding proteins (PBPs). Staphylococcal PBPs link pentapeptide side

chains of neighbouring glycan strands, via a displacement reaction where the L-lysine (third position) of one side chain is bonded to the a D-alanine (fourth position) of the other side chain via a pentaglycine bridge (87, 268). Impairing this function disrupts the cell wall and is bactericidal. As early as 1944, penicillin inactivation by what is now known as β-lactamase was being reported for resistant strains of *S. aureus* (133). Semisynthetic penicillin derivatives including methicillin (1959) and oxacillin (1961), unrecognizable to β-lactamase, offered renewed hope in the battle against *S. aureus*. This hope proved short-lived as resistance to these drugs readily emerged (125). This mechanism of resistance appeared to be nondestructive toward the antibiotic (245), and was eventually shown to be due to expression of an alternate PBP, *i.e.* PBP2a, with vastly reduced affinity for β-lactams (82). The gene encoding staphylococcal PBP2a, *mecA*, is localized in a mobile segment of DNA referred to as a staphylococcal chromosome cassette (SCC*mec*), integrated into the *S. aureus* chromosome at a site-specific location near the origin of replication (140). Seven different SCC*mec* elements have been described; compositional differences include regulatory genes for *mecA*, and the presence of additional antibiotic resistance determinants (58). Methicillin resistant *S. aureus* (MRSA) strains have become widely disseminated across the globe (100), and SCC*mec* is increasingly documented in especially aggressive community-acquired *S. aureus* infections (58).

The glycopeptide antibiotic vancomycin is the effective alternative for treating MRSA. Glycopeptides inhibit cell wall synthesis by binding the two terminal D-alanine residues of the pentapeptide side chains of peptidoglycan,

blocking transglycosylation and transpeptidation reactions (211). Vancomycin intermediate (VISA) and resistant (VRSA) strains are emerging from MRSA lineages. Intermediacy (*i.e.*, decreased sensitivity) results from a reworking of the regulatory circuitry in the *S. aureus* cell, with increased resources going toward cell wall thickening; this has the effect of saturating vancomycin. VISA is also frequently associated with altered metabolism, production of protein A, and autolysis, although the mechanisms by which these changes contributes to vancomycin tolerance are not understood; for a more comprehensive review, consult (118). Outright resistance to vancomycin in *S. aureus* is mediated by the *vanA* operon, encoding for enzymes that replace terminal D-alanine residue of peptidoglycan side chains with D-lactate (9). First reported in Japan in 1997 (115) VISA were soon documented in North America (232). The first report of VRSA in North America occurred in Michigan in 2002, and has since reemerged nine times in the USA (196). This threat to our last line of defense highlights the need for research into new antistaphylococcal drugs.

1.6.4 Response to iron starvation

Iron is a cofactor of numerous proteins involved in central metabolism and respiration. Metabolically, *S. aureus* responds to iron limitation by upregulating glycolysis and downregulating the tricarboxylic acid (TCA) cycle; extreme iron limitation results in inactivation of the electron transfer chain, leading to fermentative growth (81). The TCA cycle involves numerous enzymes that require iron as a cofactor (235). Glycolysis generates ATP independently of respiration, with accumulation of pyruvate and lactate. Lactate was shown to be

secreted by iron-starved *S. aureus*, resulting in acidification of the surrounding medium; this might contribute to liberation of iron from host sequestration proteins transferrin and lactoferrin (81).

S. aureus pathogenesis is also influenced by iron availability. Fur-Fe^{2+} indirectly contributes to coordinated repression of secreted hemolysins and cytotoxins, and under sustained conditions of iron limitation upregulation of these factors occurs. On the other hand, Fur-Fe^{2+} contributes to positive regulation of immunomodulatory proteins, including superantigens, protein A, complement inhibitory protein, and chemotaxis inhibitor (249). A *fur* inactivated mutant was shown to be less virulent in a murine pneumonia model of infection (249), suggesting that in the absence of this key regulatory protein, *i.e.* in a strain that consitutively expresses an iron starvation response, virulence is compromised due to improved clearance by immune cells despite the enhanced cytotoxicity of the mutant.

The iron restriction response of *S. aureus* also involves secretion of siderophores. A review of the scientific literature uncovers at least four named molecules: staphyloferrin A (SA, 479 Da) (134), staphyloferrin B (SB, 449 Da) (104), aureochelin (577 Da) (46), and staphylobactin (822 Da) (50). It should be noted that the first three studies involved many collection-specific isolates, whose taxonomic identification has not been corroborated using molecular techniques. Contemporary bioinformatic interrogation has uncovered only two potential NIS pathways (loci *sfa* and *sbn*) and one NRPS pathway (gene *aus*) encoded within all sequenced *S. aureus* genomes. One of the NIS pathways is also conserved broadly among CNS genomes; the NRPS pathway appears less

frequently (principally, within *Staphylococcus epidermidis*). The NRPS synthetase has been recently characterized (273, 278), and does not produce an iron-binding metabolite (N. Magarvey, personal communication). As will be discussed below, only the staphyloferrins appear to be *bona fide S. aureus* siderophores, and SB appears thus far to be exclusive to *S. aureus* within the genus.

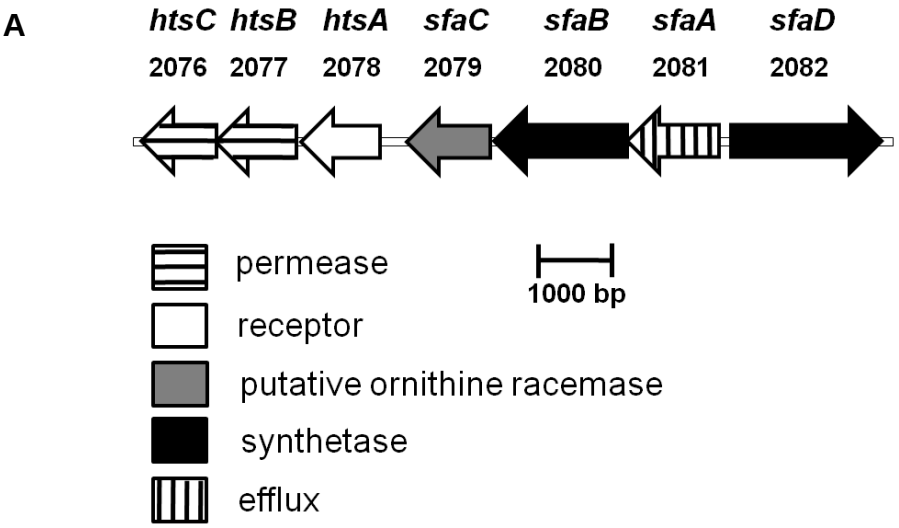
1.6.5 *S. aureus* siderophores: staphyloferrin A (SA)

SA has been identified from supernatants of both *S. aureus* and CNS (134, 149). All sequenced staphylococcal genomes contain a highly conserved locus comprising genes *sfaABC sfaD*, which encodes two NIS synthetases (Figure 1-4A). The biosynthetic pathway for synthesis of SA was recently characterized using recombinant, *S. aureus*-derived, *Escherichia coli*-expressed proteins SfaD and SfaB (Figure 1-4B) (45). SfaD condenses a molecule of citrate to the δ-amine group of D-ornithine, followed by condensation of a second citrate to the α-amine group by SfaB to yield SA. Fe^{3+} coordination by SA involves two sites provided by α-hydroxy-carboxylates derived from citrate (95), making SA a carboxylate type siderophore. The putative function of SfaC is an amino acid racemase, presumably for isomerization of ornithine from L- to D-forms. SfaA is predicted to be a member of the major facilitator family of efflux pumps.

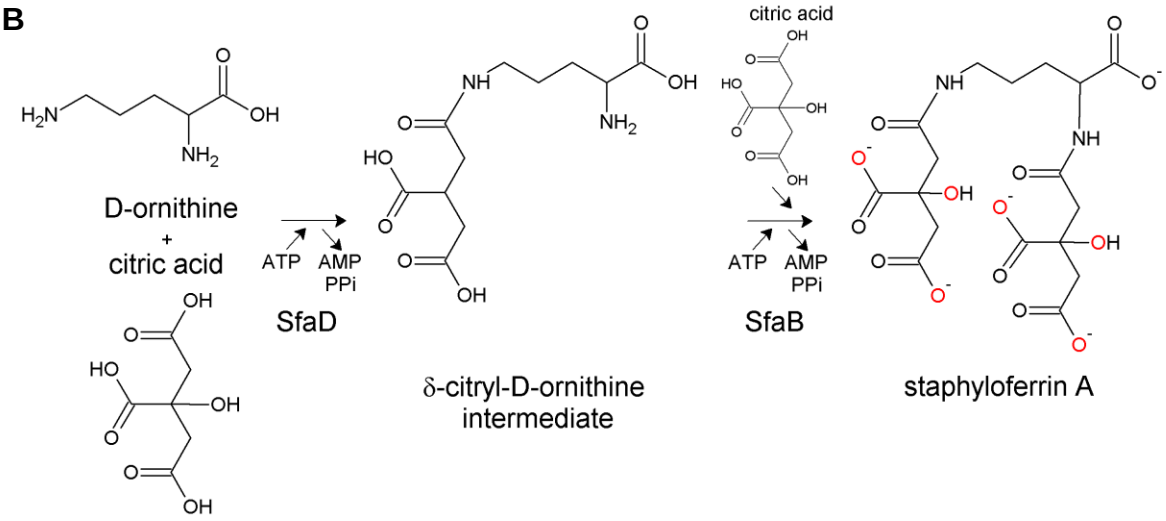
Based on sequence alignments, SfaB and SfaD cluster among B type NIS synthetases (34). Their chemistry, however, aligns them with the A-type

Figure 1-4: The *S. aureus hts/sfa* locus encodes for transport and biosynthesis of staphyloferrin A. (A) Schematic of the *hts* operon, encoding the receptor lipoprotein and permease components of an ABC transporter, and the *sfa* locus, encoding enzymes involved in biosynthesis of staphyloferrin A. Numbers refer to locus tags within the *S. aureus* strain Newman genome (*e.g.* NWMN_2076). **(B)** NIS synthetases SfaD and SfaB assemble SA from two molecules of citric acid and one molecule of D-ornithine. Proposed Fe^{3+}-coordinating atoms of SA are in red.

A


htsC htsB htsA sfaC sfaB sfaA sfaD
2076 2077 2078 2079 2080 2081 2082
1000 bp
permease
receptor
putative ornithine racemase
synthetase
efflux

B


D-ornithine
+
citric acid
ATP
AMP
PPi
SfaD
δ-citryl-D-ornithine
intermediate
citric acid
ATP
AMP
PPi
SfaB
staphyloferrin A

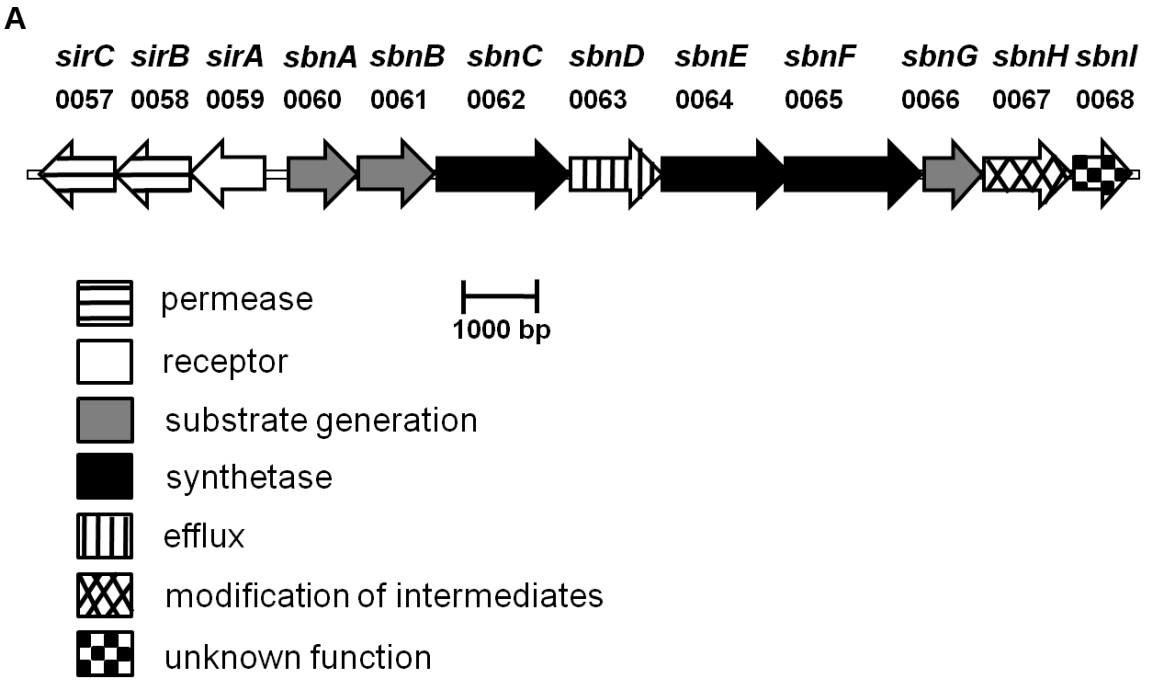
enzymes (*i.e.* specificity for citrate); they have been recently been reassigned to a subgroup within the A-type enzymes (193).

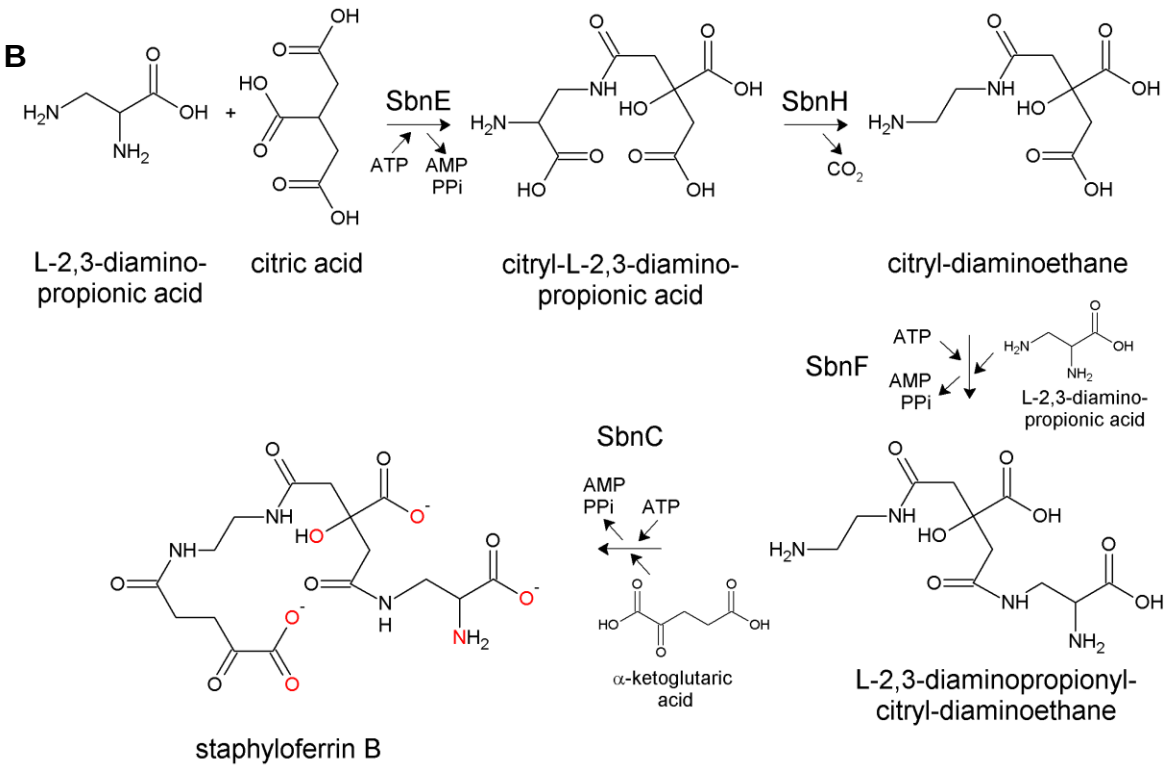
1.6.6 *S. aureus* siderophores: staphyloferrin B (SB)

SB was first purified from supernatant extracts of *Stapylococcus hyicus* (CNS) (104). The biochemical pathway for SB synthesis was recently characterized using recombinant, *S. aureus*-derived, *Escherichia coli*-expressed proteins (40). The requisite NIS synthetases include SbnC, SbnE, and SbnF, encoded within operon *sbnABCDEFGHI* (siderophore biosynthesis) (Figure 1-5A), which was previously implicated in staphylobactin production using molecular genetics techniques (50); thus, SB and staphylobactin are one and the same. The *sbn* operon has not been annotated in any sequenced CNS genome, and a host of CNS isolates tested negative for *sbn* genes (50), calling into question early reports of SB production by CNS species (104, 149); it should be noted, however, that the *S. hyicus* genome is still unsequenced. Curiously, *sbn* genes and SB production have also been described for two proteobacteria: the plant pathogen *Ralstonia solanacearum* (21), and the heavy metal tolerant saprotroph *Cupriavidus metallidurans* (124, 180).

Biosynthesis of SB can be summarized as follows (Figure 1-5B): molecules of citrate and L-diaminopropionic acid (L-Dap) are condensed by SbnE, forming citryl-Dap; SbnH decarboxylates the carboxyl group from the L-Dap moiety, creating citryl-diaminoethane (C-Dae); SbnF condenses a second molecule of L-Dap to the remaining free prochiral carbon of the citrate moiety; finally, SbnC condenses α-ketoglutarate to the free amine of the diaminoethane

Figure 1-5: The *S. aureus sir*/*sbn* locus encodes for transport and biosynthesis of staphyloferrin B. (A) Schematic of the *sir* operon, encoding the receptor lipoprotein and permease components of an ABC transporter, and the *sbn* operon, encoding enzymes involved in biosynthesis of staphyloferrin B. Numbers refer to locus tags within the *S. aureus* strain Newman genome (*e.g.* NWMN_0057). **(B)** NIS synthetases SbnE, SbnF, and SbnC, and decarboxylase SbnH, assemble SB from one molecule of citric acid, one molecule of α-ketoglutaric acid, and two molecules of L-diaminopropionic acid. Proposed Fe^{3+}-coordinating atoms of SB are in red.


A
sirC sirB sirA sbnA sbnB sbnC sbnD sbnE sbnF sbnG sbnH sbnI
0057 0058 0059 0060 0061 0062 0063 0064 0065 0066 0067 0068
permease
receptor
substrate generation
synthetase
efflux
modification of intermediates
unknown function
1000 bp


B
L-2,3-diamino-propionic acid
citric acid
SbnE
ATP
AMP
PPi
citryl-L-2,3-diamino-propionic acid
SbnH
CO2
citryl-diaminoethane
SbnF
ATP
AMP
PPi
L-2,3-diamino-propionic acid
SbnC
AMP
PPi
ATP
α-ketoglutaric acid
staphyloferrin B
L-2,3-diaminopropionyl-citryl-diaminoethane

moiety (40). In contrast to the Sfa synthetases, phylogenetic alignments of the Sbn synthetases agree well with their NIS activity class (34): SbnE has A-type activity, SbnC has B-type activity, and SbnF has C-type activity. The hexadentate coordination of Fe^{3+} by SB is unusual for a carboxylate-type siderophore; in addition to typical coordination using five oxygen atoms, the sixth coordinating atom is nitrogen from the primary amine group of the terminal L-Dap residue (94).

The roles of remaining Sbn proteins have not been published. Based on bioinformatic analyses, their putative functions include a serine sulfhydrylase (SbnA) and an ornithine cyclodeaminase (SbnB), which could work in concert to generate L-Dap; a major facilitator family protein (SbnD), possibly for efflux of aposiderophore; an aldolase (SbnG); and a protein of unknown function (SbnI).

1.6.7 Siderophore transport: the staphyloferrins

The ABC transporter for SA consists of lipoprotein receptor SirA and heterodimeric permease SirBC (18, 51). The *sirABC* (staphylococcal iron regulated) operon resides adjacent to the *sbn* operon on the *S. aureus* chromosome, is preceded by a consensus Fur box, and is upregulated under conditions of iron limitation (51). SirA has a dissociation constant for ferrated SB in the low nanomolar range (94), and is required by *S. aureus* for uptake of SB produced biologically (51) and synthetically (40). Among the CNS, only the *S. lugdunensis* genome appears to encode an ABC transporter with reasonable sequence homology to *sirABC*, albeit lacking the *sbn* operon.

An operon encoding a putative ABC transporter preceded by a Fur box is situated adjacent to the *sfa* locus in *S. aureus* and CNS chromosomes, making it an attractive candidate for the genetic component involved in SA uptake. The operon in question has been annotated *htsABC* (heme transport system) based on observations that mutations in either permease gene result in an iron source preference shift toward transferrin (231). This conclusion was derived from limited experimental analysis and no subsequent publications have contributed to the paradigm of HtsABC-mediated heme uptake. Furthermore, the crystal structure of HtsA bound to SA has recently been published (95). The HtsA binding pocket is lined with positively charged arginine residues that are quite conducive to interacting with anionic siderophores; accommodation of hydrophobic, planar heme molecules seems less likely, but has not been empirically discounted.

Notably absent from *sir* and *hts* operons is a candidate ATPase gene. Transport of both staphyloferrins is dependent on gene *fhuC*, which encodes the ATPase required for transport of exogenously produced hydroxamate siderophores via the FhuBG permease, and is transcribed from unlinked operon *fhuCBG* (further described below) (237).

1.6.8 Siderophore transport: exogenous siderophores

S. aureus uses a number of hydroxamate type siderophores, including aerobactin, ferrichrome, and desferrioxamine-B. *S. aureus* culture supernatants test negative for production of this siderophore class; thus they are considered exogenous. Transport is dependent on a permease/ATPase complex encoded

from operon *fhuCBG* (227). As mentioned previously, the FhuC ATPase is coopted by SirABC and the SA transporter (237). Two homologous receptor lipoproteins, both genetically unlinked to *fhuCBG*, are involved in substrate recognition: FhuD1 and FhuD2. The latter demonstrates higher substrate affinity and a broader proficiency for recognition of different hydroxamate-containing molecules (229). Hydroxamate-mediated iron acquisition is especially relevant to medical practices, specifically with regards to chelation therapy for treatment of iron overload disorders such as thalassemia. A frequently administered plasma chelator is desferrioxamine-B, which is distributed under various commercial names including Desferal™ (Novartis); without precaution, chelation therapy may actually predispose patients to bacterial sepsis.

S. aureus is also known to transport enterobactin as an iron ligand despite being deficient in production of catecholate siderophores (226). Prior to the body of work presented here, the transporter for this siderophore class had not been identified. An attractive candidate is encoded within the *sstABCD* operon, which includes genes for a dimeric permease (SstAB), an ATPase (SstC), and a lipoprotein sharing sequence identity with bacterial receptors for iron ligands (SstD). This operon was shown to be iron regulated *in vitro* and *in vivo* (178), but no transported substrates were identified.

1.6.9 Heme uptake

Heme uptake by *S. aureus* is mediated by the Isd system of cell wall and membrane proteins (168). Recognition of hemoproteins occurs at the outer face

of the cell wall. In Gram-positive bacteria this is a robust yet dynamic structure that acts as a scaffold for covalently linked surface proteins; for pathogens, many of these are involved in recognition of and adherence to host factors. *S. aureus* binds hemoglobin and hemoglobin-haptoglobin using wall-anchored proteins IsdB (250) and IsdH (61). These extract heme through an uncharacterized mechanism and passage it to the cytoplasmic membrane via transfers along wall-embedded proteins IsdC and IsdA, toward membrane protein IsdE (153, 182, 277). IsdA/B/C/H all contain NEAT (Near-iron transport) domains: ~120 residue motifs forming eight-stranded immunoglobulin β-sandwich folds, creating hydrophobic heme binding pockets (200). At the cytoplasmic membrane, IsdE and IsdF form the receptor and permease components, respectively, of the heme ABC transporter (98); within the cytosol, iron liberation through heme degradation is mediated by monooxygenases IsdG and IsdI (230). A model explaining the directionality of the heme relay from the surface of the cell wall to the IsdE/F transporter has been proposed, and is dependent on two factors: i) the localization of the heme binding proteins, which are situated progressively closer to the cytoplasmic membrane; ii) the dynamic rate of transfer, which is slowest at the surface of the cell wall (IsdB/H to IsdA) but becomes progressively faster approaching the membrane (IsdC to IsdE) (96). The contribution of Isd to virulence in animal models of infection is significant, suggesting this system makes a major contribution to fulfilling the nutritional iron needs of this important pathogen (201, 250). Additionally, IsdB is one of the most abundantly expressed factors on the surface of iron-starved *S. aureus*, making it an attractive candidate for antistaphylococcal vaccines (132,

139). The importance of Isd to heme uptake should not be overstated, however; even in its absence, *S. aureus* readily grows on heme as a sole iron source, and at least one alternate uptake system has recently been uncovered (D. E. Heinrichs and C. Chung, unpublished data).

1.6.10 Summary of *S. aureus* iron homeostasis genes

A review of the scientific literature pertaining to characterized *S. aureus* iron homeostasis factors, combined with a swath of annotated but uncharacterized genes from genome sequencing projects, reveals *S. aureus* is exquisitely proficient at adapting to fluctuations in iron availability. Known and putative iron homeostasis genes are summarized in Table 1-3. The redundancy and complexity of iron acquisition mechanisms suggests that therapeutics targeting the overall process may be of limited efficacy unless they are equally comprehensive.

1.7 Objective and hypotheses

Septic *S. aureus* infection can lead to numerous debilitative and lethal disease symptoms. Prior to the start of this study, it was known that *S. aureus* uses serum transferrin as an iron source, but the mechanism or mechanisms for this phenomenon were not described. It was also known that *S. aureus* produces at least two siderophores, but their contributions to infection were poorly characterized. During the course of this study, noteworthy publications describing the role of catecholamine stress hormones in liberating iron from transferrin also prompted the question of whether this interaction could

Table 1-3: *S. aureus* genes with known or putative roles in iron homeostasis

Genetic locus (*S. aureus* Newman)	Gene nomenclature	Role	Ref.
NWMN_0059-0057	*sirABC*	Staphyloferrin B aka staphylobactin transporter (receptor lipoprotein and heterodimeric permease components)	(18, 51, 94)
NWMN_0060-0068	*sbnABCDE FGHI*	Staphyloferrin B aka staphylobactin biosynthesis	(18, 40, 50)
NWMN_0111	*isdI*	Heme monooxygenase	(230)
NWMN_0336-0338	*fepABC*	Ferrous iron ABC transporter (receptor lipoprotein, putative ferrous iron peroxidase, homodimeric permease components)	(23)
NWMN_0581, 0582	*fepA*, *fepG*	Putative iron compound ABC transporter (receptor lipoprotein and homodimeric permease components)	(24)
NWMN_0603-0601	*sitABC*	Iron-regulated putative ABC transporter (heterodimeric permease components, ATPase, and lipoprotein receptor)	(114, 164)
NWMN_0604	*feoA*/*sirR*	Putative iron/divalent cation-dependent repressor	(114, 164)
NWMN_0616-618	*fhuCBG*	ATPase for hydroxamate/ staphyloferrin A/ staphyloferrin B ABC transporters; hydroxamate siderophore transporter (heterodimeric permease components)	(227, 237)
NWMN_0702-0705	*sstABCD*	Putative siderophore ABC transporter (heterodimeric permease components, ATPase, and lipoprotein receptor)	(178)

Table 1-3 continued

NWMN_0908	N/A	Iron-regulated putative receptor lipoprotein	(4)
NWMN_1040	*isdB*	Hemoglobin/haptoglobin binding; heme extraction	(250)
NWMN_1041, 1042	*isdA*, *isdC*	Heme transfer	(97, 182, 203, 277)
NWMN_1043-1045	*isdDEF*	Heme transporter (lipoprotein and heterodimeric permease components)	(97, 202)
NWMN_1047	*isdG*	Heme monooxygenase	(230)
NWMN_1320	*brnQ3*	Putative branched chain amino acid transport protein; heme transport protein	N/A
NWMN_1406	*fur*	Ferric uptake regulatory protein	(249)
NWMN_1931	*fhuD1*	Hydroxamate siderophore transporter (receptor lipoprotein)	(229)
NWMN_2043	*ftnA*	Putative bacterioferritin	(179)
NWMN_2078-2076	*htsABC*	Heme transporter? Staphyloferrin A transporter (receptor lipoprotein and heterodimeric permease components)	(18, 95, 231)
NWMN_2082, 2081-2079	*sfaD*, *sfaABC*	Staphyloferrin A biosynthesis	(18, 45)
NWMN_2185	*fhuD2*	Hydroxamate siderophore transporter (receptor lipoprotein)	(228)
NWMN_2262-2261	*hrtBA*	Heme transporter (efflux)	(239)
NWMN_2263-2264	*hssRS*	Heme-sensing two-component regulator	(241)
NWMN_2450	*feoB*	Putative ferrous iron ion transport protein	N/A
NWMN_2614	*isdH*	Hemoglobin/haptoglobin binding; heme extraction	(61)

contribute to the growth of *S. aureus* in serum. The primary objective of this study was thus to describe the ways in which *S. aureus* acquires transferrin iron. Experiments were designed to test the following hypotheses:

1) Secreted siderophores and their cognate transport systems contribute to growth of *S. aureus* on transferrin and to virulence.

2) Liberation of iron from transferrin following interaction with catecholamine stress hormones provides *S. aureus* with iron for growth on transferrin and contributes to virulence, and acquisition of this iron is dependent on a transport system encoded by the *sst* operon.

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Chapter 2

Siderophore-mediated iron uptake in *Staphylococcus aureus*

Significant portions of this chapter have been published in the following article:

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Additional portions appeared in the following articles:

Cheung, J., F. C. Beasley, S. Liu, G. A. Lajoie, and D. E. Heinrichs (2009). Molecular characterization of staphyloferrin B biosynthesis in *Staphylococcus aureus*. Molecular Microbiology, 74:594-608. This work was carried out with Dr. G. A. Lajoie's research group, Department of Biochemistry, University of Western Ontario.

Beasley, F. C., C. Marolda, J. Cheung, S. Buac, and D. E. Heinrichs (2011). *Staphylococcus aureus* transporters Hts, Sir and Sst capture iron liberated from human transferrin by staphyloferrin A, staphyloferrin B and catecholamine stress hormones, respectively, and contribute to virulence. Infection and Immunity, *in press*.

2.1 Introduction

The extracellular fluid of the mammalian host is low in iron due to this element's poor solubility at neutral pH, and through its stringent sequestration by host scavenging proteins. This is an important facet of innate resistance to disease, and is described as nutritional immunity (66). The primary sequestration factor in the serum component of plasma is the globular glycoprotein transferrin, which has the ability to bind two atoms of ferric iron with high affinity. In the healthy host, transferrin is perpetually undersaturated (approximately 30% iron loaded) (16), providing a constitutive capacity for sequestration of trace Fe^{3+} ions. Invasive pathogenic bacteria frequently target host ferroproteins, including transferrin, through secretion of siderophores: small, soluble molecules with affinities for iron approaching or exceeding the target molecule. Siderophores are often peptidic molecules assembled by cytosolic enzymes without the benefit of a ribosomal template.

The pathogenic bacterium *Staphylococcus aureus* is a leading cause of bacteremia, as it is well adapted to overcoming iron deprivation in blood. Its abilities to lyse erythrocytes (7, 8, 13, 21, 26, 28, 37) and capture hemoglobin iron through the Isd system of cell wall-anchored and transmembrane proteins (32, 45, 48, 52, 65) are well described, and are prominently touted as the strategy through which *S. aureus* acquires iron during sepsis (62). The ability to grow on transferrin as an iron source has also been noted (44, 51, 55), but studies have contributed little toward a mechanistic explanation of this process, and have not evaluated any contribution to virulence.

At the commencement of this study, *S. aureus* had previously been shown, using molecular genetic techniques, to produce at least one structurally uncharacterized siderophore, termed staphylobactin. This metabolite's production was a function of the *sbnABCDEFGHI* operon (staphylobactin or siderophore biosynthesis) (Figure 1-5A), which is induced under conditions of iron limitation (17). Uptake of Fe-staphylobactin was shown to be a function of the SirABC (staphylococal iron regulated) transporter, whose operon is situated adjacent to *sbn* on the *S. aureus* chromosome (18, 35).

The *sbn* operon encodes three NIS synthetases: SbnC, SbnE, and SbnF (12). Chromosomal inactivation of a synthetase was reported to curtail growth of *S. aureus* under conditions of severe iron chelation (17). *S. aureus* genome sequencing projects subsequently uncovered two alternate loci from *sbn* which, according to annotation, could be involved in production of nonribosomal peptides. One encodes a recently characterized multidomain NRPS (69), which does not produce metabolites with iron coordination capacities (N. Magarvey, personal communication). Annotation of the third locus (Figure 1-4A) described it to encode two NIS synthetases. Curiously, this locus is chromosomally adjacent to an operon encoding an ABC transporter implicated in heme acquisition based on limited evidence (62). Concurrently to this study, the NIS locus was annotated by another research group as *sfna* (staphyloferrin A biosynthesis) (14). It is hereby rebranded *sfa* for compliance with proper protocols in bacterial genetic nomenclature.

In this study, mutagenesis was performed to eliminate loci *sbn*, *sfa*, *sir*, and *hts* from the staphylococcal chromosome in an attempt to categorize the entire

complement of *S. aureus* siderophore biosynthesis/transport systems, as well as to dissect their individual and combined contributions toward growth of *S. aureus* in iron-restricted media such as serum, and to assess their roles in virulence in murine infection models.

2.2 Materials and methods

2.2.1 Bacterial growth conditions

All bacteria were cultured at 37 °C unless otherwise indicated. Bacterial strains used are summarized in Table 2-1. *Escherichia coli* was grown in Difco Luria-Bertani broth (BD Diagnostics). *Staphylococcus epidermidis* was grown in Gibco RPMI Medium 1640 [+] L-glutamine [+] 25 mM Hepes (Invitrogen) enriched with 1% w/v Difco casamino acids (CAA-RPMI) (Becton, Dickinson and Co.). For genetic manipulations, *S. aureus* was grown in Difco tryptic soy broth (TSB) (Becton, Dickinson and Co.). For subsequent experiments, *S. aureus* strains were grown, as specified below, in: Tris-minimal succinate broth (TMS) (61); TMS chelated with 2,2'-dipyridyl (Sigma-Aldrich) or ethylenediaminedi-N,N'-bis(2-hydroxyphenylacetic acid) (EDDHA) (LGC Standards GmbH); a 40:60 mixture of TMS and horse serum (Sigma-Aldrich) that was previously incubated at 55 °C for 2 h to inactivate complement (HoS-TMS); TMS treated for 24 h at 4 °C with 10% w/v Chelex-100 resin (Bio-Rad) prior to addition of post-autoclaving nutrients (C-TMS); or C-TMS amended with 10 to 20 µM human apotransferrin or holotransferrin (approximately 60% iron saturated) (Sigma-Aldrich). For iron repletion of restricted growth media, $FeCl_3$ was added to a final concentration of 50 to

100 µM. Solid media were prepared by incorporating 1.5% w/v low iron Difco Bacto agar (Becton, Dickinson and Co.) to specified media.

For selection of plasmids and recombinant alleles, antibiotics (BioShop, Burlington, Ontario) were added to media at the following concentrations: ampicillin (100 µg/mL), tetracycline (4 µg/mL), kanamycin (50 µg/mL), neomycin (50 µg/mL), chloramphenicol (30 µg/mL for *E. coli* and 5 µg/mL for *Staphylococcus* sp.), erythromycin (300 µg/mL for *E. coli* and 3 µg/mL for *S. aureus*), and lincomycin (20 µg/mL). All media were made with water purified through a Milli-Q water purification system (Millipore). All glassware was treated overnight in 0.1 M HCl and rinsed thoroughly in Millipore water to remove residual contaminating iron.

2.2.2 General molecular genetic methodologies

Purification of plasmid DNA was performed using a QIAPrep Mini-spin kit (QIAgen) according to the manufacturer's instructions. For preparations from *Staphylococcus*, 10 µg lysostaphin (Sigma-Aldrich) and a 37 °C incubation were incorporated into the P1 buffer treatment until cell lysis occurred. Plasmids used are summarized in table 2-1. Purification of *S. aureus* chromosomal DNA was performed as previously described (60).

Polymerase chain reaction (PCR) was performed using *Taq* polymerase (New England Biolabs) or *Pwo* polymerase (Roche Diagnostics) according to manufacturers' instructions. Primers were designed using Primer3 software (Whitehead Institute and Howard Hughes Medical Institute). Primers are summarized in table 2-1. Restriction digests were performed using enzymes

Table 2-1: Bacterial strains, plasmids, and oligonucleotides used in this study

	Description	**Ref.**
***E. coli* strains**		
DH5α	F^- ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r_k^- m_k^+) *phoA supE44 thi*-1 *gyrA96 relA1* λ^-	Invitrogen
***S. aureus* strains**		
RN4220	r_k^- m_k^+; accepts foreign DNA	(41)
RN6390	Prophage-cured laboratory strain	(56)
Newman	Wild-type clinical isolate	(22)
H306	RN6390 *sirA*::Km	(18)
H672	RN6390 *sbnF*::Km (originally described as *sbnE*::Km)	(17)
H1448	RN6390 Δ*htsABC*::Tc	This study; (6)
H1480	RN6390 *sirA*::Km Δ*htsABC*::Tc	This study; (6)
H803	Newman *sirA*::Km	(18)
H1262	Newman Δ*htsABC*::Tc	This study; (6)
H1497	Newman *sirA*::Km Δ*htsABC*::Tc	This study; (6)
H1321	RN6390 Δ*sbnABCDEFGHI*::Tc	This study; (6)
H1661	RN6390 Δ*sfaABCsfaD*::Km	This study; (6)
H1649	RN6390 Δ*sbnABCDEFGHI*::Tc Δ*sfaABCsfaD*::Km	This study; (6)
H1331	Newman Δ*sbnABCDEFGHI*::Tc	This study; (6)
H1665	Newman Δ*sfaABCsfaD*::Km	This study; (6)
H1666	Newman Δ*sbnABCDEFGHI*::Tc Δ*sfaABCsfaD*::Km	This study; (6)

Table 2-1 continued

H1074	Newman Δ*fhuCBG*::Em	(63)
H1112	Newman *fhuG*::Tn*917*	(60)
AD25	Canadian atopic dermatitis isolate	M. McGavin collection
MSSA476	American osteomyelitis/sepsis methicillin-sensitive isolate	(36)
MW2	American septic arthritis methicillin-resistant isolate	(3)
UAMS-1	American osteomyelitis isolate	(27)
USA300 LAC	American community-acquired necrotizing fasciitis isolate	(50)
***S. epidermidis* strains**		
846-1	Plasmid-cured type strain	D. Mack
1457-M10	Biofilm-deficient 846-1 mutant (*icaA*::Tn917; Em^R)	(20)
Plasmids		
pBC SK(+)	*E. coli* cloning vector; Cm^R	Stratagene
pUC19	*E. coli* cloning vector; Ap^R	(70)
pLI50	*E. coli*/*S. aureus* shuttle vector; Ap^R/Cm^R	Novagen
pDG1513	pMTL22 derivative carrying tetracycline resistance cassette; Ap^R	(33)
pDG780	BluescriptKS$^+$ derivative carrying kanamycin resistance cassette; Ap^R	(33)
pAUL-A	*E. coli*/*S. aureus* shuttle and temperature-sensitive suicide vector; Em^R	(10)
pFB10	pAUL-A derivative carrying Δ*sbnABCDEFGHI*::Tc allele; Tc^R Em^R	(6)
pEV83	pAUL-A derivative carrying Δ*htsABC*::Tc allele; Tc^R Em^R	(6)
pFhuC	pLI50 derivative carrying *fhuC*; Cm^R	(63)

Table 2-1 continued

pEV90	pLI50 derivative carrying *S. aureus sfaABCsfaD*; Cm^R	This study; (6)
pEV95	pLI50 derivative carrying *S. epidermidis sfaABCsfaD*; Cm^R	This study; (6)
pEV96	pLI50 derivative carrying *S. saprophyticus sfaABCsfaD*; Cm^R	This study; (6)
pFB50	pEV90 derivative carrying Δ*sfaABCsfaD*::Km allele with loss of function frameshift mutation in Gram-positive replicase gene *repB*; Cm^R	This study; (6)
pALC2073	*E. coli*/*S. aureus* shuttle vector; Ap^R/Cm^R	(5)
pSirABC	pBC SK(+) derivative carrying *sirABC*; Cm^R	(18)
pFB54	pALC2073 derivative carrying *S. aureus fhuC*/*sirABC* transcriptional fusion; Cm^R	This study
pFB57	pALC2073 derivative carrying *S. aureus fhuC*[K42N] (loss of function Walker motif missense mutation)/*sirABC* transcriptional fusion; Cm^R	This study
Oligonucleotides*		
*hts*5'F-*Sac*I	5' TGAGCTCTGCGATTACATTGGAGGCTG 3'	
*hts*5'R-*Bam*HI	5' GGGATCCGATAACAATTATCATTGTCAAG 3'	
	Generation of *S. aureus* Δ*htsABC*::Tc allele, 5' arm	
*hts*3'F-*Bam*HI	5' CGGATCCCTAACATATGATTAGAGTTTAAAA 3'	
*hts*3'R-*Xba*I	5' GTCTAGAGGTCTCATAACCGGTCTAAAAA 3'	
	Generation of *S. aureus* Δ*htsABC*::Tc allele, 3' arm	
sbn5'F-*Kpn*I	5' TTGCGCGGTACCAGGAAGCGCTTTTGATTGAA 3'	
sbn5'R-*Sma*I	5' TTGTGTCCCGGGATGACTGACCCTTTCGCATC 3'	
	Generation of Δ*sbnABCDEFGHI*::Tc allele, 5' arm	
sbn3'F-*Sma*I	5' TTGCGCCCCGGGCGCAATTGGAAGCAGTTTT 3'	
sbn3'R-*Xba*I	5' TTGCGCTCTAGAGATGCCAATACGATGACAGG 3'	
	Generation of Δ*sbnABCDEFGHI*::Tc allele, 3' arm	
sfa_{SA}5'	5' GTATAGATTGTATTTAATAAGTTAATGTAATCC 3'	
sfa_{SA}3'	5' TGCAAACGATATGTAGTATAACTTGTCAAC 3'	
	Cloning of *S. aureus sfaABCsfaD*	
sfa_{SE}5'-*Eco*RI	5' ATATGAATTCTTGAGCATGACGCTCAAGTGC 3'	
sfa_{SE}3'-*Sma*I	5' ATATCCCGGGGAGACGGTGCGTTGAGTTAAAG G 3'	
	Cloning of *S. epidermidis sfaABCsfaD*	

Table 2-1 continued

sfa$_{SS}$5'-*Bam*HI	5' TATAGGATCCATCTATTTTTACTTTAATCTAGCGG ACGG 3'
sfa$_{SS}$3'-*Sma*I	5' TATACCCGGGCTATGTTTCATCGTTTTCCCTCCT TAG 3'
	Cloning of *S. saprophyticus sfaABCsfaD*
*fhuC*5'-*Sph*I	5' TTGATAGCATGCCATGACAAATCGAGCTATCC 3'
*fhuC*3'-*Pst*I	5' TTGATACTGCAGTTAAGAATAAGCTCTGCGAC A 3'
	Cloning of *S. aureus fhuC* or *fhuC*[K42N] to make a transcriptional fusion with *sirABC*
sfaD-RT-PCR-F	5' CCTCTAATGCAATGCCATATTTA 3'
sfaD-RT-PCR-R	5' ACAATGAATCACCTATCGTGACA 3'
sfaA-RT-PCR-F	5' AGTCTATCATGCGCCAACAAC 3'
sfaA-RT-PCR-R	5' AACCTGTCGCCATAATCAATAA 3'
htsA-RT-PCR-F	5' TTTAAATCCAGAGCGTATGATCA 3'
htsA-RT-PCR-R	5' CAGAAGAAATTAAGCCACGAGAT 3'
gyrB-RT-PCR-F	5' ATAATTATGGTGCTGGGCAAAT 3'
gyrB-RT-PCR-R	5' AACCAGCTAATGCTTCATCGATA 3'
	Real time PCR primers (gyrase B used as internal "housekeeping gene" reference)

*underlined sequences in oligonucleotides denote restriction sites

purchased from New England Biolabs and Roche Diagnostics according to manufacturers' instructions. Blunting reactions were performed using Klenow fragment (Roche Diagnostics) according to manufacturer's instructions. Linear DNA fragments were purified directly from restriction reactions or following resolution through agarose gels using the QIAQuick PCR purification kit (QIAgen) according to the manufacturer's instructions. Ligation reactions were performed using T4 DNA ligase (New England Biolabs). Sequencing reactions were performed by the London Regional Genomics Centre.

2.2.3 Cloning and mutagenesis of *S. aureus*

Plasmids generated and used in this study are summarized in Table 2-1. Allelic replacement was used to delete the majority of operons *sbnABCDEFGHI* and *htsABC*, and locus *sfaABCsfaD*, from the chromosome of *S. aureus*. The Δ*sbnABCDEFGHI*::Tc knockout allele contained arms homologous to *sbnA* and to noncoding DNA downstream of *sbnI*. The arms were cloned sequentially to plasmid pBC SK(+), *Xba*I to *Sma*I and *Kpn*I to *Sma*I. A tetracycline resistance cassette was excised from plasmid pDG1513 with restriction enzymes *Ssp*I and *Nae*, blunted with Klenow fragment, and cloned between the arms at the *Sma*I site. The knockout allele was excised and cloned to the temperature sensitive *E. coli*/*S. aureus* shuttle vector pAUL-A, *Kpn*I to *Xba*I, creating plasmid pFB10. This was electroporated into *S. aureus* strain RN6390 after passaging through strain RN4220. The RN6390 recipient was grown in TSB to mid-log phase at 30 °C, shifted to 42 °C for 16 h, then plated to TSB agar plates containing kanamycin and neomycin. Resistant clones were screened for sensitivity to

erythromycin, indicating a loss of pAUL-A backbone DNA following integration of the knockout allele to the chromosome via homologous recombination on either side of the tetracycline resistance cassette.

To generate the Δ*htsABC*::Tc allele, 5' and 3' arms targeting the noncoding region flanking the operon were PCR amplified and sequentially cloned to plasmid pUC19, *Sac*I to *Bam*HI then *Bam*HI to *Xba*I. A tetracycline resistance cassette was excised from plasmid pDG1513 with restriction enzymes *Bam*HI and *Bgl*II and ligated between the flanking arms at the *Bam*HI site. The Δ*htsABC*::Tc allele was excised and cloned to plasmid pAUL-A, *Sac*I to *Xba*I, generating plasmid pEV83. Subsequent passaging into *S. aureus* and integration into the chromosome was performed as described above for *sbn* mutagenesis.

To generate the Δ*sfaABCsfaD*::Km knockout allele, the *sfa* cluster was PCR amplified, restricted at a *Sac*I site downstream of the *sfaD* stop codon, and cloned *Sac*I to *Sma*I to the *E. coli*/*S. aureus* shuttle vector pLI50, making plasmid pEV90. Plasmid pEV90 was digested with *Spe*I and *Eco*RV internally to *sfaD* and *sfaC*, excising most of the gene cluster, which was replaced by the *Xba*I/*Pvu*II excised kanamycin resistance cassette from plasmid pDG780. *Nsi*I restriction followed by Klenow fragment fill-in introduced a frameshift mutation into the *repB* gene of the pLI50 backbone, inactivating the Gram positive replicase protein, and creating plasmid pFB50. Plasmid pFB50 was electroporated to *S. aureus* strain RN4220 carrying unmodified pLI50, enabling propagation of pFB50 by complementation *in trans* with wild type RepB. Plasmid pFB50 was transduced to *S. aureus* strain RN6390 using phage 80α as

described below, and recipient culture was plated to TSB agar containing kanamycin and neomycin. Resistant clones were screened for sensitivity to chloramphenicol, indicating a loss of pLI50 backbone DNA following integration of the knockout allele to the chromosome via homologous recombination on either side of the kanamycin resistance cassette.

Mobilization of *S. aureus* plasmids and mutated chromosomal alleles between strains was performed using transducing ϕ80α as follows. Cultures of donor strains were grown overnight in TSB then diluted 1:2 into fresh TSB containing 2.5 mM $CaCl_2$. Cultures were incubated for 10 min then infected with $>10^8$ plaque forming units of bacteriophage. Cultures were incubated for 15 min then added to 20 mL fresh TSB containing 2.5 mM $CaCl_2$. Cultures were incubated with gentle shaking through several rounds of bacterial replication followed by culture lysis. Debris was pelleted by centrifugation at 3 krpm and supernatant was passaged through a 0.2 µM filter. Lysate was serially diluted in 10-fold increments in bacteriophage buffer (50 mM Tris pH 7.8, 0.1 M NaCl, 1 mM $MgSO_4$, 4 mM $CaCl_2$) and 100 µL aliquots were combined with 100 µL aliquots of recipient strains grown to early exponential phase in TSB containing 2.5 mM $CaCl_2$. Following a 20 min incubation at 37 ^oC, recipient/lysate mixtures were spiked with 50 mM sodium citrate and plated on selective TSB agar.

2.2.4 Real time PCR

S. aureus cells grown to stationary phase in TMS with 100 µM 2,2-dipyridyl were washed twice in saline buffer and diluted 1:100 into HoS-TMS. Cultures were grown at 37 °C with ample aeration to mid-logarithmic phase (OD_{600} ~ 1.0),

then partitioned into two aliquots of equal volume. One aliquot from the pair was spiked with 50 µM $FeCl_3$. Growth was allowed to resume for 1 h, then 20 mL culture aliquots normalized to OD_{600} of 1.0 were pelleted by centrifugation at 4 °C. RNA was extracted using RNeasy kits (QIAgen) according to the manufacturer's instructions. RNA extraction was preceded by mechanical cell disruption through two 20 s treatments in 500 µL RLT buffer in a FastProtein Blue #6550-500 tube using a FastPrep disruptor at a setting of 5.0 (Qbiogene).

100 ng RNA was reverse transcribed and PCR amplified using the iScript™ One-Step RT-PCR kit with SYBR Green (Bio-Rad), as directed, with primers listed in Table 2-1, in a Rotor-Gene 6000 Real Time Analyzer (Corbett Life Sciences). Quantification relative to housekeeping gene *gyrB* was performed using the comparative C_T method according to User Bulletin #2: ABI Prism 7700 Sequence Detection System (2001) (Applied Biosystems). For each representative RNA extraction, one-step reactions were performed in triplicate.

2.2.5 Heterologous expression of SirABC in *S. epidermidis*

In previous attempts to make a *sirABC*-expressing vector, numerous attempts to clone the operon into *S. epidermidis*-compatible shuttle vectors along with its native operator region, or under control of an inducible vector-borne promoter, proved unsuccessful. Thus, the promoterless *sirABC* operon from the previously constructed pSirABC (18) was excised using restriction enzymes *Sac*I and *Pst*I and cloned to pALC2073, replacing the *tetR*/*O* region. To enable expression of *sirABC*, and to provide the potentially requisite *S. aureus* FhuC ATPase for proper SirABC function (63), *fhuC* and its operator

region were PCR amplified from the *S. aureus* chromosome with engineered flanking *Pst*I and *Sph*I restriction sites and cloned to the pALC2073 derivative within the *Pst*I located at the 5' end of *sirABC*. The resulting plasmid, pFB54, thus carried a transcriptional fusion of *sirABC* to *fhuC* under control of the *fhuC* operator. Alternately, a nonfunctional *fhuC* allele harbouring a K42N Walker A domain mutation, characterized previously (63), was cloned in similar fashion to create a transcriptional fusion for plasmid pFB57. After passaging through *S. aureus* RN4220, pFB54 and pFB57 were transformed to sucrose-competent *S. epidermidis* 1457-M10 via electroporation.

2.2.6 Complementation of the *sfa* deletion mutation

The Δ*sfaABCsfaD*::Km mutation in the *S. aureus* RN6390 chromosome was complemented using plasmid pEV90, described above. Additionally, complementation was performed using the annotated *sfaABCsfaD* locus from *S. epidermidis*. This was PCR amplified and cloned to pLI50 between restriction sites *Eco*RI and *Sma*I.

2.2.7 Bacterial growth curves

S. aureus cells grown to stationary phase in TMS with 100 µM 2,2-dipyridyl were washed twice in saline buffer and diluted 1:100 into HoS-TMS broth. Culture medium was left unamended (iron deplete) or amended with 50 µM $FeCl_3$ (iron replete). Cultures were grown at under constant medium amplitude shaking in a Bioscreen C machine (Oy Growth Curves Ab Ltd.), with absorbance measured at 600 nm.

2.2.8 Concentration of siderophore from culture supernatants

S. aureus cells grown to stationary phase in TMS with 100 µM 2,2-dipyridyl were washed twice in saline buffer and diluted 1:100 into TMS containing 250 nM EDDHA in acid washed glass flasks with generous head space. Cultures were grown to the postexponential phase (approximately 30 h). Culture aliquots were extracted and cells were removed by centrifugation at 5 krpm. Supernatants were frozen at -80 °C and liquid was removed under a vacuum at -80 °C. Lyophilized extract was resuspended in 1/5 the original supernatant volume in methanol with gentle shaking for 30 min at room temperature. Insoluble debris was removed by ultracentrifugation. Methanol was removed by rotary evaporation and residue was redissolved in 1/20 the original supernatant volume in water. Further purification included passage across an amberlite IRA-402 (Fluka) anion exchange column. The column was washed extensively with water before elution with 3 M formic acid. Siderophore active fractions (see next section) were subjected to a desalting step using a C18 cartridge (Applied Separations).

2.2.9 Chrome-azurol S assay for siderophore activity

Siderophore activity of solutions was measured using the chrome azurol S (CAS) assay (59). Dilutions of sample were mixed with equal volumes of CAS shuttle solution and incubated in darkness at room temperature with gentle shaking for 45 min. With an empty cuvette serving as the blank, siderophore units were calculated as follows:

$$\frac{100 \times \text{dilution factor} \times (A_{630} \text{ of control solution} - A_{630} \text{ of sample})}{A_{630} \text{ of control solution}}$$

For describing siderophore activity of raw culture supernatants, siderophore units were normalized to a culture OD_{600} of 1.0.

2.2.10 Mass spectrometry

Spectrometric analyses were performed by the staff of the laboratory of G. A. Lajoie (Department of Biochemistry, University of Western Ontario). Eluent desalted across a C18 cartridge (see above) was dried and re-dissolved in 100 µl of water containing 100 mM EDTA and 0.1% formic acid. Addition of EDTA greatly increased the detection level of metabolites, since contaminating metal ions have been shown to interfere with detection (46). 20 µL was injected into a liquid chromatography mass spectrometry/tandem mass spectrometry (LC-MS/MS) system comprised of a Waters CapLC with a Phenomenex Jupiter Proteo 90 A column (150 x 1.0 mm), 4 µm coupled to a Micromass Q-TOF micro mass spectrometer (Waters). Separation was carried out at a flow rate of 40 µl/min with a gradient starting at 0% B for 4 min, ramping to 15% B in 4 min, then ramping to 90% B in 4 min and holding for 4 min. Solvent A was water and solvent B was 95% acetonitrile, both with 0.1% formic acid. LC-MS was performed with electrospray in negative ion mode with a scan range 200-950 *m/z*. Collision-induced dissociation was performed with a mass range 60-500 *m/z* using Argon as collision gas. Variable collision energy of 20-30 V

was applied to obtain an informative fragmentation spectrum. Data were analyzed using MassLynx 4.0 (Micromass).

2.2.11 Siderophore plate bioassays

Staphylococcal cells were successively cultured in TMS then TMS containing 100 µM 2'2-dipyridyl (*S. aureus*) or CAA-RPMI then CAA-RPMI containing 100 µM 2'2-dipyridyl (*S. epidermidis*). Cells were pelleted and washed twice in saline buffer. Cells were seeded into cooled molten agar media to densities of 5×10^8 CFU/mL. Media used included TMS agar containing 10 µM EDDHA (*S. aureus*) and CAA-RPMI agar containing 5 µM EDDHA (*S. epidermidis*). Media were dispensed into Petri dishes in precise 25 mL aliquots. Plates were allowed to gel and dry in the dark at room temperature for approximately 6 h. Sterile 6 mm diameter paper discs (Becton, Dickinson and Co.) impregnated with up to 20 µL of an iron-ligand solution were placed on the surface of the agar. Growth promotion was measured as the diameter of a halo of bacterial colonies forming around the paper disc after 36 h of growth.

2.2.12 Mouse models of *S. aureus* infection

Mouse sepsis experiments were performed as follows. *S. aureus* cultures were grown overnight in TMS or TSB, pelleted by centrifugation, washed twice in saline buffer, and diluted 1:50 (OD_{600} = 1.0) into fresh TMS or TSB. These cultures were grown to midexponential phase (OD_{600} ~ 1 to 3), pelleted by centrifugation, washed twice in saline buffer, and resuspended in saline buffer. After a 30 min equilibration incubation at room temperature, cells were diluted to

an optical density correlating to a predetermined CFU/mL titre. Seven week old female BALB/c mice (Charles River Laboratories or Jackson Laboratories) housed in microisolator cages (maximum four per cage) were infected via tail vein injection with 100 µL bacterial suspension containing 0.1 to 5 x 10^7 CFU. Mice were euthanized if the infection rendered them immobilized, incapable of eating or drinking, or if they lost greater than 25% body mass. Four days post infection, mice were euthanized via intraperitoneal injection of sodium pentobarbitol. During dissection, organs including hearts, kidneys (as a pair), livers, spleens, and/or lungs (as a pair) were removed and placed in sterile phosphate-buffered solution containing 0.1% v/v Tween-20. Organs were homogenized. Homogenates were serially diluted and four 10 µL aliquots were spotted onto TSB agar plates. After 24 h growth, CFU were counted and bacterial loads (log CFU/organ or organ pair) were calculated. The Student's *t*-test was used to calculate statistical significance for differences in data sets.

Bacteria were cultured and prepared in a similar fashion for skin lesion models of infection. Seven week old male BALB/c or nude SKH-1 mice were purchased from Charles River. The backs of BALB/c mice (Charles River Laboratories) were shaved 24 h prior to infection. Mice were injected subcutaneously on the right flank with 200 µL bacterial suspension containing 5 x 10^8 CFU. Lesions were measured at 24 h intervals until sacrifice using electronic calipers. Lesion area was calculated as 0.5 x length x width x π (47). The Student's unpaired *t*-test was used to calculate statistical significance for differences in data sets.

2.3 Results

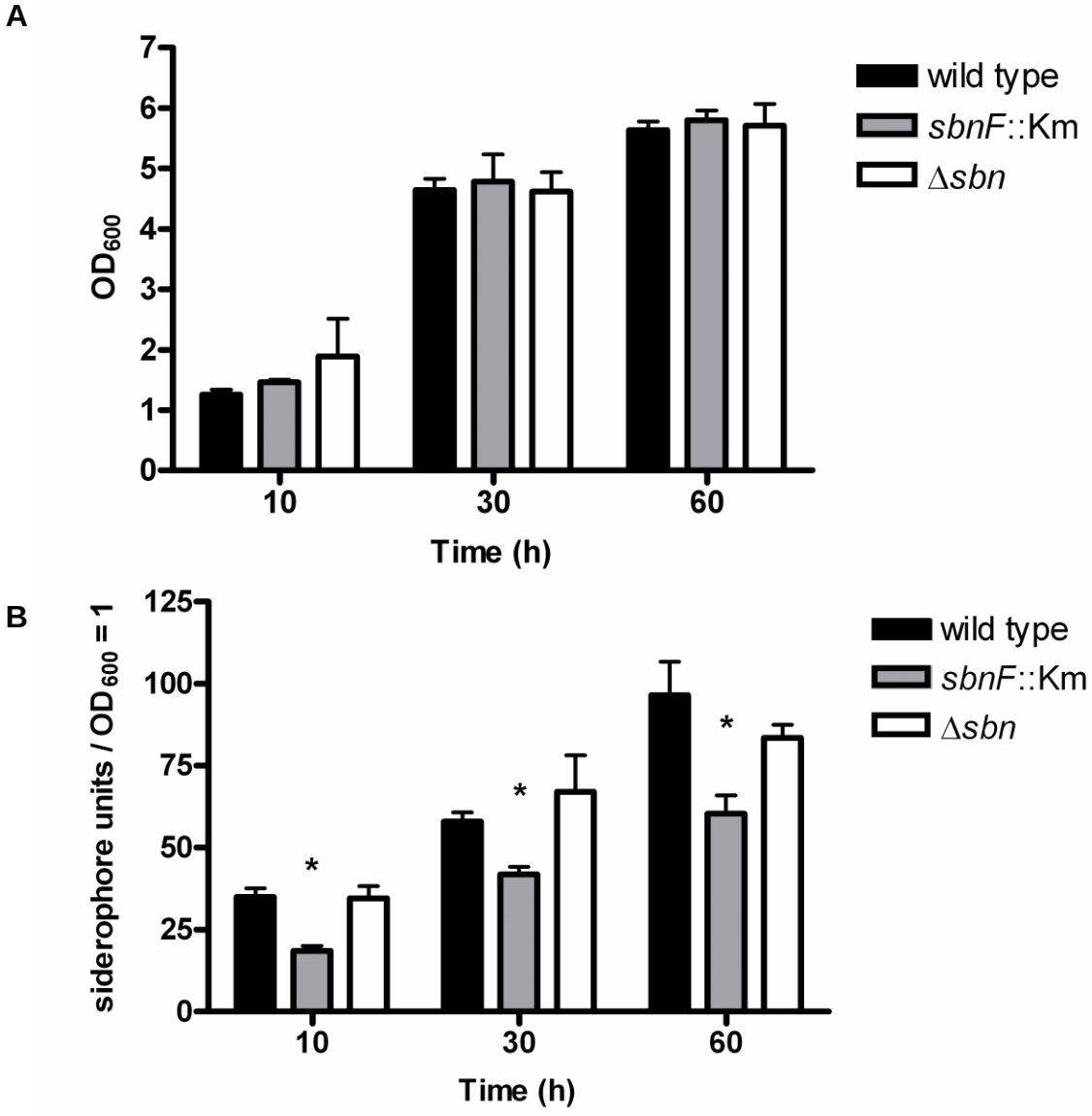
2.3.1 A Δ*sbn* mutant secretes siderophore(s)

It was previously reported that a *S. aureus* strain RN6390 staphylobactin synthetase mutant (*sbnF*::Km, erroneously described as *sbnE*::Km in the report) was severely impaired for growth in chelated basal medium (TMS containing 10 µM EDDHA). This defect was attributed to lack of siderophore production (17). Attempts were made to reproduce this experiment using an operon-spanning Δ*sbn* deletion mutant, also in *S. aureus* strain RN6390. These proved unsuccessful as even the wild type parental strain was severely growth impaired in this medium (data not shown). As EDDHA is a very strong iron chelator, the assay was repeated using a lower concentration sufficient to create conditions of moderate iron restriction (0.25 µM).

In this revised growth medium, both the *sbnF*::Km and Δ*sbn* mutants grew equally capably as their isogenic wild type parent (Figure 2-1A). Culture supernatant siderophore activity was sampled at three intervals (10, 30, and 60 h). While the *sbnF*::Km mutant was moderately but significantly impaired for siderophore output at all three sampling times, it nevertheless secreted quantifiable amounts of (an) alternate iron chelator(s), while its Δ*sbn* counterpart performed at a level statistically comparable to the wild type parent (Figure 2-1B). From this pilot study, it was concluded that at least one alternate genetic locus to the *sbn* operon mediates *S. aureus* siderophore production.

Figure 2-1: *S. aureus sbn* operon mutants produce siderophore(s).

(A) *S. aureus* RN6390 derivatives with mutations in the *sbn* operon (*sbnF*::Km and Δ*sbn*) were grown in low iron medium. **(B)** Mutants and wild type alike secreted iron-chelating metabolite(s) into the supernatant, detected using the CAS assay at sampling times of 10, 30, and 60 hours. Error bars represent standard deviation from the mean. Statistical differences between mutants and the wild type parental strain were calculated using the Student's unpaired *t*-test (* = $P < 0.01$).


A
7
6
5
4
3
2
1
0
OD600
10
30
60
Time (h)
wild type
sbnF::Km
Δsbn
B
125
100
75
50
25
0
siderophore units / OD600 = 1
*
*
*
10
30
60
Time (h)
wild type
sbnF::Km
Δsbn

2.3.2 *sfa* encodes putative NIS synthetases and is conserved among pathogenic staphylococci

The *sfa* locus is conserved across all sequenced *Staphylococcus* genomes, as is an adjacently transcribed operon encoding a putative iron compound ABC transporter, *htsABC* (Figure 1-4A). BLAST searches uncovered similarities between predicted *sfa* and *sbn* siderophore synthetases, and with aerobactin synthetase genes from *E. coli*, suggesting the *sfaABCsfaD* locus may be involved in production of a staphylococcal siderophore (Table 2-2).

2.3.3 *sfa* is repressed by iron via Fur

Previous work demonstrated that the *sbn* operon is expressed under conditions of iron restriction in a Fur/Fe-dependent fashion (17); it was of interest to determine if *sfa* would be regulated in such a fashion as well. The intergenic region between divergently transcribed *sfaA* and *sfaD* as well as the operator region of *htsABC* contain sequence motifs resembling canonical Fur protein recognition sites (42) (Figure 2-2), suggesting these genes play roles in the iron deprivation response of staphylococci. Real-time PCR analysis confirmed that, consistent with this prediction, all three transcripts are upregulated in an iron deprivation-dependent fashion, *i.e.* they are repressed by iron saturation of the culture medium, but continue to be readily expressed regardless of iron availability in a *fur*-inactivated mutant (Figure 2-3).

Having established the *sfa* locus is a component of the Fur-mediated iron deprivation transcriptional response, and that it encodes putative siderophore synthetases, a Δ*sfa* mutant knockout allele was constructed in the *S. aureus*

Table 2-2: BLAST matches to putative *S. aureus* siderophore biosynthesis/transport proteins

Protein/ locus (strain Newman genome)	Match	Bacterium	Identity (%)	Similarity (%)	E
SfaA/ 2081	Predicted major facilitator family transporter (SERP1780)	*S.epidermidis* RP62A	75	91	1e-167
	Predicted major facilitator family transporter (SSP0706)	*S.saprophyticus* ATCC15305	62	81	1e-131
SfaB/ 2080	Predicted siderophore biosynthesis protein (SERP1779)	*S.epidermidis* RP62A	65	79	0.0
	Predicted siderophore biosynthesis protein (SSP0707)	*S.saprophyticus* ATCC15305	60	78	0.0
	SbnC	*S. aureus* Newman	24	43	7e-16
	Aerobactin biosynthesis protein IucC	*E. coli* APEC O1	22	42	2e-6
SfaC/ 2079	Predicted amino acid racemase (SERP1778)	*S. epidermidis* RP62A	64	82	3e-126
	Predicted amino acid racemase (SSP0708)	*S.saprophyticus* ATCC15305	60	81	8e-122

Table 2-2 continued

SfaD/ 2082	Predicted siderophore biosynthesis protein (SERP1781)	*S. epidermidis* RP62A	60	76	0.0
	Predicted siderophore biosynthesis protein (SSP0705)	*S.saprophyticus* ATCC15305	54	70	0.0
	SbnE	*S. aureus* Newman	24	41	4e-21
	Aerobactin biosynthesis protein IucC	*E. coli* APEC O1	23	43	2e-12
HtsA/ 2078	Predicted iron compound-binding protein (SERP1777)	*S. epidermidis* RP62A	73	85	8e-129
	Predicted iron compound-binding protein (SSP0709)	*S.saprophyticus* ATCC15305	63	79	4e-105
HtsB/ 2077	Predicted iron compound transporter, permease component (SERP1776)	*S. epidermidis* RP62A	73	90	2e-132
	Predicted siderophore transport system, permease component (SSP0710)	*S.saprophyticus* ATCC15305	69	84	6e-119
HtsC/ 2076	Predicted iron compound transporter permease component (SERP1775)	*S. epidermidis* RP62A	76	91	2e-119
	Predicted siderophore transport system, permease component (SSP0711)	*S.saprophyticus* ATCC15305	68	87	9e-109

Figure 2-2: Operator regions of the *sfalhts* locus. Nucleotide sequences of the operator regions of the *htsABC* operon and the *sfaABCsfaD* locus. Predicted start codons are in bold; predicted Shine-Dalgarno sequences (S.D.) are underlined; putative Fur box sequences are between two lines. Nucleotide positions along the *S. aureus* strain Newman chromosome are indicated.

Pos. 2315220 to 2315319 (*S. aureus* Newman)

ACCTCTCATCGTTCCACTCCTTAATATGTATAACTTCATTTATTATTTTA
TGGAGA**GTA**GCAAGGTGAGGAATTATACATATTGAAGTAAATAATAAAAT
Met S.D.
←*htsA*

TTGATAACAATTATCATTGTCAAGTAACGTTCAATCTTTTTTATATTTCT
AACTATTGTTAATAGTAACAGTTCATTGCAAGTTAGAAAAAATATAAAGA
Fur box

Pos. 2319611 to 2319660 (*S. aureus* Newman)

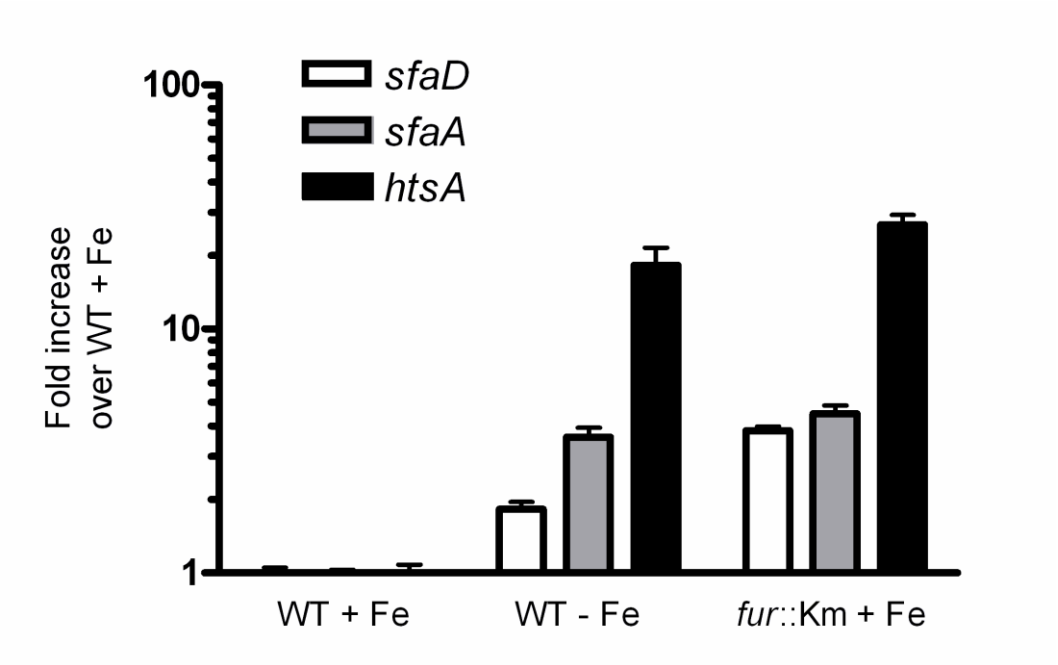
ACTGCTAAAAAAATATTTTGTCATAAAACTTACACCCGCATTCTGTTGAT
TGACGATTTTTTTATAAAACA**GTA**TTTTGAATGTGGGCGTAAGACAACTA
Met S.D.
←*sfaA*

AATAAGAATCATTATCATTAATATGGATTATACAAATCTATCTTGCTTTT
TTATTCTTAGTAATAGTAATTATACCTAATATGTTTAGATAGAACGAAAA
Fur box

GTAAAATATTTCATAGAGGTGAATT**ATG**AACTTGAACTTAATTTTTAAAG
CATTTTATAAAGTATCTCCACTTAATACTTGAACTTGAATTAAAAATTTC
S.D. Met
sfaD→

Figure 2-3: Expression of *sfa* and *hts* genes is regulated by Fur-Fe.

Regulation of *sfaD*, *sfaA*, and *htsA* by iron and Fur in *S. aureus* strain Newman. Real-time PCR of wild type and a *fur* inactivated mutant demonstrates upregulation in the absence of iron, or in the absence of Fur protein when iron is present. Error bars represent standard deviation from the mean (n = 3). Strains were grown in HoS-TMS medium. + Fe denotes cultures spiked with 100 µM $FeCl_3$ for one hour at mid-log phase prior to cell harvesting.


sfaD
sfaA
htsA
100
10
1
Fold increase over WT + Fe
WT + Fe
WT - Fe
fur::Km + Fe

chromosome for the purpose of characterizing its contribution to siderophore iron uptake. When combined with the Δ*sbn* mutation, this resulted in a *S. aureus* strain devoid of NIS synthetases.

2.3.4 The *sbn* operon mediates SB biosynthesis; staphylobactin is SB

Low iron culture supernatant concentrates of the *S. aureus* strain RN6390 Δ*sbn* Δ*sfa* mutant and its isogenic Δ*sfa* counterpart were examined for differences in mass ion composition, especially for those correlating to the known structures of staphylococcal siderophores (*i.e.* SA and SB), using LC-ESI-MS. Shown in Figure 2-4 are the selected ion chromatograms and mass spectra for the two samples. A peak with *m/z* of 447.14, corresponding to the published structure of SB (34), eluted from the Δ*sfa* preparation at 5.09 min (Figure 2-4A), while no peak for that mass above background levels of detection was observed for the Δ*sfa* Δ*sbn* preparation (Figure 2-4B). To further confirm that the peak at 447.14 *m/z* represented SB, LC-MS/MS with collision-induced dissociation (CID) was carried out, generating a fragmentation pattern consistent with the chemical structure of SB (data not shown).

2.3.5 The *sfa* locus mediates SA biosynthesis

LC-ESI-MS was also used to determine the molecular identity of the Sfa-synthesized metabolite. Low iron culture supernatant concentrates of the *S. aureus* strain RN6390 Δ*sbn* Δ*sfa* mutant and its isogenic Δ*sbn* counterpart were examined for differences in mass ion composition. Shown in Figure 2-5 are the selected ion chromatograms and mass spectra for the two samples. A

Figure 2-4. Identification of staphyloferrin B production by *S. aureus* strain RN6390. (A) Concentrated spent culture supernatant from the iron-starved Δ*sfa* mutant was subjected to LC-ESI-MS analysis. Shown is the selected ion chromatogram for the ion at *m*/*z* 447.1 from LC-ESI-MS, with the ESI-MS spectrum of that peak displayed in the inset. **(B)** Similar analysis to **(A)**, using concentrated spent culture supernatant from the iron-starved Δ*sbn* Δ*sfa* mutant.

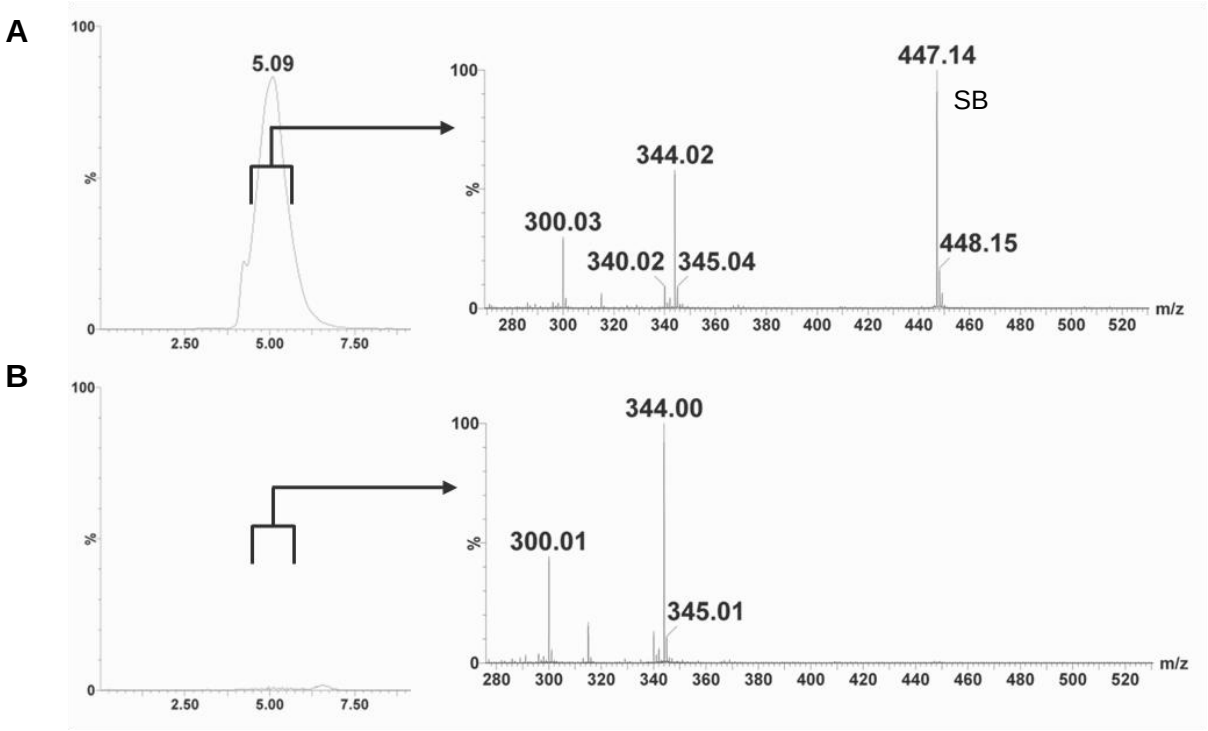
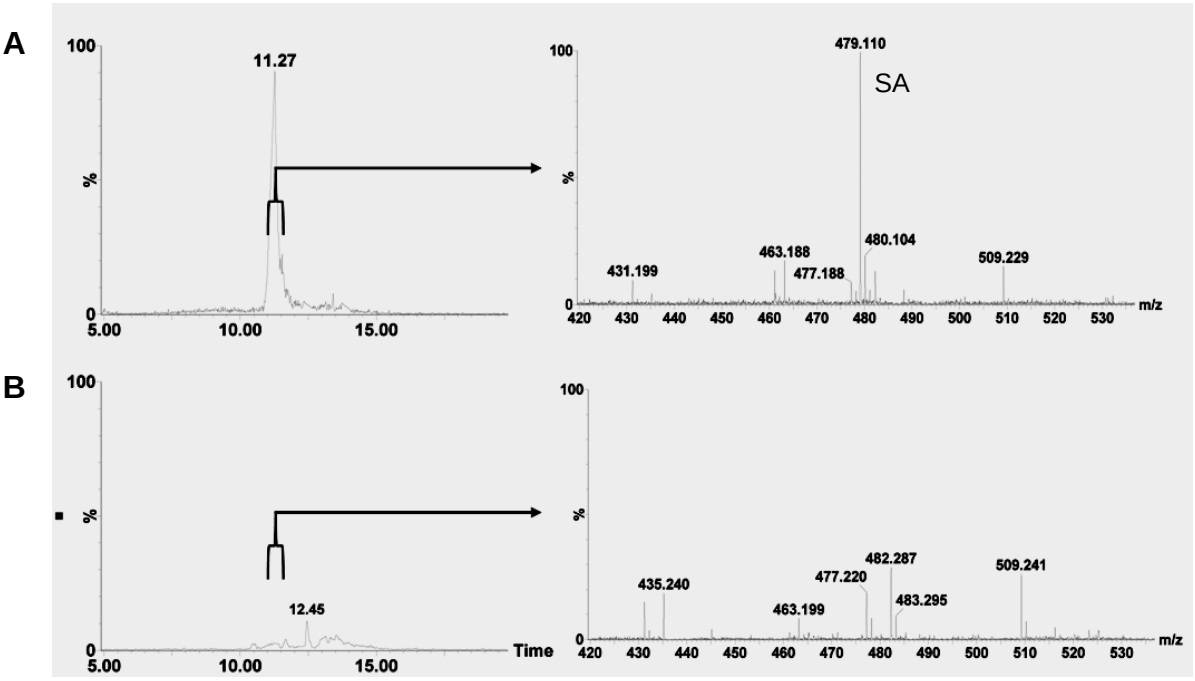

A
100
%
0
5.09
2.50
5.00
7.50
100
%
0
447.14
SB
344.02
300.03
340.02
345.04
448.15
280
300
320
340
360
380
400
420
440
460
480
500
520
m/z
B
100
%
0
2.50
5.00
7.50
100
%
0
344.00
300.01
345.01
280
300
320
340
360
380
400
420
440
460
480
500
520
m/z

Figure 2-5. Identification of staphyloferrin A production by *S. aureus* strain RN6390. (A) Concentrated spent culture supernatant from the iron-starved Δ*sbn* mutant was subjected to LC-ESI-MS analysis. Shown is the selected ion chromatogram for the ion at *m*/*z* 479.1 from LC-ESI-MS, with the ESI-MS spectrum of that peak displayed in the inset. **(B)** Similar analysis to **(A)**, using concentrated spent culture supernatant from the iron-starved Δ*sbn* Δ*sfa* mutant.

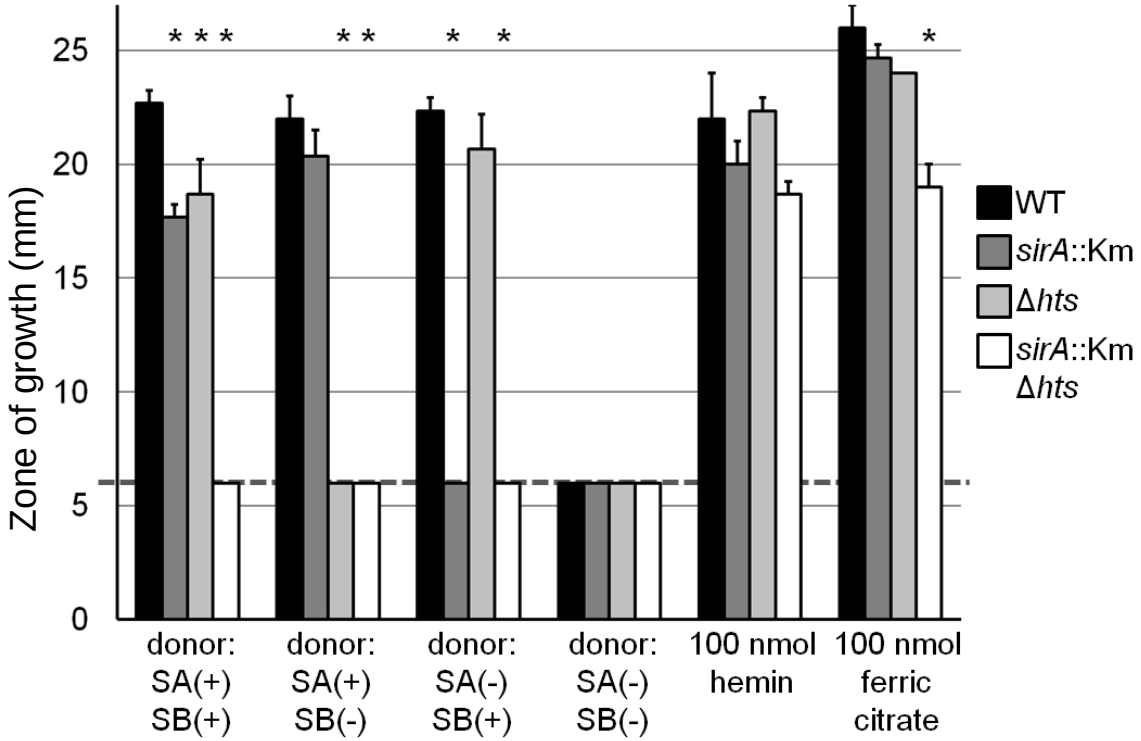

A
B
100
%
0
11.27
5.00
10.00
15.00
479.110
SA
480.104
463.188
477.188
431.199
509.229
420
430
440
450
460
470
480
490
500
510
520
530
m/z
12.45
Time
482.287
477.220
435.240
463.199
483.295
509.241

peak with *m/z* of 479.11, corresponding to the published structure of SA (39), eluted from the Δ*sbn* preparation at 11.27 min (Figure 2-5A), while no peak for that mass above background levels of detection was observed for the Δ*sbn* Δ*sfa* preparation (Figure 2-5B). To further confirm that the peak at 479.110 *m/z* represented SA, LC-MS/MS with collision-induced dissociation (CID) was carried out, generating a fragmentation pattern consistent with the chemical structure of SA (data not shown).

2.3.6 SA and SB are transported by HtsABC and SirABC, respectively and exclusively

Given the chromosomal proximity of operon *htsABC* to the *sfa* locus, it was hypothesized that HtsABC is responsible for transport of SA, despite an earlier report that it contributes to staphylococcal heme acquisition (62). To characterize the role of HtsABC in staphyloferrin transport, a Δ*htsABC* mutant allele was constructed. It has previously been demonstrated that SirABC is involved in transport of staphylobactin/SB using a *sirA* insertional inactivation mutant (18). Disc diffusion agar plate assays were used to test the interaction between staphyloferrins and transporters, by measuring the growth promotion caused by siderophore concentrates obtained from isogenic RN6390 staphyloferrin biosynthesis mutants toward a series of isogenic transporter mutants seeded into an otherwise prohibitively iron chelated medium. Results (Figure 2-6) can be summarized as follows. The ability to grow on SA was solely dependent on HtsABC; the ability to grow on SB was solely dependent on

Figure 2-6. Growth on staphyloferrins A and B is dependent on ABC transporters HtsABC and SirABC, respectively. *S. aureus* strain RN6390 wild type (WT) and isogenic transporter mutants were seeded in agar for a disc diffusion assay to compare growth on concentrated culture supernatants from donors that produce both, one, or neither of the staphyloferrins. Plates contained TMS agar chelated with 10 µM EDDHA, suppressing growth without addition of an exogenous iron source. Asterisks denote statistically significant differences in diameter of growth around the disc relative to wild type treated with the same iron amendment ($P < 0.001$), as calculated using the Student's unpaired *t*-test (n = 3). The dashed grey line denotes the diameter of the disc. Error bars represent standard deviation from the mean.


Zone of growth (mm)
0
5
10
15
20
25
* * *
* *
*
*
donor: SA(+) SB(+)
donor: SA(+) SB(-)
donor: SA(-) SB(+)
donor: SA(-) SB(-)
100 nmol hemin
100 nmol ferric citrate
WT
sirA::Km
Δhts
sirA::Km Δhts

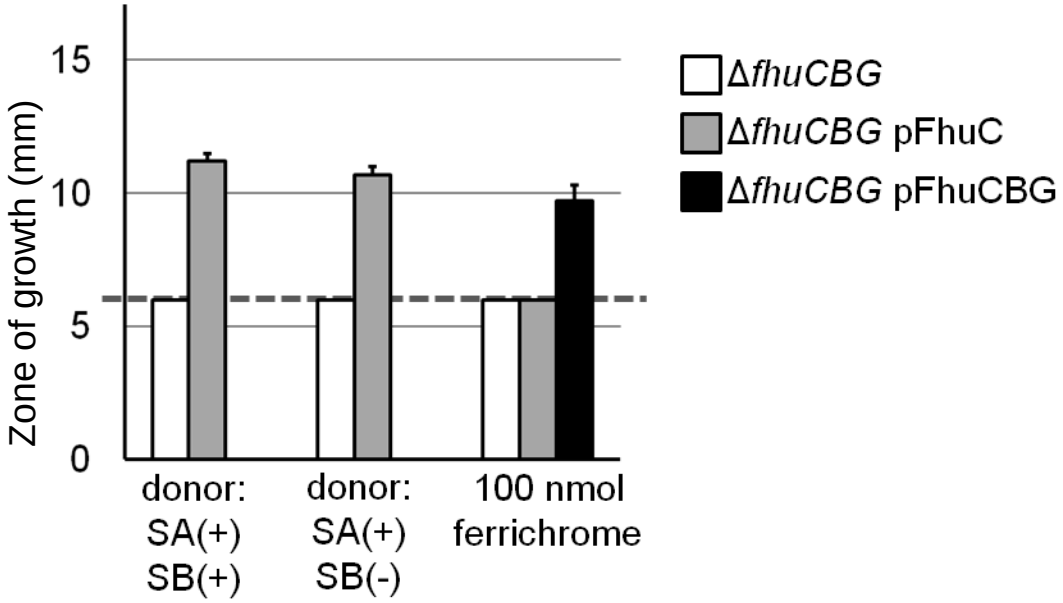
SirABC; in the absence of SA and SB, *S. aureus* produced no detectable growth-promoting metabolites; and, the double transporter mutant strain was insensitive to application of a preparation replete for both staphyloferrins. It was also observed that inactivation of *hts* had no effect on growth promotion by positive controls including hemin.

2.3.7 FhuC is required for staphyloferrin transport by HtsABC and SirABC

By definition, ABC transporters harness the energy of ATP hydrolysis to carry out translocation of a substrate across the cell membrane; however, annotation of both *htsABC* and *sirABC* operons (Figures 1-4A and 1-5A) reveals they are lacking candidate genes for ATP-hydrolyzing enzymes. It has been shown that FhuC, the requisite ATPase for transport of staphylobactin (*i.e.* SB), is coopted from the FhuCBG ABC transporter for hydroxamate type siderophores of exogenous origin (63). Agar plate bioassays were used to demonstrate that FhuC is also required for SA-dependent growth promotion in iron-restricted medium (Figure 2-7). *S. aureus* strain Newman Δ*fhuCBG* was unable to grow on exogenously applied *S. aureus* supernatant concentrates containing SA, or on the hydroxamate siderophore ferrichrome. Complementation with a vector encoding native FhuC protein restored growth promotion on SA, while complementation with the entire *fhuCBG* operon, including permease genes, was required to restore growth promotion on the hydroxamate siderophore ferrichrome.

Figure 2-7. Growth on staphyloferrins is dependent on ATPase FhuC.

S. aureus strain RN6390 wild type (WT) and its isogenic Δ*fhuCBG* mutant complemented with vectors encoding *fhuC* (pFhuC) or the full *fhu* operon (pFhuCBG) were seeded in agar for a disc diffusion assay to compare growth on concentrated culture supernatants from donors that produce SA. Plates contained TMS agar chelated with 10 µM EDDHA, suppressing growth without addition of an exogenous iron source. The dashed grey line denotes the diameter of the paper disc spotted with concentrate. Error bars represent standard deviation from the mean (n = 3).


15
10
5
0
Zone of growth (mm)
donor:
SA(+)
SB(+)
donor:
SA(+)
SB(-)
100 nmol
ferrichrome
ΔfhuCBG
ΔfhuCBG pFhuC
ΔfhuCBG pFhuCBG

2.3.8 Staphyloferrins enable growth of *S. aureus* in serum

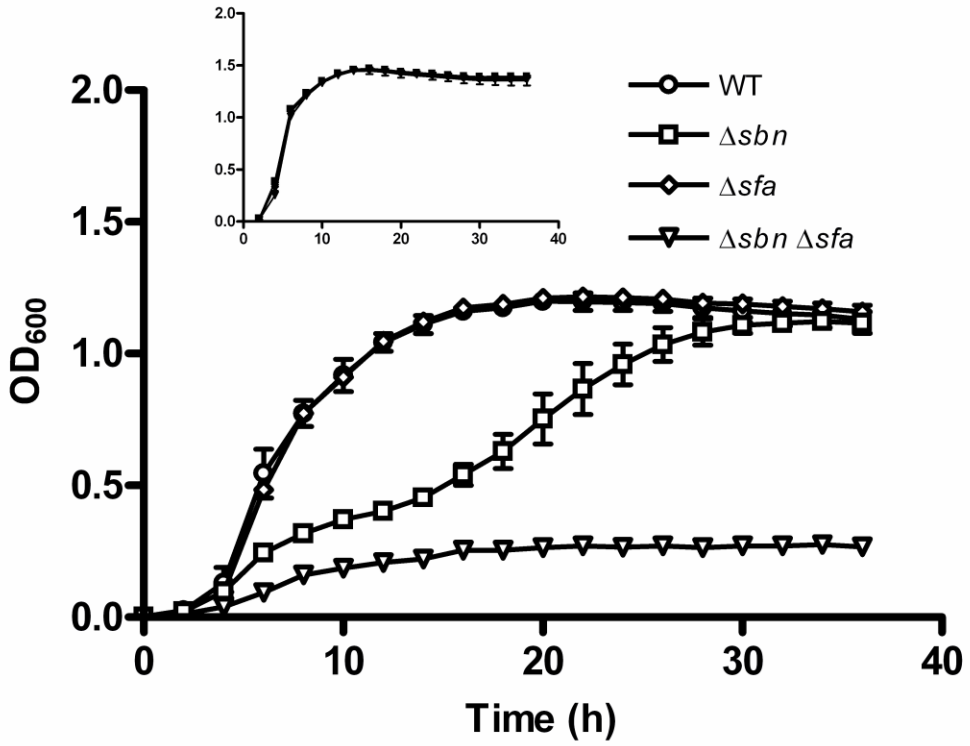
Several studies have demonstrated that microbial siderophores contribute to the growth of pathogens in the iron-restricted milieu of serum (Table 1-1), prompting the hypothesis that staphyloferrins might play a similar role for *S. aureus*. To test this, *S. aureus* mutants inactivated for production of one or both of SA and SB were grown in a medium comprised of 60% complement-deactivated horse serum and 40% low-iron TMS broth (HoS-TMS).

A previous characterization of a *S. aureus* RN6390 *sbnF* mutant (SB-deficient) described this strain as severely defective for growth in TMS due to a lack of siderophore output (17). In this study, a strain Newman Δ*sbn* mutant demonstrated markedly impaired growth compared to its wild type parent within the first 15 h of incubation, but subsequently recovered to reach an approximately equivalent 30 h cell density (Figure 2-8A). Supplementation of the medium with free iron obviated this defect. Despite an absence of SB, end-point supernatant siderophore activity of the Δ*sbn* mutant was equivalent to that of its parent strain, as measured using the CAS assay (Figure 2-8B), implying SA might account for this recovery. To ensure these results were not specific to strain Newman, growth experiments were repeated using RN6390 and its isogenic staphyloferrin biosynthetic mutants, and similar growth dynamics were observed (Figure 2-9).

In contrast to Δ*sbn* mutants, Δ*sfa* mutants were not noticeably impaired for growth in horse serum (Figures 2-8A and 2-9A), despite a decrease in overall output of siderophore into the culture supernatant (Figures 2-8B and 2-9B). Strains lacking both sets of staphyloferrin loci, however, were severely

Figure 2-8. Staphyloferrin production promotes growth of *S. aureus* Newman in serum. (A) *S. aureus* strain Newman (WT) and isogenic SA and SB deficient mutants (Δ*sfa*, Δ*sbn* respectively) were cultured in Hos-TMS medium. Production of SA alone resulted in delayed growth kinetics, toward an eventual culture density equivalent to wild type. Production of SB alone was sufficient for wild type growth. Lack of staphyloferrin production severely restricted growth. All mutant kinetics were ameliorated to wild type with addition of 50 µM $FeCl_3$ (inset). **(B)** Culture supernatant siderophore activity, measured using the CAS assay, was dependent on intact *sfa* or *sbn* loci, and repressed by addition of 50 µM $FeCl_3$. Error bars represent standard deviation from the mean (n = 3).

A


WT
Δsbn
Δsfa
Δsbn Δsfa
OD600
Time (h)
0.0
0.5
1.0
1.5
2.0
0
10
20
30
40

B

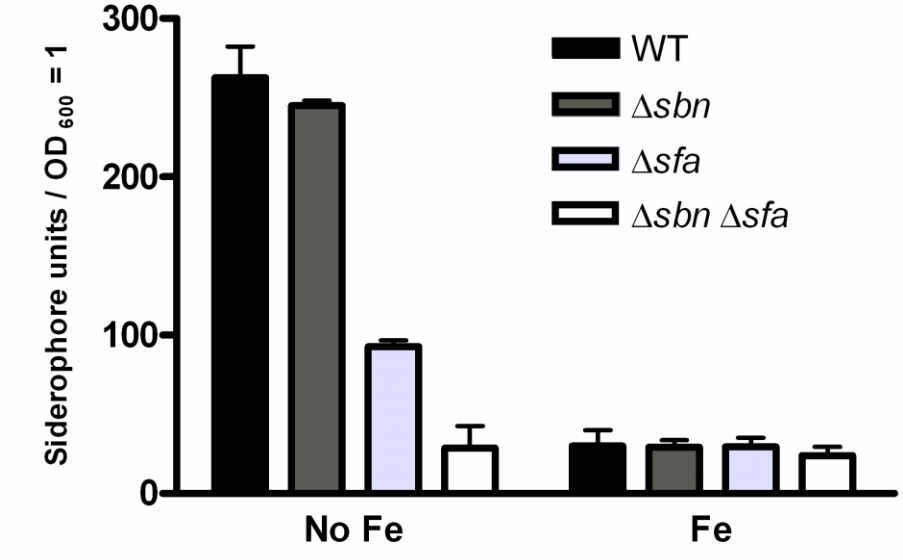
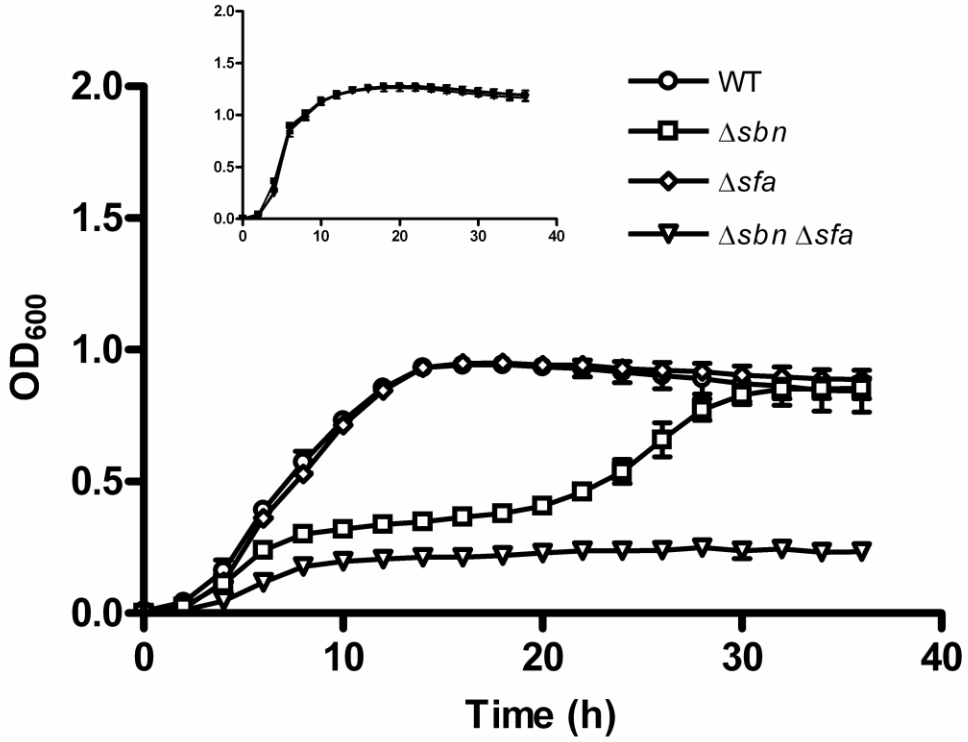
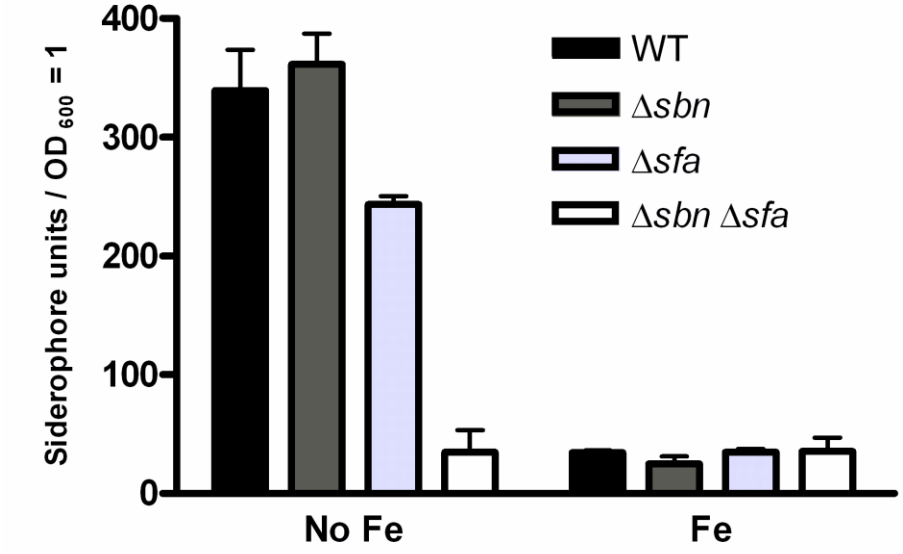

Siderophore units / OD600 = 1
WT
Δsbn
Δsfa
Δsbn Δsfa
0
100
200
300
No Fe
Fe

Figure 2-9. Staphyloferrin production promotes growth of *S. aureus* RN6390 in serum. (A) *S. aureus* strain RN6390 (WT) and isogenic SA and SB deficient mutants (Δ*sfa*, Δ*sbn* respectively) were cultured in Hos-TMS medium. Production of SA alone resulted in delayed growth kinetics, toward an eventual culture density equivalent to wild type. Production of SB alone was sufficient for wild type growth. Lack of staphyloferrin production severely restricted growth. All mutant kinetics were ameliorated to wild type with addition of 50 µM $FeCl_3$ (inset). **(B)** Culture supernatant siderophore activity, measured using the CAS assay, was dependent on intact *sfa* or *sbn* loci, and repressed by addition of 50 µM $FeCl_3$. Error bars represent standard deviation from the mean (n = 3).

A


WT
Δsbn
Δsfa
Δsbn Δsfa
OD600
Time (h)
0.0
0.5
1.0
1.5
2.0
0
10
20
30
40

B


Siderophore units / OD600 = 1
WT
Δsbn
Δsfa
Δsbn Δsfa
No Fe
Fe
0
100
200
300
400

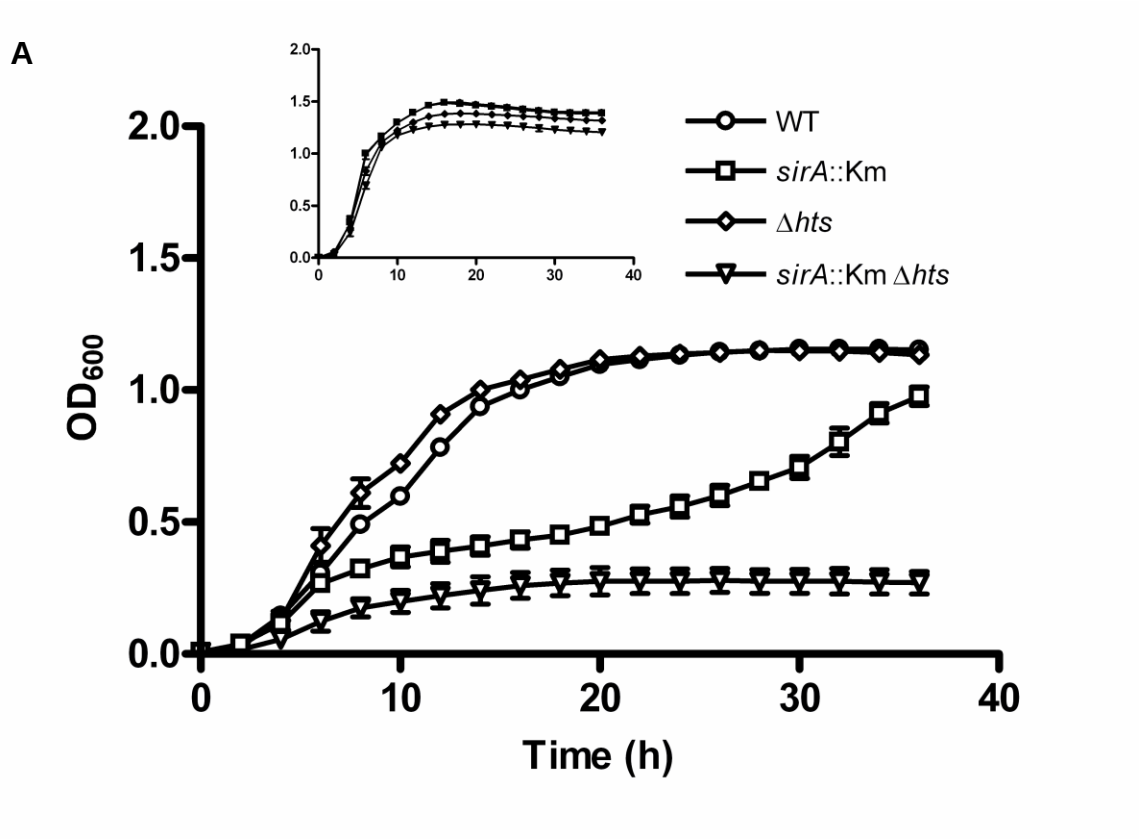
attenuated for growth (Figures 2-8A and 2-9A). This defect was presumably due to their inability to produce siderophore above background detection levels (Figures 2-8B and 2-9B), and growth could be corrected to wild type levels if the medium was saturated with free iron. Taken together with data presented above describing the inability of Δ*sbn* Δ*sfa* supernatant concentrates to promote the iron-restricted growth of wild type *S. aureus*, these results strongly suggest that total siderophore production is mediated by the *sbn* and *sfa* loci.

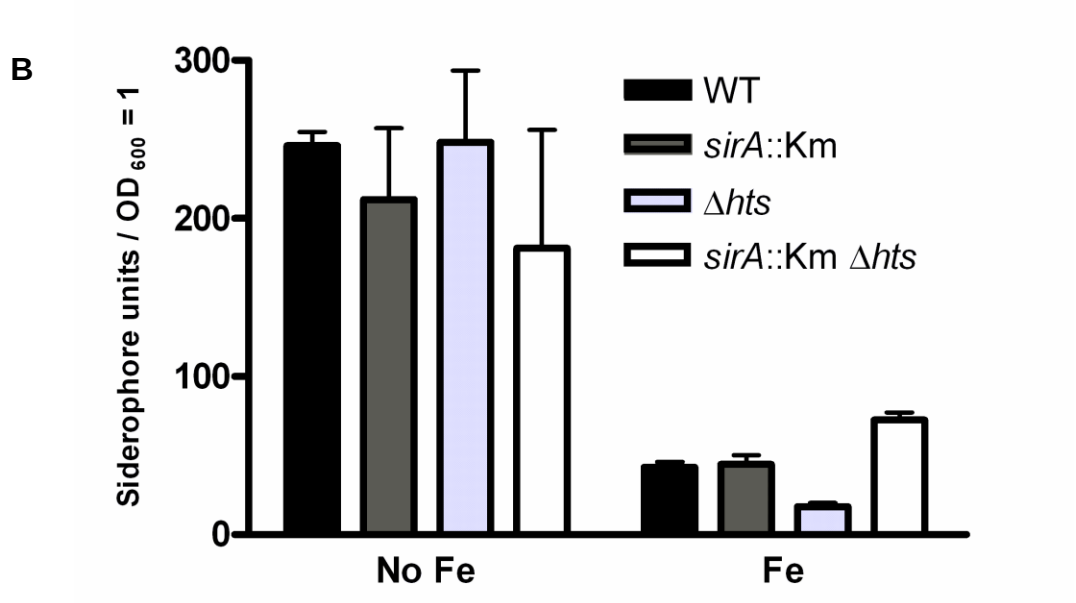
It was also of interest to determine if inactivation of staphyloferrin transporters would result in comparable growth defects to mutating biosynthetic loci (Figure 2-10A). Similar to the lack of a growth defect for the Δ*sfa* mutant, a Δ*hts* knockout strain was not impaired for growth in horse serum relative to the wild type parent, while a *sirA* insertional mutant demonstrated growth kinetics characterized by extended lag and slower logarithmic growth, mimicking its Δ*sbn* counterpart. More importantly, a double *sirA*::Km Δ*hts* mutant was equally attenuated for growth to its Δ*sbn* Δ*sfa* counterpart. Interestingly, siderophore output by transporter mutants did not appear compromised, as endpoint supernatant activities of all cultures tested were roughly equivalent to the wild type parent if normalized to cell density (Figure 2-10B).

2.3.9 Staphyloferrins enable growth of *S. aureus* on transferrin

Numerous microbial siderophores contribute to the extraction of iron from the serum sequestration protein transferrin (Table 1-1), prompting the hypothesis that staphyloferrins might play a similar role for *S. aureus*. To test this, *S. aureus* staphyloferrin mutants were grown in TMS broth that had

Figure 2-10. Staphyloferrin transport promotes growth of *S. aureus* Newman in serum. (A) *S. aureus* strain Newman (WT) and isogenic SA and SB transporter mutants (Δ*hts*, *sirA*::Km respectively) were cultured in Hos-TMS medium. Transport of SA alone resulted in delayed growth kinetics, toward an eventual culture density equivalent to wild type. Transport of SB alone was sufficient for wild type growth. Lack of staphyloferrin transport severely restricted growth. All mutant kinetics were ameliorated to wild type with addition of 50 µM $FeCl_3$ (inset). **(B)** Culture supernatant siderophore activity, measured using the CAS assay, was not dependent on intact *hts* or *sir* operons, and was repressed by addition of 50 µM $FeCl_3$. Error bars represent standard deviation from the mean (n = 3).


A
WT
sirA::Km
Δhts
sirA::Km Δhts
OD600
Time (h)
0.0
0.5
1.0
1.5
2.0
0
10
20
30
40


B
Siderophore units / OD600 = 1
WT
sirA::Km
Δhts
sirA::Km Δhts
0
100
200
300
No Fe
Fe

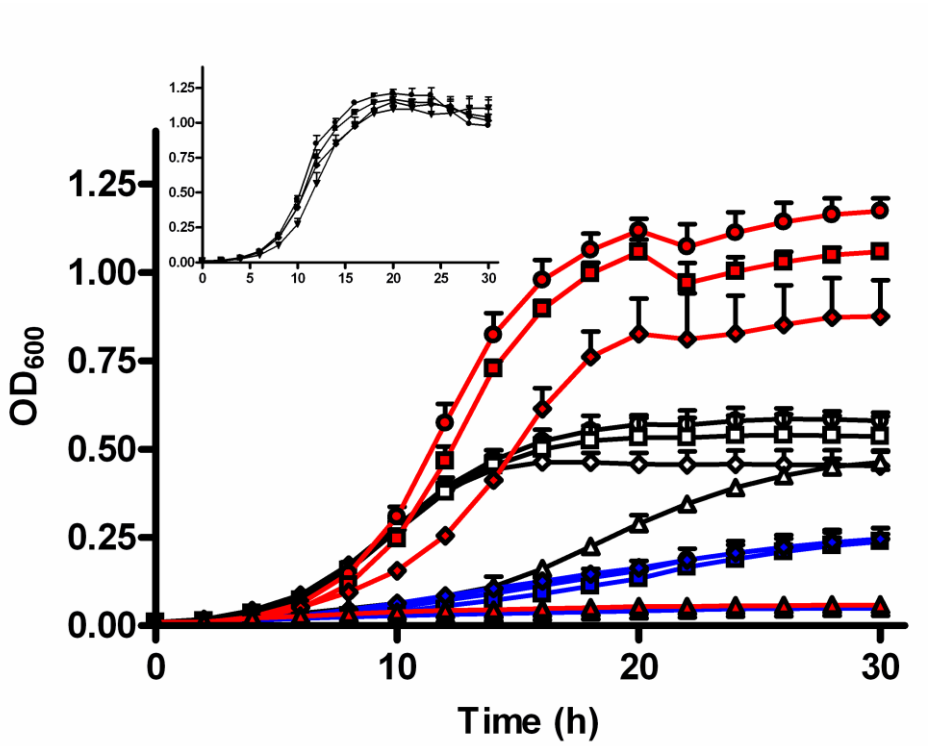
previously been deferrated by application of the chelating resin, Chelex-100 (C-TMS), and reconstituted for iron with 10 μM human transferrin. According to the manufacturer's claims, this was approximately 60% iron saturated, thus serving as both a source of iron for strains with suitable extraction strategies, but also as a sink of iron for strains lacking these strategies.

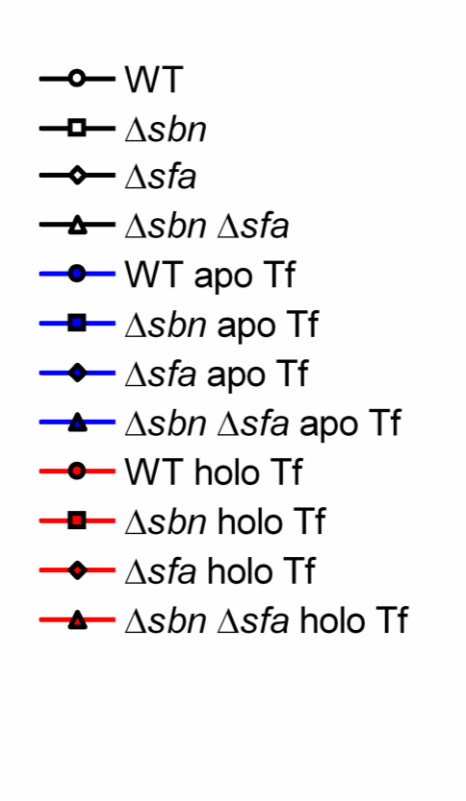
Despite Chelex treatment, enough contaminating iron persisted to promote some growth of *S. aureus* in the absence of a deliberately applied amendment (*i.e.* transferrin) (Figure 2-11). Obtaining this iron did not require staphyloferrin production, since it was observed for the Δ*sbn* Δ*sfa* mutant, although either staphyloferrin accelerated the onset of exponential growth for single locus mutants. Holotransferrin was sufficient to promote otherwise iron-restricted growth of *S. aureus* producing at least one staphyloferrin, to roughly equivalent levels (Figure 2-11), suggesting iron extraction from this mammalian sequestration factor is a redundant process mediated by both SA and SB. The siderophore-deficient growth defect could be rescued with addition of sufficient free $FeCl_3$ to saturate transferrin (inset). In contrast to holotransferrin, apotransferrin was detrimental to growth of all strains tested, suggesting competition occurred for binding to free contaminating iron with staphylococcal siderophores.

2.3.10 The *sfalhts* locus is conserved in CNS

To determine whether coagulase-negative staphylococcal (CNS) *sfa* loci are analogous to their *S. aureus* counterparts, attempts were made to correct the *S. aureus* Δ*sbn* Δ*sfa* growth defect to a Δ*sbn* mode of growth through

Figure 2-11. Staphyloferrin production promotes growth of *S. aureus* Newman on human transferrin. *S. aureus* strain Newman (WT) and isogenic SA and SB deficient mutants (Δ*sfa*, Δ*sbn* respectively) were cultured in C-TMS medium supplemented with 10 µM human transferrin (Tf), in apo- or holo- (approximately 60% saturated) forms. Production of either staphyloferrin permitted growth on holotransferrin approaching that of the wild type parent, while lack of staphyloferrin production severely restricted growth. All mutant kinetics on holotransferrin were ameliorated to wild type with addition of 50 µM $FeCl_3$ (inset). Error bars represent standard deviation from the mean (n = 3).


OD600
Time (h)
0.00
0.25
0.50
0.75
1.00
1.25
0
10
20
30

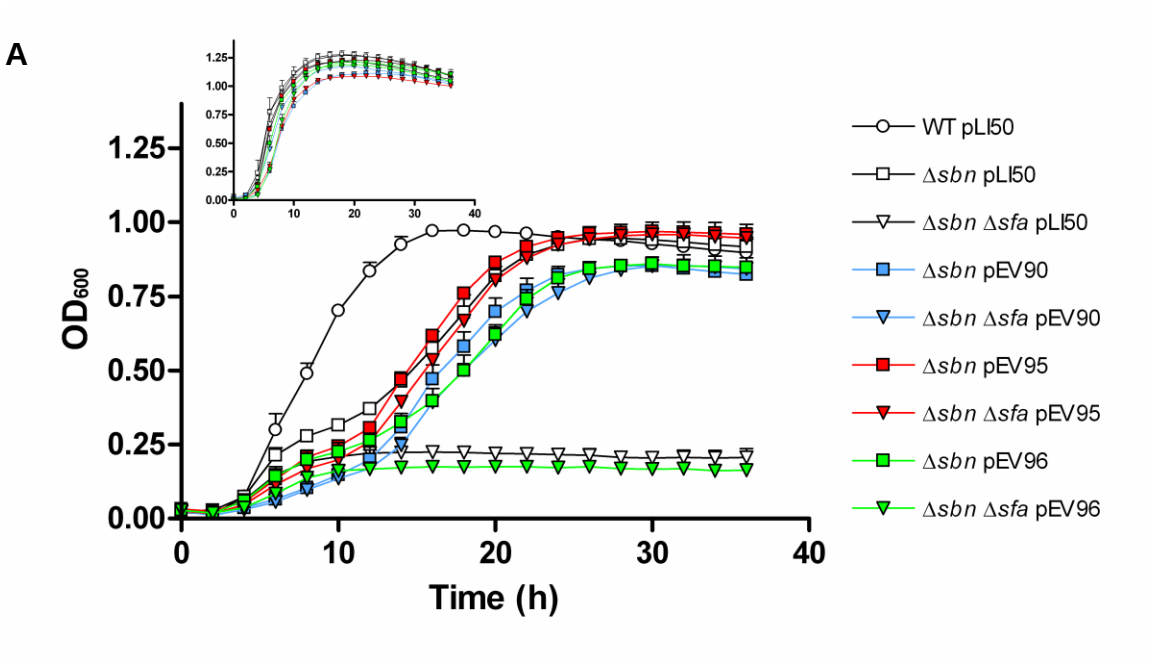

WT
Δsbn
Δsfa
Δsbn Δsfa
WT apo Tf
Δsbn apo Tf
Δsfa apo Tf
Δsbn Δsfa apo Tf
WT holo Tf
Δsbn holo Tf
Δsfa holo Tf
Δsbn Δsfa holo Tf

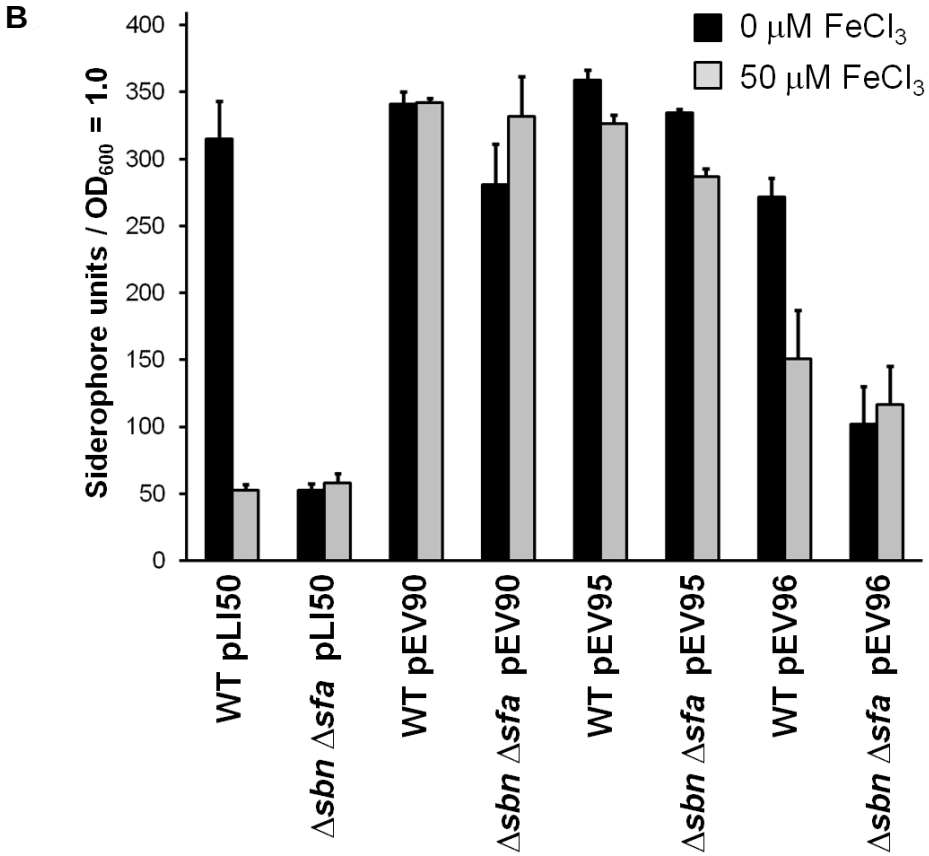
expression of cloned CNS *sfa* loci *in trans*. These loci were cloned to vector pLI50 such that expression of *sfaABC* and *sfaD* operons remained reliant on the promoter-operator region between ORFs *sfaA* and *sfaD* (Figures 1-4A and 2-2). For *S. aureus* staphyloferrin mutants grown in HoS-TMS medium, complementation was achieved using pEV90 (*sfa aureus*) and pEV95 (*sfa epidermidis*), but could not be achieved using pEV96 (*sfa saprophyticus*) (Figure 2-12A). CAS assays performed on exponential stage culture supernatants revealed that siderophore output was markedly high for strains carrying pEV90 and pEV95, regardless of the iron status of the medium (Figure 2-12B). This was likely the result of Fur protein titration due to the high copy number of the Fur-box encoding *sfa* plasmids. Curiously, this was not sufficient to compensate for SB and correct Δ*sbn* growth kinetics to wild type. Despite failing to contribute to the iron-restricted growth of the Δ*sbn* Δ*sfa* mutant, pEV96 carriage nonetheless improved CAS activity of this strain beyond basal levels (relative to pLI50), and subsequent studies have demonstrated that *S. saprophyticus* produces a siderophore available to *S. aureus* in a HtsABC-dependent fashion; the reason for a lack of growth promotion through heterologous genetic expression of *S. saprophyticus sfa* genes remains unresolved.

2.3.11 Heterologous expression of SirABC in *S. epidermidis* enables growth on SB

To date, no *sbn*-like genetic loci have been annotated in any sequenced CNS genomes, and with the exception of *S. lugdunensis*, no *sir*-like loci have

Figure 2-12. *sfa* is conserved in *S. epidermidis*. (A) *S. aureus* strain RN6390 growth defects in HoS-TMS arising from *sfa* inactivation could be rescued *in trans* by *sfa* genes expressed from vector pLI50. Chromosomal genotypes included wild type (WT), SB-inactivated (Δ*sbn*), and SA/SB-inactivated (Δ*sbn* Δ*sfa*). pLI50 derivatives contained *sfa* loci from *S. aureus* (pEV90), *S. epidermidis* (pEV95), and *S. saprophyticus* (pEV96); the latter failed to rescue the Δ*sfa* growth defect. Presence of empty complementation vector (pLI50) did not contribute to iron restricted growth. Growth defects could also be rescued by addition of 50 µM $FeCl_3$ (inset). **(B)** Expression of *sfa* from high copy number vector pLI50 derivatives (pEV90, pEV95, pEV96) resulted in increased siderophore production even in the presence of 50 µM $FeCl_3$; siderophore production was measured using the CAS assay. Error bars represent standard deviation from the mean (n = 3).


A
OD600
Time (h)
WT pLI50
Δsbn pLI50
Δsbn Δsfa pLI50
Δsbn pEV90
Δsbn Δsfa pEV90
Δsbn pEV95
Δsbn Δsfa pEV95
Δsbn pEV96
Δsbn Δsfa pEV96


B
Siderophore units / OD600 = 1.0
0 μM FeCl3
50 μM FeCl3
WT pLI50
Δsbn Δsfa pLI50
WT pEV90
Δsbn Δsfa pEV90
WT pEV95
Δsbn Δsfa pEV95
WT pEV96
Δsbn Δsfa pEV96

been annotated. *S. aureus* is exquisitely adapted to surviving in low iron niches; during its evolutionary history, its genome has acquired multiple determinants endowing transport of exogenously derived siderophores that facilitate such a lifestyle. Certain CNS species also profit from exogenous siderophores; for instance, *S. epidermidis*, whose genome encodes an annotated Fhu-like ABC transporter, is known to use hydroxamate siderophores (19), despite not synthesizing these molecules (49). It was thus of interest to determine if a directed genetic acquisition event could render a CNS species capable of using SB, which could foreshadow the emergence of more virulent strains capable of exploiting a wider range of clinically relevant iron ligands.

This was achieved through heterologous expression of *sirABC* in *S. epidermidis* strain 1457-M10. A biofilm deficient mutant was selected for this experiment because of its improved transformability relative to its biofilm proficient parental strain. Previous attempts to construct *sirABC*-expressing vectors have been hindered by the apparent incompatibility of the *sir-sbn* operator region in *E. coli* strains used for genetic manipulations, necessitating the transcriptional fusion of this operon to an alternate staphylococcal iron-regulated promoter. To forestall the possibility that *S. epidermidis* FhuC might be unsuitable for proper functioning of SirABC, *sirABC* was cloned downstream of *S. aureus fhuC* and its native operator region in shuttle vector pALC2073, forming plasmid pFB54. To determine if *S. epidermidis* FhuC is an adequate surrogate for its *S. aureus* homologue, a transcriptional fusion to a nonfunctional Walker domain mutant allele of *S. aureus fhuC* was also made, generating plasmid pFB57. Either vector rendered strain 1457-M10 responsive

to application of a supernatant concentrate from a SB-producing, SA-deficient *S. aureus* donor strain (Figure 2-13), demonstrating that genetic transfer events between *S. aureus* and *S. epidermidis* can augment the iron uptake repertoire of the latter's genome.

2.3.12 Inactivating siderophore biosynthesis has a minimal impact on virulence in a mouse sepsis model of infection

It was previously shown that *S. aureus* with a chromosomal inactivation of one of the three SB synthetases was attenuated in a five to six day mouse kidney abscess model of infection (17). In this model, *S. aureus* is injected to mice intravenously but rarely persists in the blood more than a few hours; rather, it localizes to soft tissues and seeds within organs (11). Because of their exquisite susceptibility to bloodborne *S. aureus*, kidneys are the most commonly investigated organs for quantification of bacterial load, with the hallmark of severe infection being the formation of bacteria-laden renal abscesses. In contrast to the previous study, numerous pilot studies with injected Newman Δ*sbn* and Δ*sfa* mutants failed to demonstrate significant reductions in kidney bacterial burden resulting from inactivation of staphyloferrin production (data not shown); thus subsequent studies were expanded to include other organs, chiefly livers and hearts. Spleens and lungs were also examined in some experiments, but yielded data with extreme variability, and will not be discussed further. Additionally, different infective loads were tested.

The results of four infection studies are presented in Figure 2-14 and Appendix Figure A1. At a lower infective dose a significant attenuation of

Figure 2-13. Heterologous expression of *sirABC* enables *S. epidermidis* to grow on SB. *S. epidermidis* strain 1457-M10 expressing *S. aureus fhuCsirABC* as a transcriptional fusion from plasmid pALC2073 (pFB54) seeded in agar for a disc diffusion assay grew in response to addition to *S. aureus* culture concentrate containing SB. A Walker domain null mutant allele of *S. aureus fhuC* in the fusion (pFB57) did not compromise this growth promotion. Plates contained RPMI agar chelated with 5 µM EDDHA, suppressing growth without addition of an exogenous iron source. The dashed grey line denotes the diameter of the paper disc spotted with concentrate. Error bars represent standard deviation from the mean (n = 3).

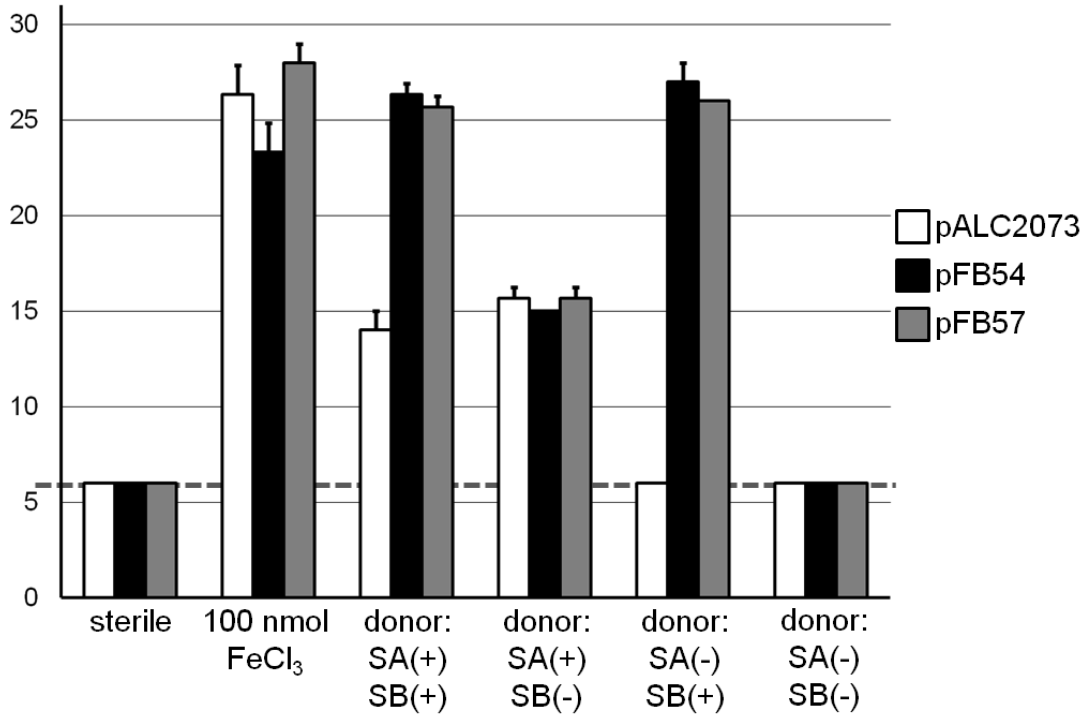
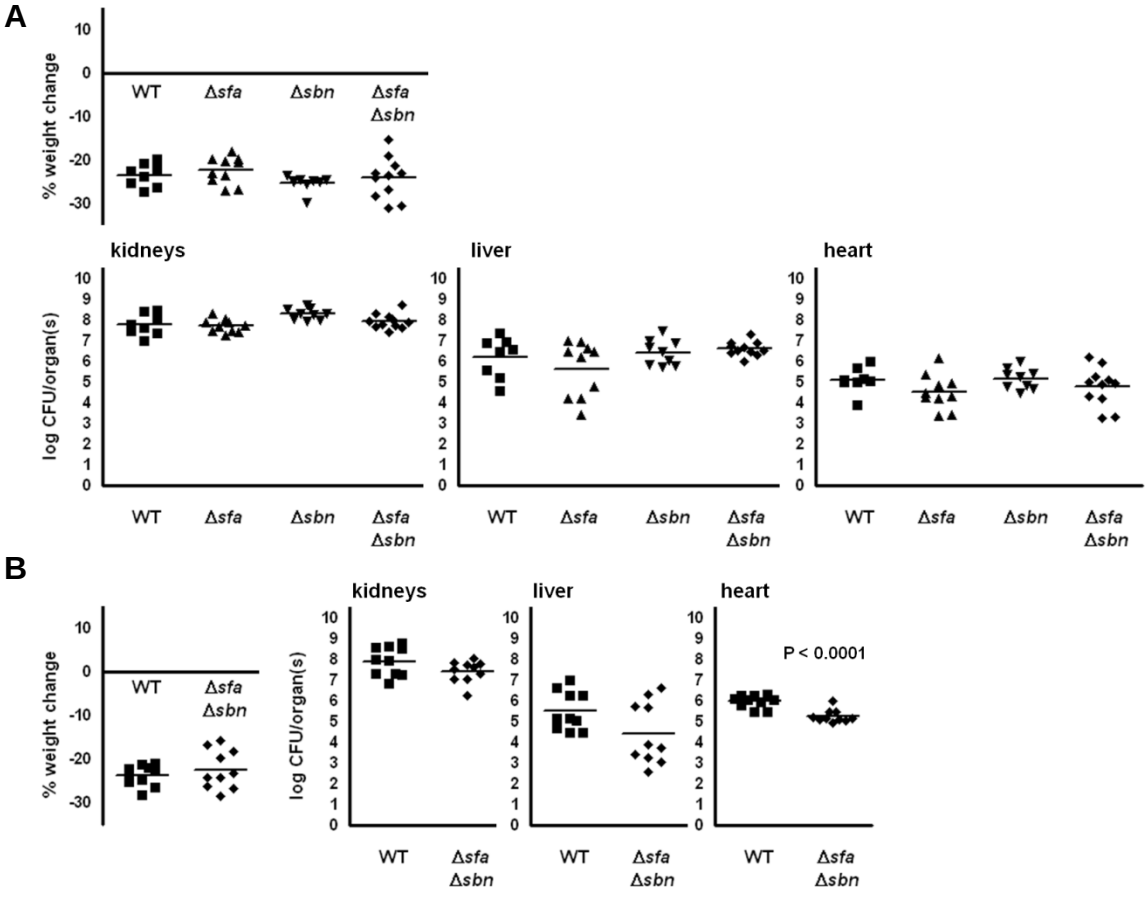

30
25
20
15
10
5
0
sterile
100 nmol FeCl3
donor: SA(+) SB(+)
donor: SA(+) SB(-)
donor: SA(-) SB(+)
donor: SA(-) SB(-)
pALC2073
pFB54
pFB57

Figure 2-14. *S. aureus* Newman strains mutated in *sfa* and *sbn* loci are minimally attenuated in a mouse systemic model of infection.

Approximately 8 x 10^6 CFU **(A)** or 5 x 10^6 CFU **(B)** *S. aureus* Newman and isogenic staphyloferrin biosynthetic mutant derivatives were injected intravenously to Charles River BALB/c immunocompetent mice. Percentage of mouse body mass lost over four days and CFU recovered from target organs are plotted. Statistical analysis was performed using the Student's unpaired *t*-test. *P* values denote significant differences from wild type (WT). Calculated infective doses, in millions of CFU injected, were as follows: wild type, 8.000 **(A)** and 6.000 **(B)**; Δ*sfa*, 8.625; Δ*sbn*, 8.625; Δ*sfa* Δ*sbn*, 8.750 **(A)** and 6.000 **(B)**.


A
% weight change
WT
Δsfa
Δsbn
Δsfa Δsbn
kidneys
liver
heart
log CFU/organ(s)
B
P < 0.0001

bacterial load in hearts was observed in Charles River mice (Figure 2-14B); this was not reproducible using mice purchased from Jackson Laboratories (Appendix Figure A1). No significant differences in bacterial loads between strains were observed for other organs. These results suggest siderophore production is not important for virulence in four-day mouse sepsis models.

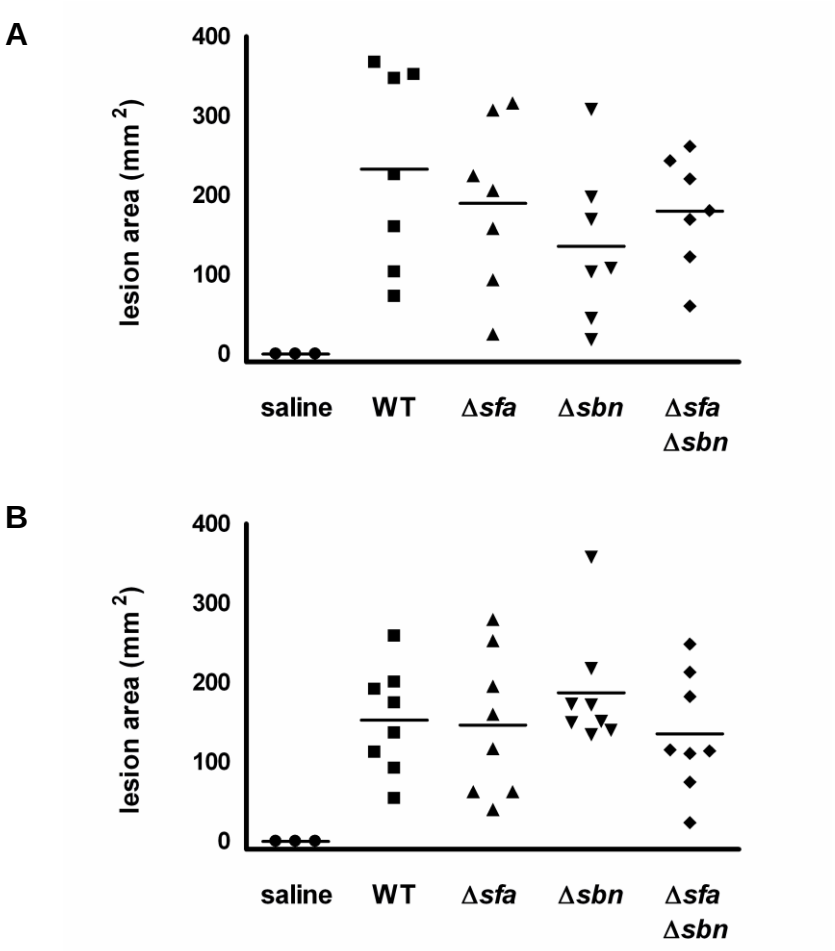
2.3.13 Inactivating siderophore biosynthesis has no impact on virulence in a mouse skin abscess model of infection

Siderophore mutants were also examined for attenuation in a mouse skin abscess model. *S. aureus* Newman saline cell suspensions were injected subcutaneously into the rear flank of Charles River nude SKH-1 or shaved BALB/c mice (approximately 5 x 10^8 CFU). Pus-filled lesions appeared within 24 to 36 h, and surface area was measured at 24 h intervals for up to a week. Lesion size generally stabilized by the second day, and data are presented for that day (Figure 2-15). Lesion sizes were highly variable and no significant differences were noted between wild type and isogenic siderophore biosynthetic mutant strains.

2.3.14 Inactivating siderophore transport has a significant impact on virulence in a mouse sepsis model of infection

The mouse sepsis infection model was approached again to assess virulence phenotypes associated with isogenic *S. aureus* Newman siderophore transport mutants. The Δ*fhuCBG* mutant was also tested, as a strain lacking the FhuC ATPase is impaired for Hts and Sir-mediated transport functions. To

Figure 2-15. *S. aureus* Newman strains mutated in *sfa* and *sbn* loci are not significantly attenuated in a mouse skin lesion model of infection. Approximately 5 x 10^8 CFU *S. aureus* Newman and isogenic staphyloferrin biosynthetic mutant derivatives were injected subcutaneously to nude SKH-1 **(A)** or shaved BALB/c **(B)** immunocompetent mice. An equivalent volume of sterile saline suspension buffer was injected as a negative control. Two day lesion areas are plotted. Statistical analysis was performed using the Student's unpaired T-test. Calculated infective doses, in billions of CFU injected, were as follows: wild type (WT), 0.519 **(A)** and 0.319 **(B)**; Δ*sfa*, 0.437 **(A)** and 0.325 **(B)**; Δ*sbn*, 0.458 **(A)** and 0.350 **(B)**; Δ*sfa* Δ*sbn*, 0.460 **(A)** and 0.303 **(B)**.


A
lesion area (mm 2)
400
300
200
100
0
saline
WT
Δsfa
Δsbn
Δsfa Δsbn
B
lesion area (mm 2)
400
300
200
100
0
saline
WT
Δsfa
Δsbn
Δsfa Δsbn

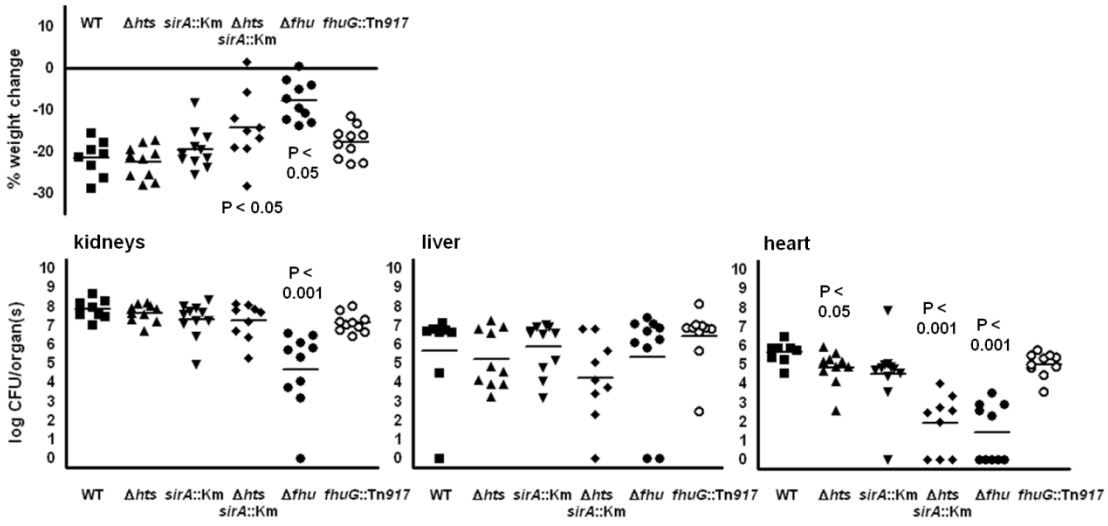
discount any Δ*fhu* phenotypes being attributed to loss of the FhuBG permease, rather than the promiscuous FhuC ATPase, a *fhuG* transposon insertion mutant (*fhuC*$^{+}$) was used as a control.

In contrast to the sepsis trial involving biosynthetic mutants, strains wholly deficient for staphyloferrin transport—*i.e.* the Δ*hts sirA*::Km and the Δ*fhu* mutants—generated robust attenuation phenotypes in BALB/c mice. Significant reductions ($P < 0.05$ using the Student's *t*-test) were observed for weight loss, kidney load, and heart load for at least one of these two mutants (Figure 2-16). The Δ*fhu* mutant was likely attenuated due to lack of ATPase FhuC for HtsABC/SirABC-mediated transport processes, rather than a lack of FhuD1/FhuD2FhuBG-mediated ferric hydroxamate transport, as the *fhuG*$^{-}$ mutant performed comparably to wild type (Figure 2-16).

2.3.15 Inactivating siderophore transport enhances siderophore production by *S. aureus*

Transcriptional regulation of *sfa*, *hts*, *sbn*, and *sir* is described as iron-regulated in the paradigmatic sense, *i.e.* through a mechanism whereby transcriptional derepression occurs when cellular iron levels decrease and Fur-Fe^{2+} dissociates from the operator regions of genes within its regulon (Figure 2-3 and (17, 18)). By the rules of this model it would be reasonable to predict that a genetic mutation compromising iron uptake, including one affecting staphyloferrin transport, would exacerbate iron starvation, consequently enhancing expression of unchanged iron acquisition genes, including those involved in staphyloferrin production. It has been shown that inactivation of Sir

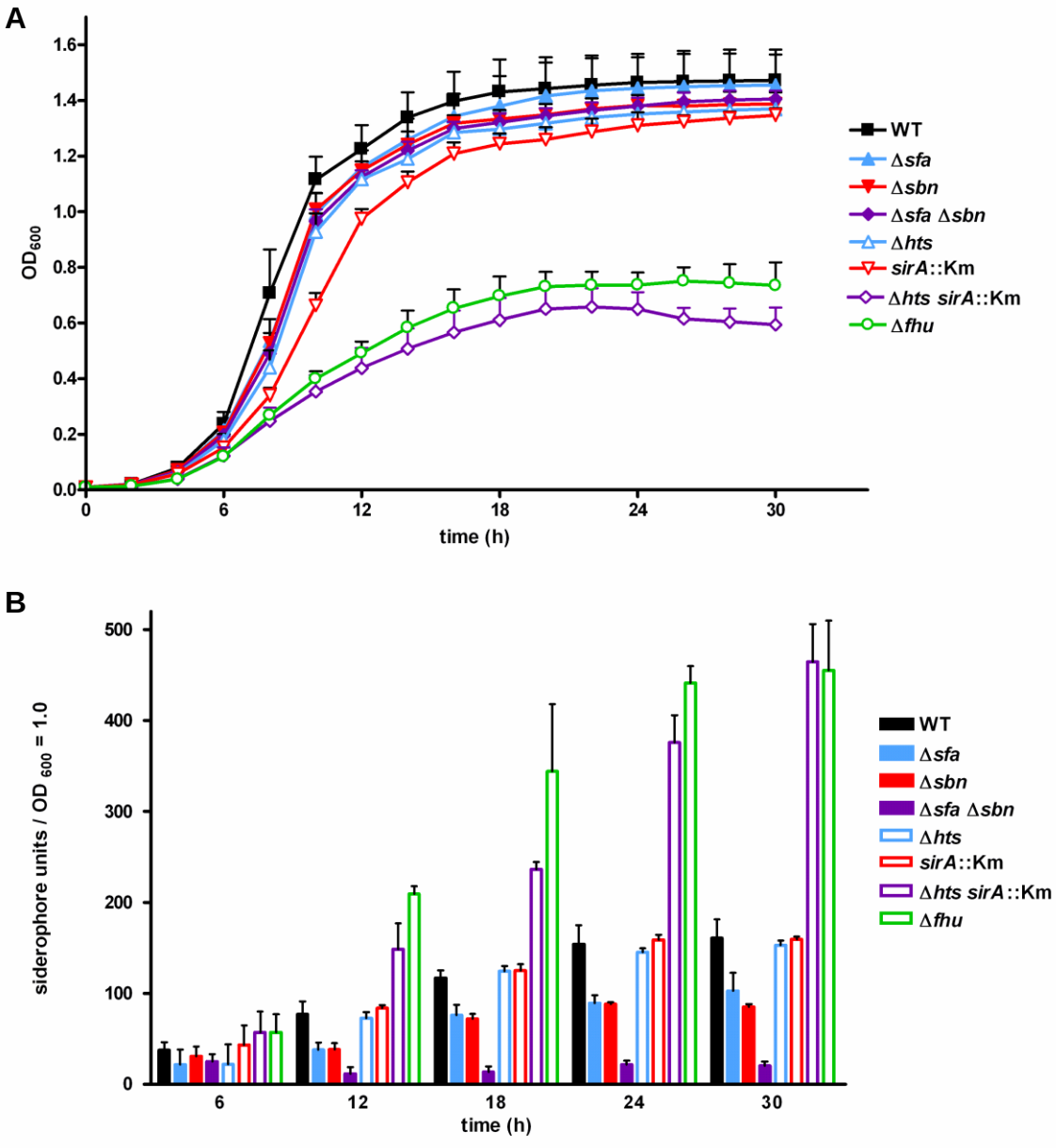
Figure 2-16. *S. aureus* Newman strains mutated in *hts*, *sir*, and *fhu* operons are significantly attenuated in a mouse systemic model of infection. Approximately 9 x 10^6 CFU *S. aureus* Newman and isogenic siderophore transport mutant derivatives were injected intravenously to Charles River immunocompetent mice. Percentage of mouse body mass lost over four days and CFU recovered from target organs are plotted. Statistical analysis was performed using the Student's unpaired T-test. *P* values denote significant differences from wild type (WT). Calculated infective doses, in millions of CFU injected, were as follows: WT, 9.750; Δ*hts*, 9.750; *sirA*::Km, 10.625; Δ*hts sirA*::Km, 8.875; Δ*fhu*, 9.250; *fhuG*::Tn*917*, 8.750.


% weight change
WT
Δhts
sirA::Km
Δhts sirA::Km
Δfhu
fhuG::Tn917
P < 0.05
P < 0.05
kidneys
liver
heart
log CFU/organ(s)
P < 0.001
P < 0.05
P < 0.001
P < 0.001

leads to upregulation of *sbn* (18). In an attempt to explain why the virulence attenuation phenotype of staphyloferrin transport mutants was much more pronounced than that of corresponding biosynthetic mutants, the relative total siderophore output of the host of strains used in the mouse infection models was examined. These were grown in TMS broth and supernatant siderophore activity was sampled at six hour intervals. In this culture medium, the only source of iron stems from trace contamination of the other ingredients; thus it is readily depleted by exponentially growing cells.

This medium sustained growth of all strains tested. Single-locus mutants and the Δ*sfa* Δ*sbn* mutant all grew comparably to the isogenic wild type parental strain (Figure 2-17A), and this growth was apparently not dependent on siderophores, as the Δ*sfa* Δ*sbn* mutant did not render its supernatant CAS-positive above background levels of detection (Figure 2-17B). While the Δ*hts sirA*::Km and Δ*fhu* strains (*i.e.* strains wholly deficient for transport of staphyloferrins) also experienced a normal lag phase and some exponential growth, they entered stationary phase at lower culture density. Their siderophore output, however, continued to rise drastically well into stationary phase, well exceeding output of the parental strain. This is dissimilar to siderophore output measured in HoS-TMS medium (Figure 2-9B), but different results might be attributable to differences in carbon availability between HoS-TMS and TMS. This would suggest that, at least under certain iron-restricted growth conditions, impairing siderophore transport does not affect siderophore production by *S. aureus* and may cause the organism to hyperchelate its own surroundings.

Figure 2-17. Inactivation of siderophore transport does not impair siderophore production by *S. aureus*. (A) *S. aureus* strain Newman (WT) and isogenic mutant derivatives inactivated for staphyloferrin biosynthesis (Δ*sfa*, Δ*sbn*, Δ*sfa* Δ*sbn*) or transport (Δ*hts*, *sirA*::Km, Δ*hts sirA*::Km, Δ*fhu*) were grown in TMS medium. **(B)** Siderophore activity of culture supernatants was sampled at 6 h intervals using the CAS assay. The mutant wholly impaired for biosynthesis (Δ*sfa* Δ*sbn*) failed to generate siderophore activity above baseline levels, while mutants wholly impaired for transport (Δ*hts sirA*::Km and Δ*fhu*) demonstrated markedly enhanced siderophore output. Error bars denote standard deviation from the mean (n = 3).


A
OD600
time (h)
WT
Δsfa
Δsbn
Δsfa Δsbn
Δhts
sirA::Km
Δhts sirA::Km
Δfhu
B
siderophore units / OD 600 = 1.0
time (h)
WT
Δsfa
Δsbn
Δsfa Δsbn
Δhts
sirA::Km
Δhts sirA::Km
Δfhu

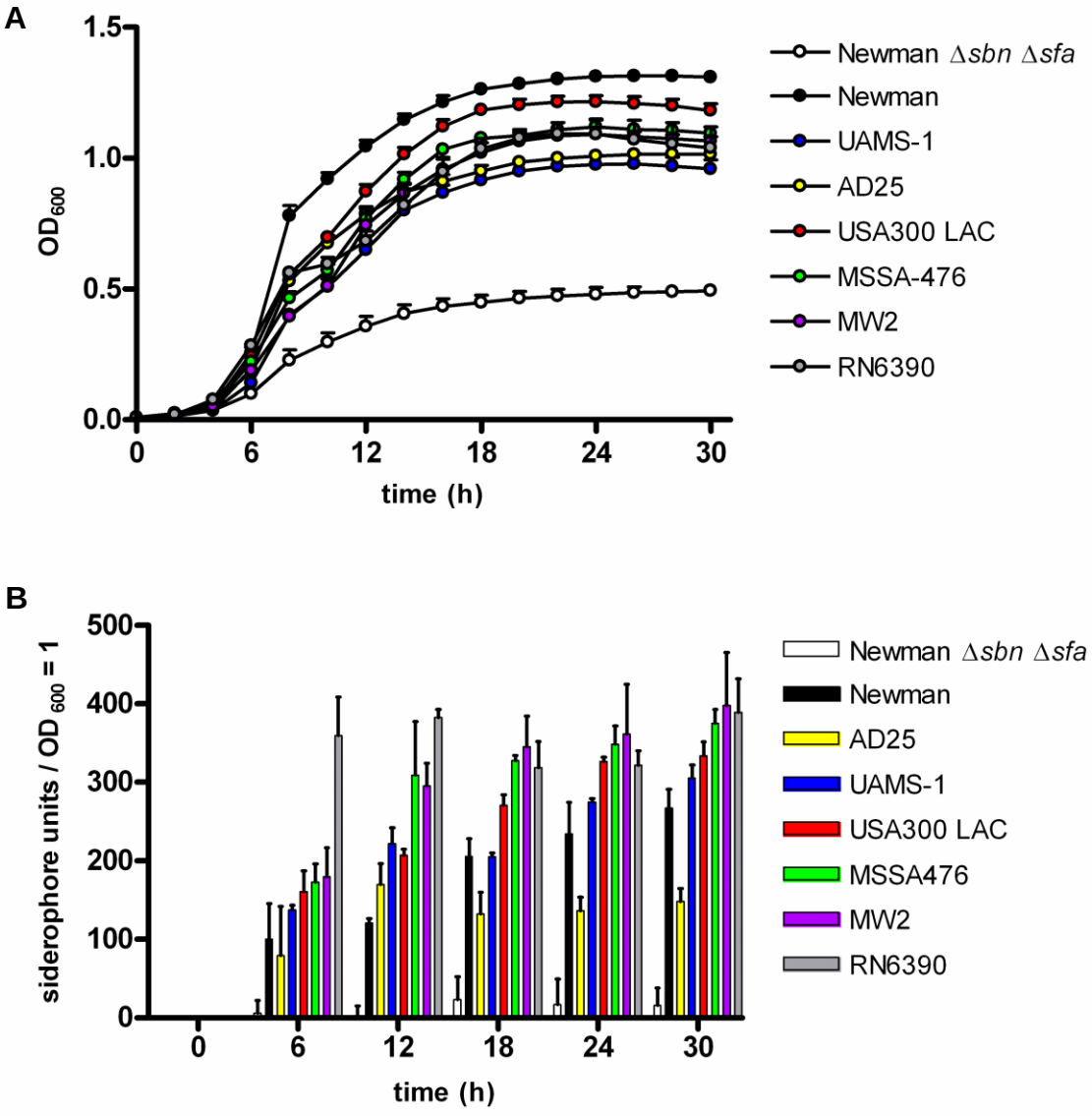
2.3.16 *S. aureus* strain Newman is an efficient producer of siderophores

From the mouse infection models, it was apparent that inactivation of *sbn* and *sfa* had minimal impact on the virulence of this strain compared to the wild type parent, suggesting minimal reliance on siderophore-mediated iron uptake *in vivo*. It was therefore of interest to compare siderophore production of Newman to other clinically relevant *S. aureus* strains. These were grown in HoS-TMS media with periodic sampling of culture supernatant siderophore activity using the CAS assay. Growth dynamics for all wild type strains were comparable; notably, Newman achieved higher stationary phase density than all other isolates (Figure 2-18A). At all intervals, Newman supernatant siderophore activity was lower than all other *S. aureus* strains tested, with the exception of dermatitis-associated strain AD25 (Figure 2-18B), with the most marked differences noted within the first 12 hours of growth. Despite apparently secreting less siderophore than other strains, Newman output was sufficient to counter the bacteriostatic effects of serum.

2.4 Discussion

Decades-old surveys of *Staphylococcus* culture collections described members of this genus to produce at least two carboxylate siderophores: SA and SB (34, 39, 43). More recently, Dale *et al.* (17) provided the first insight into the molecular and biochemical mechanisms underlying staphylococcal siderophore synthesis in demonstrating that the *sbn* operon of *S. aureus* encodes NIS synthetases involved in the production of a secreted ferric iron chelator. Lacking structural information linking the molecule to either

Figure 2-18. Siderophore production by *S. aureus* strain Newman is comparatively lower than other laboratory strains and clinical isolates. **(A)** *S. aureus* strain Newman, its isogenic mutant derivative inactivated for staphyloferrin biosynthesis (Δ*sfa* Δ*sbn*), and an array of laboratory and clinical *S. aureus* isolates were grown in HoS-TMS medium. **(B)** Siderophore activity of culture supernatants was sampled at 6 h intervals using the CAS assay. Strain Newman exhibited lower amounts of siderophore production than most of the other strains, nevertheless achieving the highest endpoint density. Error bars denote standard deviation from the mean (n = 3).


A
1.5
1.0
0.5
0.0
OD600
0
6
12
18
24
30
time (h)
Newman Δsbn Δsfa
Newman
UAMS-1
AD25
USA300 LAC
MSSA-476
MW2
RN6390
B
500
400
300
200
100
0
siderophore units / OD 600 = 1
0
6
12
18
24
30
time (h)
Newman Δsbn Δsfa
Newman
AD25
UAMS-1
USA300 LAC
MSSA476
MW2
RN6390

staphyloferrin, researchers tentatively termed the metabolite staphylobactin. Data presented in this study (Figure 2-4) and by collaborators (12) formally described the role of *sbn*-encoded enzymes in synthesis of SB. In characterizing a synthetase mutant, Dale *et al*. reported that *S. aureus* does not produce another siderophore and cannot grow in conditions of severe iron eprivation. Genome sequencing projects revealed that the *sbn* operon is unique to *S. aureus* among the staphylococci; however, a second locus encoding putative NIS synthetases, *sfaABCsfaD*, is found on the chromosome of *S. aureus* and among all other members of the genus. This annotation prompted reinvestigation of the findings of Dale *et al*., to test the hypothesis that the *sfa* locus encodes for synthetases involved in production of a second siderophore.

The current study has revealed that under conditions of iron restriction, SB-deficient mutants, including the strain used by Dale *et al.*, do in fact readily secrete siderophore (Figure 2-1). The discrepancy between studies might be attributed to methodological differences not described in the published protocols. Chromosomal deletion of the *sfa* locus abolished production of the second siderophore, and combining this mutation with an *sbn* operon deletion rendered *S. aureus* devoid of siderophore output. Cotton *et al.* (14) concurrently demonstrated assembly of SA to be mediated *in vitro* by synthetases SfaD and SfaB, and in this study *sfa* mutants were generated to confirm this genetic locus is responsible for SA production (Figure 2-5). It was also shown that an orthologous locus is responsible for production of the same metabolite for other pathogenic staphylococci, including *S. epidermidis* (Figure 2-12).

A central dogma in *S. aureus* physiology is that its preferred iron source during infection is heme (62). The most abundant reservoir of iron in the mammalian host is within heme groups of hemoglobin (23); in *S. aureus*, the Isd complex of cell wall and membrane associated proteins extract and transport heme as detailed in numerous reports (for a review, see (31)). Expression of *isd* genes is directly regulated by iron and comprises part of the Fur-mediated iron deprivation response (48). However, uncompartmentalized serum hemoglobin (*i.e.* outside of erythrocytes) is relatively scarce, circulating at a concentration of approximately 2.5 µM (53), representing 10 µM Fe. In contrast to *isd* genes, expression of *S. aureus* haemolysins is under direct control of multiple two-component regulatory systems, and most importantly the Agr quorum-sensing system, such that their production occurs after the initial stages of infection, once attachment has occurred (for a review, see (54)). Enhanced production of lytic toxins including α- and γ-hemolysin has been documented for a *S. aureus fur*$^{-}$ mutant (64), but mechanistic data directly linking this phenotype to an absence of Fur repression is lacking, and this phenotype is probably a symptom of a more global disruption of regulatory processes; in another study, transcriptional upregulation of hemolysin genes by wild type *S. aureus* was not noted for cells *in vitro* under conditions of iron limitation (2). Thus, haemolysin secretion is not directly dependent on the intracellular iron status of the bacterial cell and is likely preceded by siderophore production at the onset of invasive infection. In comparison to hemoglobin, transferrin is found in serum at a concentration of approximately 25 µM (4); assuming 30% saturation, this represents a concentration of 7.5 µM Fe, rivalling the serum hemoglobin pool as

a reservoir for iron during initial stages of staphylococcal sepsis. In fact, in sufferers of hemochromatosis, who demonstrate above average transferrin saturation, this protein's reservoir may constitute a more abundant serum iron pool than uncompartmentalized hemoglobin; hemochromatosis is well documented as a predisposing factor to infection (38).

In this study, it was shown that the ability of *S. aureus* to withstand the bacteriostatic effects of serum is alleviated through production of either staphyloferrin (Figure 2-9), and these siderophores are required for *S. aureus* proliferation on holotransferrin as a sole iron source (Figure 2-10). This is in good agreement with the role attributed to siderophores for a range of pathogenic bacteria (Table 1-1). However, inactivation of staphyloferrin biosynthesis had marginal effects on *S. aureus* virulence in a mouse bacteremia model of infection (Figure 2-14). With cursory consideration, this would suggest against describing siderophores as important players in the *S. aureus* repertoire of virulence factors. Other findings of this study, however, suggest further investigation into the contributions of siderophores toward staphylococcal pathogenesis is merited. Most importantly, it was noted that inactivation of siderophore transport without concomitant inactivation of biosynthesis did result in significant and reproducible attenuation of infection in the model (Figure 2-16). This may have been a consequence of transporter mutants continuing to secrete staphyloferrins (Figures 2-10, 2-17), to the point they enhanced the iron-withholding capacity of their milieu. This phenomenon hints that therapeutics incapacitating siderophore transport might be efficacious in combating *S. aureus* infection. With an improved understanding of Sfa/Sbn

synthetase biochemistry, it might be feasible to design staphyloferrin derivatives that compete with the natural ligands for binding to receptor proteins SirA and HtsA, without benefiting the *S. aureus* cell through subsequent transport and release of iron.

A reevaluation of the mouse as a model for studying infective processes of a predominantly primate and livestock-adapted pathogen is merited. Mice exhibit a remarkable innate immunity toward *S. aureus* infection, and the requirement for an infective dose exceeding one million CFU, directly injected into the bloodstream, is hardly representative of the opportunistic infective process in humans, whereby many orders of magnitude fewer infective units enter the bloodstream through a traumatic breach in the endothelium. To some degree, murine innate resistance reflects the lack of coevolution between pathogen and surrogate host; *S. aureus* virulence factors have evolved for enhanced specificity toward human factors, at the expense of specificity toward homologous factors in other genera. Pertinently, it was recently shown that humanizing mice for hemoglobin vastly enhanced *S. aureus* virulence phenotypes associated with Isd-mediated heme uptake (58). While the ability of a siderophore to extract iron from transferrin largely reflects the higher affinity for Fe^{3+} exhibited by the former, this phenomenon may also result from a more dynamic interaction. The enteric bacterial siderophore aerobactin has an *in vitro*-calculated affinity for iron well below that of transferrin (40), yet mediates extraction of iron regardless (25), and is a noted virulence factor (67); this is necessarily the result of an uncharacterized facet of the interaction between the two molecules, which might further be influenced by the species from which the

transferrin is derived. The difficulties in generating robust attenuation phenotypes for siderophore mutants in mice may reflect decreased compatibility of murine transferrin with staphyloferrins. It was of interest to evaluate the growth of *S. aureus* on mouse transferrin, but purchasing sufficient quantities of this molecule proved prohibitively expensive.

The adaptive significance of producing two staphyloferrins with redundant functions is not clear, though *S. aureus* is certainly not unique among bacteria in secreting multiple siderophores; this has been documented for pathogens including *Pseudomonas aeruginosa*, *Escherichia coli*, and *Bacillus anthracis*, to name a few (9, 15, 68). In certain cases, production of a second siderophore is an adaptive mechanism countering the innate immune factor siderocalin (24). This host protein sequesters a range of iron ligands with 2,3-catecholate iron coordination groups, such as bacillibactin (1). To compensate for the sequestration of this chelator during infection, *B. anthracis* also produces petrobactin, which coordinates iron using one carboxylate and two 3,4-catecholate groups, and is not recognized by siderocalin; based on this virtue, it is has been called a “stealth” siderophore (71). Siderocalin evasion is not likely to have been a selective pressure influencing the acquisition of a second siderophore biosynthetic locus by *S. aureus*, as neither staphyloferrin incorporates catechol groups; the ability of siderocalin to sequester SA and SB, however, has not been examined. Acquisition of SB biosynthesis may contribute to the higher virulence of *S. aureus* over other CNS, by giving it a competitive advantage in the battle for iron in mixed population niches such as

skin and mucosa; as demonstrated in Figure 2-13, SB iron is unavailable to *S. epidermidis* lacking heterologously expressed SirABC.

The availability of either staphyloferrin was exclusively contingent on the presence of its cognate transport system; no overlapping functionality between Hts and Sir was observed (Figure 2-6). The crystal structures of HtsA bound to Fe-SA and SirA bound to Fe-SB have been elucidated (29, 30). Both receptors have conserved sequence and structure; both have extremely high affinities for their ferrated ligands (K_d values in the lower nanomolar range); and for both, ligand binding is characterized by localized conformational changes within the binding pockets, acting to encase the siderophore, in lieu of larger, hinge-like conformational change encompassing the greater protein structure, as occurs in the *B. subtilis* tris-catecholate binding protein FeuA (57). Neither receptor has notable affinity for the other's ligand. Specificity is dictated in large part by basic residues within either protein's binding pocket positioned to cater to the overall charge of either staphyloferrin and the geometrical distribution of iron-coordinating atoms across either molecule's structure. The HtsA binding pocket is characterized by six arginine residues which act to neutralize the large negative charge of SB (net charge of -5 when deferrated; Figure 1-4B), and are oriented to target closely clustered Fe^{3+}-coordinating oxygen molecules. The SirA binding pocket is also characterized by key arginine residues; in contrast to HtsA, these are fewer in number (three, to accommodate the -3 charge of SA; refer to Figure 1-5B), and located on different loops of the binding pocket to cater to the wider dispersion of Fe^{3+}-coordinating atoms across the SB molecule.

This is the first study to characterize phenotypes of a siderophore-deficient mutant of *S. aureus*. We have demonstrated that siderophores are major factors contributing to the noteworthy resistance *S. aureus* toward the bacteriostatic effects of transferrin. These, however, are not the only means this pathogen has of extracting iron from transferrin; Chapter 3 is dedicated to the characterization of an alternate mechanism, whose discovery was facilitated by the mutant strains described in this chapter.

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Chapter 3

Catecholamine and catechol-mediated iron uptake in *Staphylococcus aureus*

Significant portions of this chapter have been published in the following article:

Beasley, F. C., C. Marolda, J. Cheung, S. Buac, and D. E. Heinrichs (2011). *Staphylococcus aureus* transporters Hts, Sir and Sst capture iron liberated from human transferrin by staphyloferrin A, staphyloferrin B and catecholamine stress hormones, respectively, and contribute to virulence. Infection and Immunity, *in press*. Mouse surgeries were performed by Martha Harding, Animal Care and Veterinary Services, University of Western Ontario.

3.1 Introduction

Circulatory catecholamine stress hormones, including norepinephrine, epinephrine, dopamine, and L-3,4-dihydroxyphenylalanine (L-DOPA) (Figure 1-2), interfere with the extracellular iron scavenging functions of host immune proteins transferrin and lactoferrin. This occurs through direct binding of catecholamines to transferrin/lactoferrin-complexed Fe^{3+}, with resultant reduction to the Fe^{2+} valence, for which serum sequestration proteins have minimal affinity (31). Released ferrous iron can be captured by pathogenic bacteria (9). Alternatively, catecholamines compete with serum proteins for coordination of Fe^{3+} in solution using their 3,4-dihydroxybenzoyl moieties, such that three catecholamine molecules can complex around one iron atom in a geometrical arrangement evocative of the enterobactin-Fe complex (see 2,3-dihydroxybenzoic acid, Figure 1-2). It was shown that a siderophore biosynthetic mutant of *E. coli* was further compromised for catecholamine-mediated capture of transferrin iron in a contact-independent fashion (across a dialysis membrane) if the strain was also defective for enterobactin transport (*entA*$^-$), implicating the enterobactin transporter in the additional function of capturing the Fe-catecholamine complexes (2).

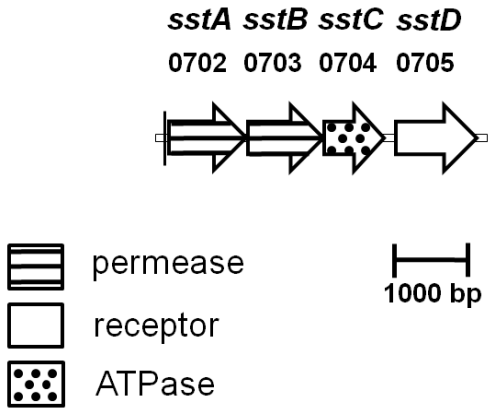
Catecholamine-dependent promotion of bacterial growth has been proposed as a contributory factor in the development of gut sepsis following trauma with associated increases in circulating stress hormone levels (10) and with localized destruction of norandrenergic neurons (16). Catecholamines have also been shown to enhance growth of pathogenic bacteria on serum (1, 9, 19, 31) but empirical data linking this phenomenon to virulence is lacking. Studies

on the stimulatory effects of catecholamines toward the growth of staphylococci on transferrin or in serum have described a positive response by *S. epidermidis* (16, 22, 31) but no response or a marginally positive response by *S. aureus* (22, 26, 31).

In Chapter 2, the ability of *S. aureus* to obtain iron from bulk serum and from the serum iron sequestering protein transferrin was demonstrated. This mode of growth was dependent on production of at least one of two staphyloferrins: siderophore molecules that are assembled from amino acid and organic acid precursors. Surveys of culture collections have thus far demonstrated that staphylococcal isolates do not produce two other classes of siderophore molecules, namely hydroxamates and catecholates (5, 7, 18). In Chapter 2, the ability of *S. aureus* to capture xenosiderophores (siderophores produced by other organisms) was also introduced, in the context of FhuC, the ATPase required by ABC transporters HtsABC and SirABC for active uptake of SA and SB, respectively. The original characterization of FhuC, however, pertained to its role in driving the FhuDBG ABC transporter, required for growth of *S. aureus* on hydroxamate-coordinating iron ligands, such as the fungal siderophore ferrichrome (32, 35). It is also known that *S. aureus* uses catecholate siderophores, such as enterobactin, for growth under iron restriction (32), through an undescribed transport system.

Bioinformatic analysis of the *S. aureus* genome has uncovered operon *sstABCD* (Figure 3-1), encoding a putative ABC transporter sharing sequence identity with bacterial transport systems for various iron ligands, including catecholate siderophores. SstD is a member of the class III substrate binding

Figure 3-1: The *S. aureus sstABCD* operon. Schematic of the *sstABCD* operon, encoding a predicted heterodimeric permease (SstAB), a predicted ATPase (SstC), and a lipoprotein receptor (SstD). Numbers refer to locus tags within the *S. aureus* strain Newman genome (*e.g.* NWMN_0702).


sstA sstB sstC sstD
0702 0703 0704 0705
permease
receptor
ATPase
1000 bp

protein family, a family which includes the *S. aureus* Fe^{3+}-staphyloferrin receptors HtsA and SirA, and shares significant sequence identity ($E = 9e^{-59}$) with the *Bacillus subtilis* petrobactin receptor YclQ (39). The *sst* operon is transcriptionally upregulated under conditions of iron deprivation (21), suggesting a role in the adaptive response to restrictive environments such as serum; the same study, however, did not identify any ligands for receptor lipoprotein SstD, and genomic inactivation of the transporter did not compromise virulence in a rat chamber implant model of infection. In this study, it was shown that *S. aureus* is in fact capable of catecholamine-mediated acquisition of serum iron, but this phenomenon is secondary to staphyloferrin-mediated iron uptake *in vitro* and can only be observed in the absence of siderophore production using *sfa*/*sbn* mutants. Mutants lacking Sst were unable to grow on xenosiderophores using catechol iron coordinating groups exclusively, including enterobactin, suggesting Sst is a broad specificity transporter of catecholate iron-coordination ligands. Inactivation of *sstABCD* enhanced the attenuation phenotype of siderophore biosynthesis and transporter mutants in the mouse sytemic model of infection, suggesting the ability of *S. aureus* to acquire iron from host serum is the sum of multiple mechanisms and not exclusively limited to staphyloferrin-mediated uptake.

3.2 Materials and methods

3.2.1 Bacterial growth conditions

Bacterial strains include those summarized in Table 2-1 and additional strains summarized in Table 3-1. Growth conditions, including temperature,

composition of media, and selective concentrations of antibiotics used were as described in Section 2.2.1. An additional growth medium used consisted of 80% C-TMS with 20% human serum (HuS-TMS). Human serum was collected from healthy volunteers without use of heparin sulfate; serum was separated from blood cells immediately upon collection by centrifugation at 2000 X *g* for 20 min at 4 ^{o}C, and complement was deactivated through incubation at 55 ^{o}C for 2 h.

3.2.2 General molecular genetic methodologies

DNA purification, manipulation, and recombination was performed essentially as described in Sections 2.2.2 and 2.2.3, with additions/modifications described below.

3.2.3 Mutagenesis of *S. aureus*

Oligonucleotides and plasmids strains used in this study are summarized in Tables 2-1 and 3-1. Allelic replacement was used to delete the majority of operon *sstABCD* from the chromosome of *S. aureus*. The operon and flanking regions were PCR amplified from strain RN6390 and cloned into plasmid pBAD24 using restriction enzymes *Sal*I and *Xba*I. The majority of the operon was excised via *Pvu*II digestion. A Klenow-blunted *Bam*HI restriction fragment containing an erythromycin resistance cassette was prepared from plasmid pDG646 and cloned into the *Pvu*II restriction site. The Δ*sstABCD*::Em knockout allele was excised using restriction enzymes *Sal*I and *Xba*I, and cloned to vector pAUL-A-Km, generating pSB10. Subsequent passage through *S. aureus*

Table 3-1: Bacterial strains, plasmids, and oligonucleotides used in this study

	Description	**Ref.**
***S. aureus* strains**		
H1861	RN6390 Δ*sstABCD*::Em	This study
H2221	Newman Δ*sstABCD*::Em	This study
H2224	Newman *sirA*::Km Δ*htsABC*::Tc Δ*sstABCD*::Em	This study
H2228	Newman Δ*sbnABCDEFGHI*::Tc Δ*sfaABCsfaD*::Km Δ*sstABCD*::Em	This study
***Bacillus subtilis* strains**		
HB5800	Bacillibactin-producing strain	(27)
***Escherichia coli* strains**		
BL21λ(DE3)	F^- *omp*T *hsd*S_B(r_B^-, m_B^-) *dcm gal* λ(DE3)	Novagen
AN102	Enterobactin-producing strain	(6)
Plasmids		
pBAD24	*E. coli* cloning vector; Ap^R	(15)
pDG646	*E. coli* vector containing the *ermC* gene; Ap^R	(14)
pGEX-2T-TEV	*E. coli* vector for overexpression of recombinant proteins with tobacco etch virus protease cleavable glutathione-S-transferase (GST) tags; Ap^R	(33)
pJB1	pGEX-2T-TEV derivative for overexpression of SstD with a TEV-cleavable GST; Ap^R	This study
pSB5	pLI50 derivative carrying *sstABCD*; Cm^R	This study
pSB10	pAUL-A derviative carrying ΔsstABCD::Em allele; Em^R	(3)

Table 3-1 continued

Oligonucleotides*	
*sst*5'-*Sal*I	5' AAAAGTCGACGGAATCACTGAAGATGTG 3'
*sst*3'-*Xba*I-1	5' GGGGTCTAGAGGTGAACATCCAAAGGAATCGTA 3'
	Generation of Δ*sstABCD*::Em allele
*sst*3'-*Xba*I-2	5' CCCCTCTAGACAATGATTAAGACCTTTAACCAT 3'
	Cloning of *sstABCD* to pLI50 for complementation (with *sst*5'-*Sal*I)
sstD-28/343-5'-*Bam*HI	5' TTGGATCCCAATCAAAATCAGAAACTAAAGG 3'
sstD-28/343-3'	5' CCTTTAACCATTGTTCCCCTCTTT 3'
	Cloning of soluble portion of *sstD* (codons 28-343) to pGEX-2T-TEV for overexpression

* underlined sequences in oligonucleotides denote restriction sites

strains, recombination into the chromosome with colour-based selection, and transduction between strains was performed as described in Section 2.2.3.

3.2.4 Complementation of the Δ*sst* mutation

The chromosomal Δ*sstABCD*::Km mutation was complemented *in trans* using plasmid pSB5, which was generated by cloning PCR-amplified, *Sal*I/*Xba*I-digested *sstABCD* into plasmid pLI50.

3.2.5 Bacterial growth curves and plate bioassays

For generation of growth curves, *S. aureus* cells grown to stationary phase in TMS with 100 µM 2,2-dipyridyl were washed twice in saline buffer and diluted 1:100 into HuS-TMS or C-TMS containing 10 µM human transferrin. Culture medium was left iron depleted or enriched with 100 µM $FeCl_3$ (iron replete). Other culture medium amendments included 50 to 200 µM catecholamine hormones (norepinephrine, epinephrine, dopamine, or L-DOPA) (Sigma-Aldrich). Norepinephrine and dopamine were purchased as hydrochlorides and dissolved in water to generate stock solutions; dopamine and L-DOPA were dissolved in equimolar and twice equimolar concentrations of HCl, respectively.

Disc diffusion plate availability bioassays were performed as described in Section 2.2.11. Discs were spotted with 20 µL of 750 µM stocks of catecholamines (described above) or 2,3-dihydroxybenzoic acid (Sigma-Aldrich), or 250 µM stocks of the following catecholate siderophores: enterobactin-Fe, petrobactin-Fe, salmochelin S-4-Fe (EMC Microcollections

GmbH), or bacillibactin (described below). All ligands were ferrated with 50 µM $FeCl_3$ if not described as ferrated by the vendor.

Bacillibactin was purified from *B. subtilis* strain HB5800 as described (11), with modifications. Briefly, *B. subtilis* was grown in enterobacterial minimal culture medium (23) with modifications as described (4) for 48 h. Cells were removed by centrifugation and culture supernatant was acidified to pH 3 with HCl then extracted three times with 200 mL volumes of ethyl acetate. Pooled ethyl acetate fractions were dried over $NaSO_4$, filtered, and dried in a rotary evaporator. Residue was dissolved in 1 mL methanol and added dropwise into 50 mL stirred ether. Precipitate was pelleted by centrifugation, air dried, and resuspended in dimethyl sulfoxide. Bacillibactin concentration was calculated by measuring absorbance at 318 nm as described (24).

3.2.6 Generation of anti-SstD antiserum

The soluble portion of the *sstD* gene (downstream of the lipoprotein consensus sequence) was PCR-amplified and cloned into plasmid pGEX-2T-TEV using restriction sites *Bam*HI and *Sma*I, generating plasmid pJB1 in *E. coli* strain BL21λ(DE3). For overexpression of SstD-GST, *E. coli* cells were grown at 30 °C to mid log phase, induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside, and grown for another 16 h. Cells were collected by centrifugation and ruptured using a French press. Insolubles and cellular debris were removed following centrifugation at 5000 x *g* for 15 min then at 164000 x *g* for 60 min. Filtered supernatant was passaged over a 5 mL GSTrap FF column (GE Healthcare) and eluted in buffer containing 50 mM Tris and

10 mM reduced glutathione, pH 8.0. SstD-GST was digested with recombinant hexahistidine tagged TEV protease (home brewed) overnight at 4 °C. GST and uncleaved SstD-GST were removed with a second passage over the GSTrap FF column and TEV protease was removed by passaging over a 1 mL HisTrap column (GE Healthcare). SstD concentration was calculated using a Bio-Rad protein assay as instructed (Bio-Rad).

Polyclonal antibodies against soluble SstD were generated in New Zealand white rabbits by ProSci Inc. using custom antibody production protocol 1.

3.2.7 Immunoblot analysis of whole cell lysates

S. aureus strains precultured as described in Section 3.2.5 were grown in HuS-TMS to mid-log phase. Cells from approximately 1.5 mL of culture were pelleted by centrifugation and incubated for 30 min at 37 ^{o}C in 100 µL cell wall digestion buffer containing 0.3 g/L raffinose, 50 µM Tris-Cl (pH 7.5), 145 mM NaCl, 5 mM iodoacetamide, 0.1 mM phenylmethanesulfonylfluoride, and 1 µg lysostaphin (Sigma-Aldrich). Sample total protein concentrations were calculated using the Bio-Rad protein assay following the manufacturer's instructions (Bio-Rad). Samples were boiled for 10 min, and sample volumes normalized to contain 10 µg total protein were resolved through SDS-polyacrylamide gel electrophoresis (12% acrylamide resolving gel) then transferred to 45 µm nitrocellulose membrane, according to standard protocols (30). Detection of SstD on nitrocellulose was performed after the following steps: 12 h of blocking at 4 ^{o}C in phosphate-buffered saline (PBS) containing 20% horse serum (Sigma-Aldrich) and 10% w/v skim milk; 2 h exposure to the

primary antibody at room temperature in PBS containing 0.05% Tween-20 and 2% w/v skim milk (1:7500 dilution of rabbit antiserum); 1 h exposure to secondary α-rabbit IgG antibody conjugated to IRDye-800 (LI-COR Biosciences) at room temperature in PBS/0.05% Tween/2% skim milk (1:10000 dilution of antibody). Immunoblot fluorescence was imaged using a LI-COR Odyssey Infrared Imager (LI-COR Biosciences).

3.2.8 SstD ligand binding assays

SstD was adjusted to 1 μM in 100 mM NaCl, 10 mM Tris pH 8.0. Equimolar bovine serum albumin (Sigma Aldrich) was used as a protein negative control. Ligand stocks were added in twofold concentration increments ranging between 0 and 40 μM. Ligands included enterobactin, salmochelin, petrobactin, bacillibactin, 2,3-DHBA, norepinephrine, epinephrine, dopamine, L-DOPA and Desferal^{TM}. For ferration, ligands were incubated for 5 min at room temperature with $FeCl_3$ at a ratio of 1:3 (Fe:catecholamines) or 1:1 (Fe:siderophores). Ligand affinity was measured at room temperature using intrinsic tryptophan fluorescence quenching in a Cary Eclipse (Agilent Technologies). Excitation was performed at 280 nm and fluorescence was detected at 345 nm using a excitation slit width of 5 nm and emission slit width of 5 nm. For ferrated catechol siderophores, fluorescence data were corrected for nonspecific tryptophan quenching by ligands after analogous titration with 1 μM *N*-acetyl-tryptophanamide, as described (37). The volume of starting protein solutions were 500 μL and data were corrected for changes in

fluorescence due to changes in sample volume due to ligand additions. Fluorescence data were fitted to nonlinear regression analysis using a one-site binding model and data were analyzed using Microsoft Excel.

3.2.9 Mouse models of *S. aureus* infection

Mouse infection experiments were performed using Charles River or Jackson Laboratories BALB/c mice as described in Section 2.2.12. Additionally, an infection study was performed on mice carrying surgically implanted norepinephrine dispensers to evaluate the contribution that elevated circulatory catecholamine levels make on the progression of staphylococcal disease. 48 h prior to infection, mice purchased from Charles River were anaesthetized with isofluorane gas and administered with 1 mg/kg of the analgesic meloxicam (Boehringer Ingelheim) in 400 µL saline solution via intraperitoneal injection. The right flank of each mouse was shaved and disinfected with a routine three step scrub. A dorsoventral incision was made near the shoulder and a region of skin orienting toward the hips was undermined. Alzet 2001 osmotic pumps (Durect Corp., dispensing rate of 1 µL/h) loaded with 1 mg/mL epinephrine in 0.8% buffered saline, pH 4 (Bioniche Life Sciences, Inc.), were inserted into the fold. Drug dosage and osmotic pump model were selected based on a previous report (36). pH-adjusted sterile saline-loaded pumps were inserted for mice in a drug-free control group. Incisions were closed with sutures. 24 h prior to infection, mice were administered a second dose of meloxicam. Mice were monitored daily for symptoms of adverse reactions to the surgical procedure, prior to and during the staphylococcal infection trial; none were noted.

3.3 Results

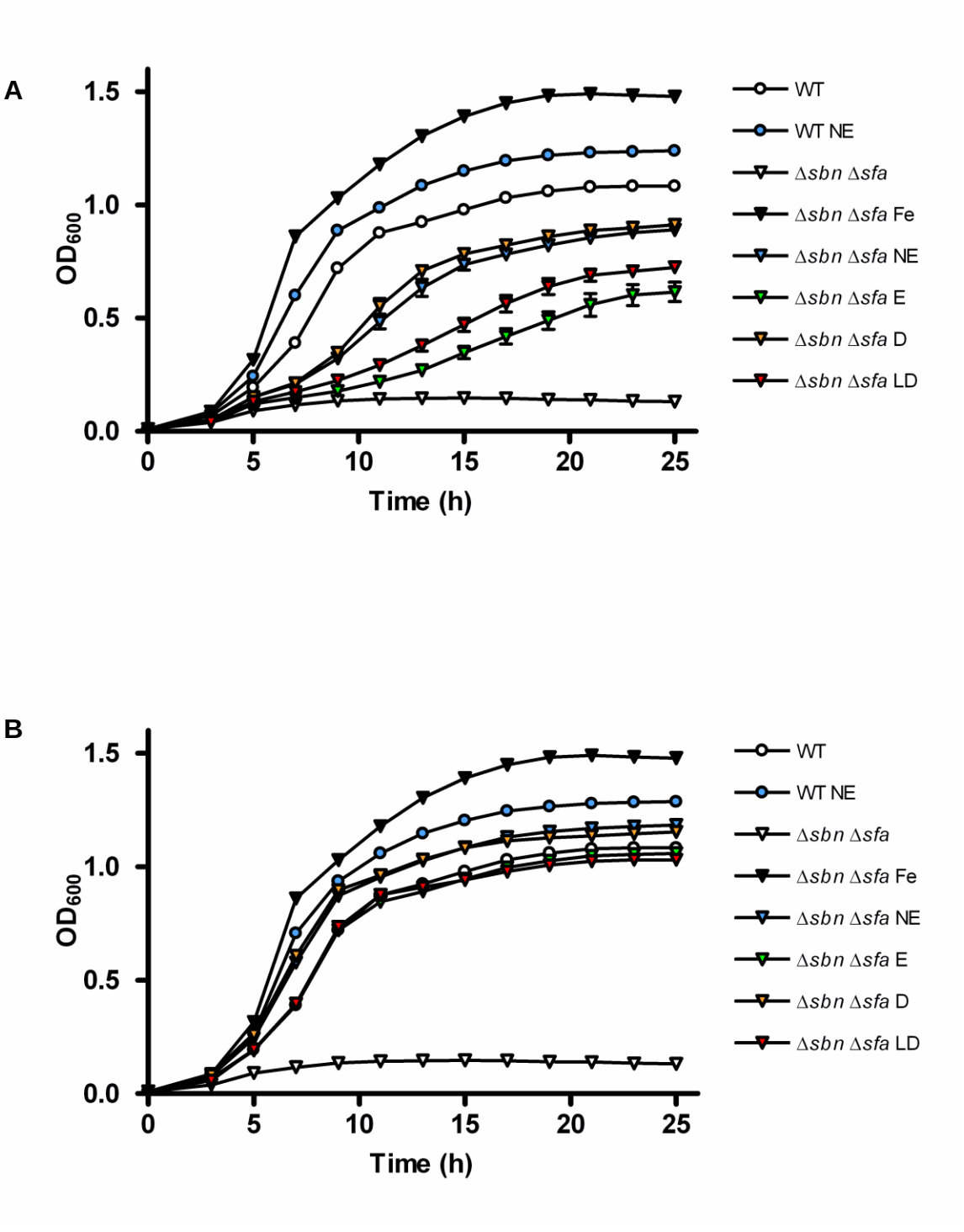
3.3.1 Catecholamine stress hormones promote the growth of *S. aureus* in serum and on transferrin in the absence of siderophore production

Catecholamines stimulate the growth of *S. epidermidis* on transferrin (17, 22), although a similar effect was not noted for *S. aureus* (22, 26, 31). In this investigation, four catecholamine hormones, including norepinephrine, epinephrine, dopamine, and L-DOPA, did not enhance growth of wild type *S. aureus* on human serum or human holotransferrin when added at a concentration of 50 µM. They did, however, partially restore the growth of the siderophore-deficient strain at the same concentration (Figures 3-2A, 3-3). Serum growth promotion up to levels conferred by siderophore production could be achieved at catecholamine concentrations of 200 µM (Figure 3-2B). Catecholamine-stimulated growth promotion in basal culture medium was negligible in the absence of holo-transferrin or whole serum, ruling out the possibility that the commercially obtained molecules were preloaded with iron (data not shown).

3.3.2 Uptake of catecholamine-liberated serum iron occurs through transporter SstABCD

The proposed model for the 3:1 molar complexation of a catecholamine hormone with Fe^{3+} in solution resembles the mechanism by which the three coordinating moieties of bacterial catechol siderophores orient around a central Fe^{3+} atom (31). Following the observation that catecholamine hormones stimulate growth in serum in the absence of siderophores, it was of interest to

Figure 3-2: Catecholamine stress hormones enhance the growth of siderophore-deficient *S. aureus* on human serum. Wild type *S. aureus* strain Newman (WT) and its siderophore nonproducing isogenic mutant (Δ*sbn* Δ*sfa*) were grown in HuS-TMS. Catecholamine stress hormones (NE = norepinephrine; E = epinephrine; D = dopamine; LD = L-DOPA) were added to concentrations of 50 **(A)** or 200 µM **(B)**. $FeCl_3$ (Fe) was added to a concentration of 100 µM. Hormones only marginally improved the growth of the wild type strain (NE used as a representative curve). Error bars represent standard deviation from the mean (n = 3).


A
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Time (h)
WT
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Δsbn Δsfa Fe
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Δsbn Δsfa LD
B
1.5
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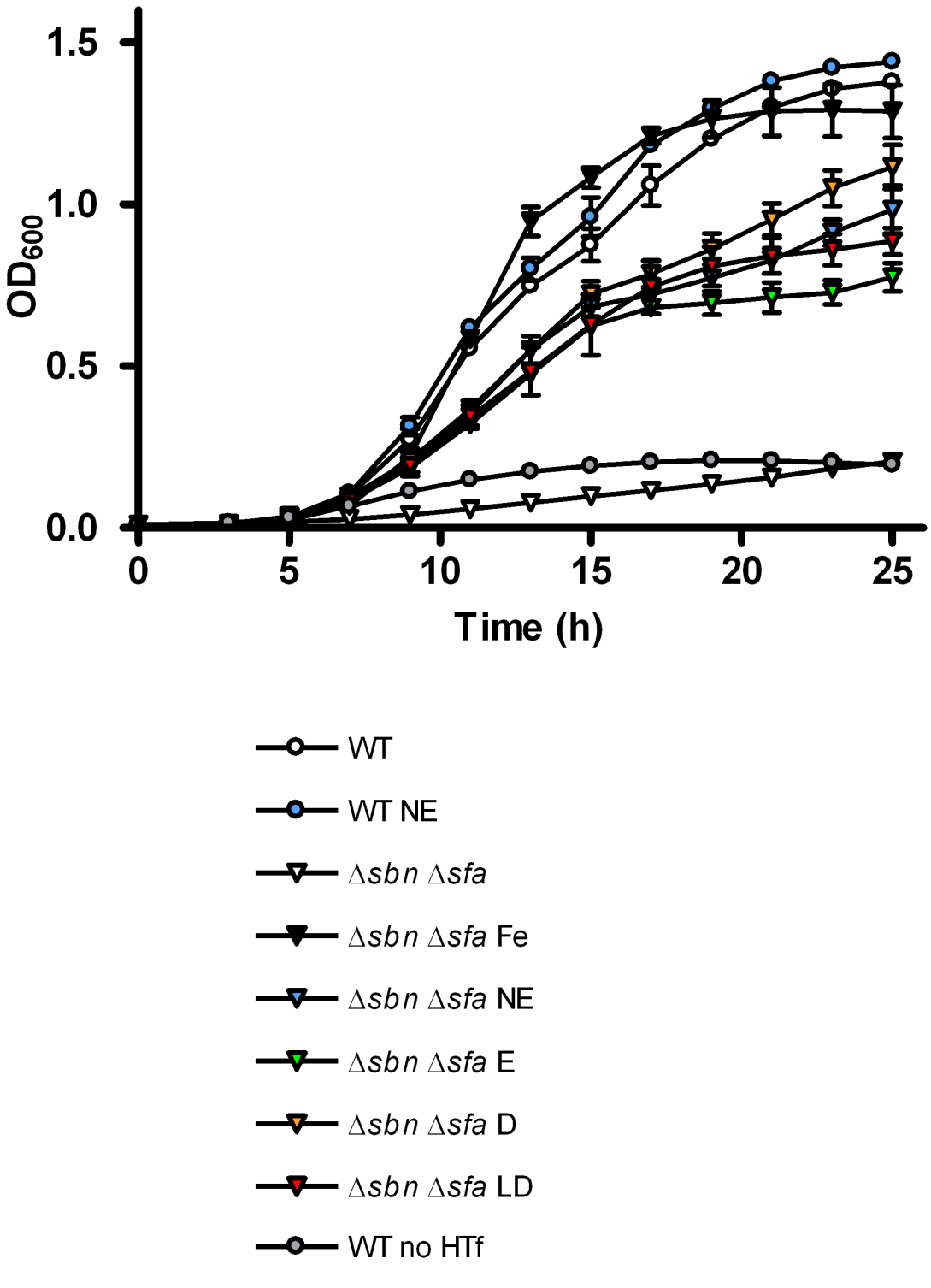
Time (h)
WT
WT NE
Δsbn Δsfa
Δsbn Δsfa Fe
Δsbn Δsfa NE
Δsbn Δsfa E
Δsbn Δsfa D
Δsbn Δsfa LD

Figure 3-3: Catecholamine stress hormones enhance the growth of siderophore-deficient *S. aureus* on human transferrin. Wild type *S. aureus* strain Newman (WT) and its siderophore nonproducing isogenic mutant (Δ*sbn* Δ*sfa*) were grown in C-TMS with 10 µM human holo-transferrin (~60% iron-saturated). Catecholamine stress hormones (NE = norepinephrine; E = epinephrine; D = dopamine; LD = L-DOPA) were added to concentrations of 50 µM. $FeCl_3$ (Fe) was added to a concentration of 100 µM. Hormones only marginally improved the growth of the wild type strain (NE used as a representative curve). Error bars represent standard deviation from the mean (n = 3).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Δsbn Δsfa Fe
Δsbn Δsfa NE
Δsbn Δsfa E
Δsbn Δsfa D
Δsbn Δsfa LD
WT no HTf

determine what transport system might be involved in catecholamine iron uptake. Based on the shared sequence identity between the components of SstABCD and other bacterial iron transporters, and a report describing its genes to be iron regulated (21), the *sst* operon was chosen for chromosomal deletion through allelic replacement. With production of staphyloferrins, a Δ*sstABCD* mutation did not have adverse effects on growth in human serum compared to an isogenic wild type parental strain (Figure 3-4A). The mutation, however, rendered a siderophore deficient mutant insensitive to the growth promoting effects of catecholamines, at concentrations as high as 200 µM. Similar defects were observed on human transferrin as a sole iron source (data not shown). The mutant phenotype was fully complemented by expression of wild type *sstABCD in trans* (Figure 3-4B).

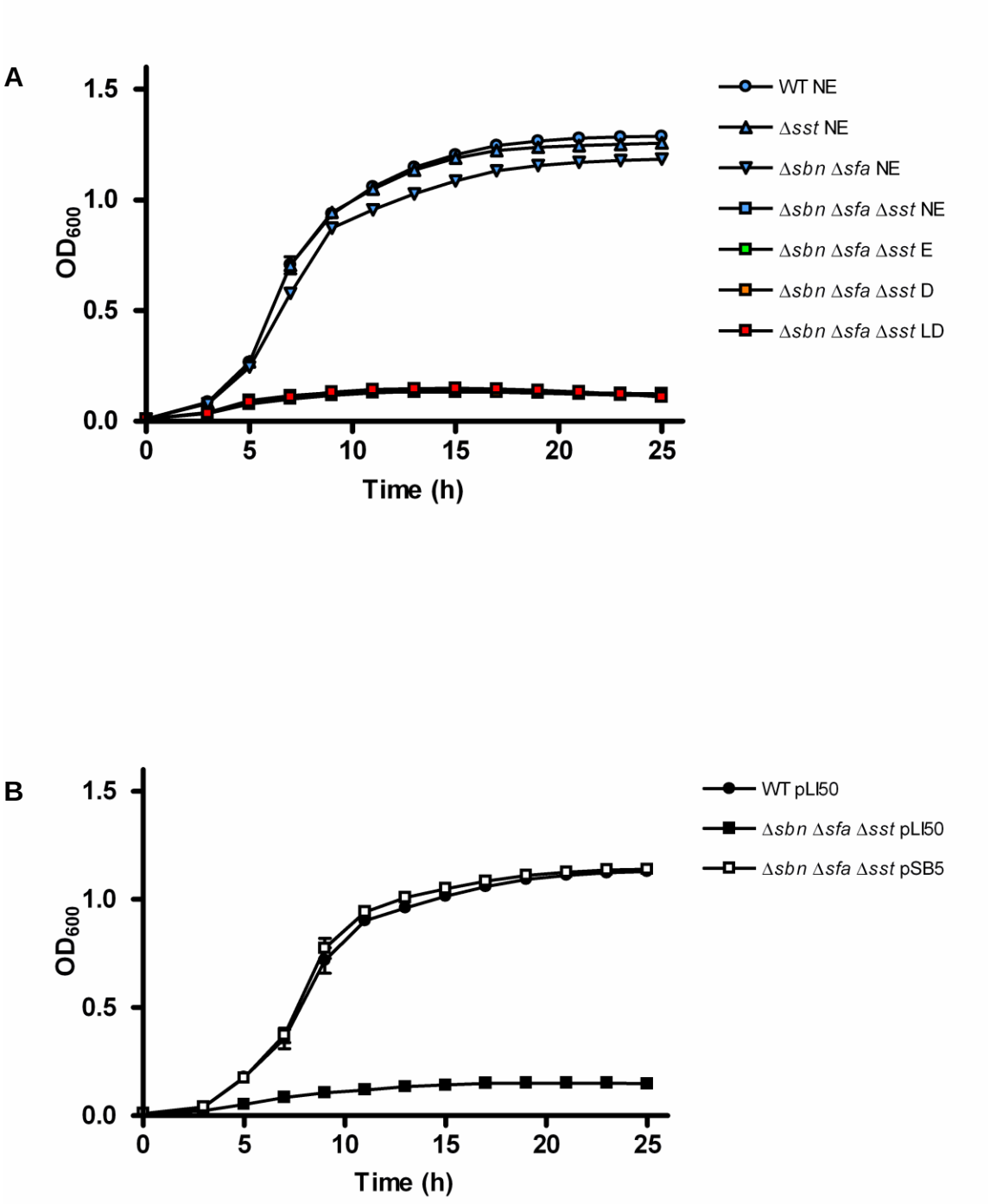
3.3.3 SstD is expressed by laboratory and clinical strains of *S. aureus*

Western blots against whole cell lysates using polyclonal SstD antiserum confirmed a lack of detectable SstD expression in the Newman Δ*sstABCD* mutant strain (Figure 3-5A). Conserved expression of SstD through a range of commonly used laboratory and clinical strains, including three community-acquired strains, was also noted (Figure 3-5C).

3.3.4 SstD binds a range of catechol-coordinating Fe^{3+} ligands

Fluorescence quenching assays were used to measure the affinity of purified SstD for catecholamines and catechol siderophores. Titration with iron-free hormone or siderophore ligands showed no quenching of the

Figure 3-4: SstABCD is required for catecholamine-mediated growth on serum. (A) Catecholamine stress hormone-mediated growth promotion of a siderophore nonproducing mutant of *S. aureus* strain Newman (Δ*sbn* Δ*sfa*) was negated by concomitant mutagenesis of *sstABCD* (Δ*sbn* Δ*sfa* Δ*sst*). Strains were grown in HuS-TMS. Catecholamine stress hormones (NE = norepinephrine; E = epinephrine; D = dopamine; LD = L-DOPA) were added to concentrations of 200 µM. $FeCl_3$ (Fe) was added to a concentration of 100 µM. **(B)** Expression of *sstABCD* from vector pSB5 restored growth with 200 µM norepinephrine in HuS-TMS. Error bars represent standard deviation from the mean (n = 3).


A
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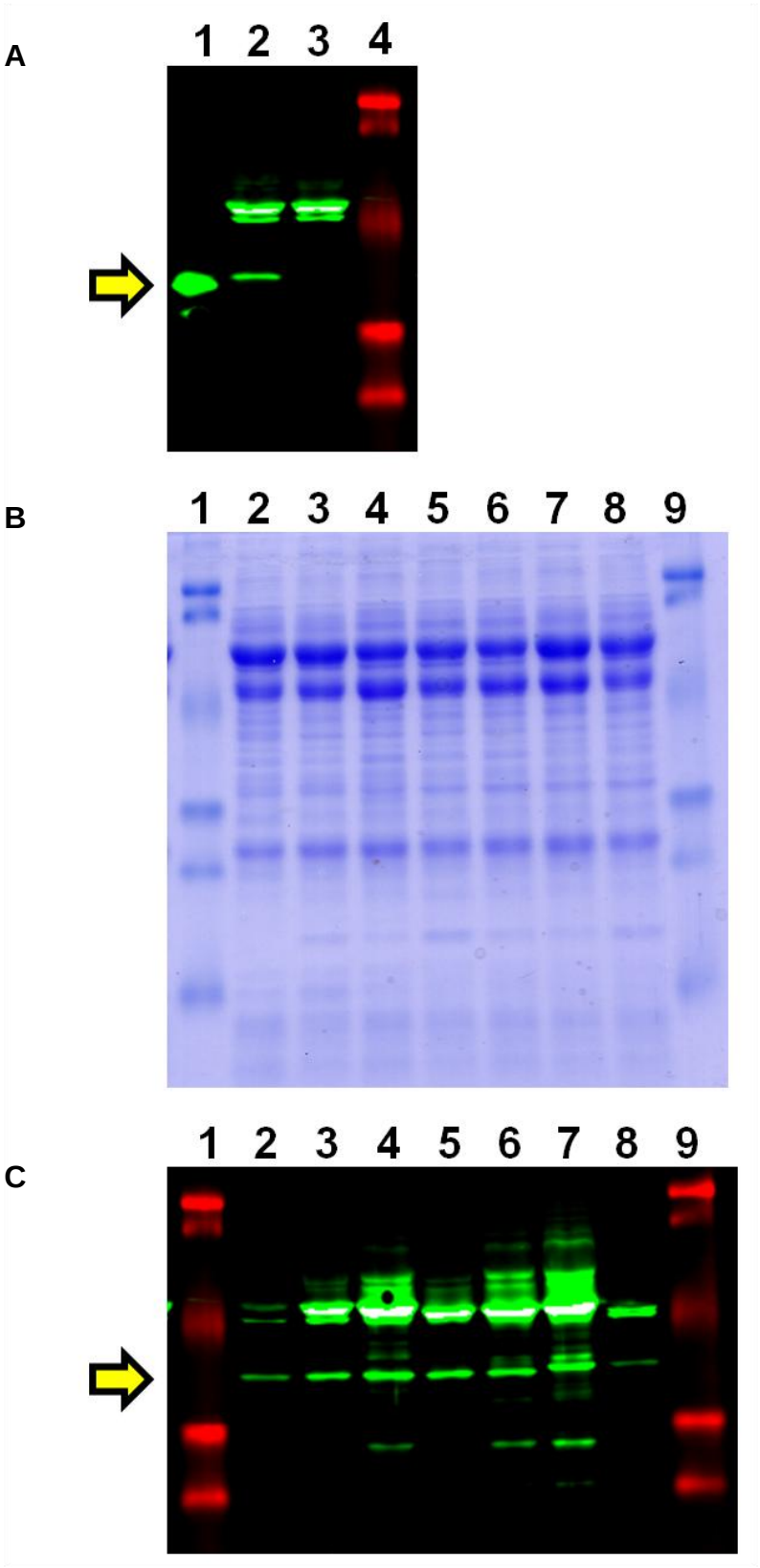
Time (h)
WT NE
Δsst NE
Δsbn Δsfa NE
Δsbn Δsfa Δsst NE
Δsbn Δsfa Δsst E
Δsbn Δsfa Δsst D
Δsbn Δsfa Δsst LD
B
1.5
1.0
0.5
0.0
OD600
0
5
10
15
20
25
Time (h)
WT pLI50
Δsbn Δsfa Δsst pLI50
Δsbn Δsfa Δsst pSB5

Figure 3-5: Detection of SstD in *S. aureus* whole cell lysates using polyclonal antiserum. (A) Polyclonal anti-SstD rabbit antibodies were used to confirm mutagenesis of the *sst* operon in *S. aureus* strain Newman in a Western blot. 1 = 1 µg purified SstD (soluble portion); 2 = wild type Newman; 3 = Newman Δ*sst* mutant; 4 = SDS-PAGE low molecular weight ladder. **(B)**, **(C)** A selection of *S. aureus* strains' whole cell lysates normalized to total protein content, confirmed with a Coommassie stain of a SDS-PAGE gel **(B)**, were probed with anti-SstD serum in a Western blot **(C)**. 1, 9 = SDS-PAGE low molecular weight ladder; 2 = RN6390; 3 = Newman; 4 = USA300 LAC; 5 = MW2; 6 = MSSA476; 7 = UAMS-1; 8 = AD25. The yellow arrows denote which band corresponds to SstD.


A
1 2 3 4
B
1 2 3 4 5 6 7 8 9
C
1 2 3 4 5 6 7 8 9

tryptophan/tyrosine fluorescence of SstD (data not shown). The fluorescence of bovine serum albumin, a protein negative control, was not quenched with any of the ligands tested (data not shown). SstD fluorescence was quenched by all four ferrated catecholamines tested ferrated catechol siderophores. Dissociation constants are reported in Table 3-2.

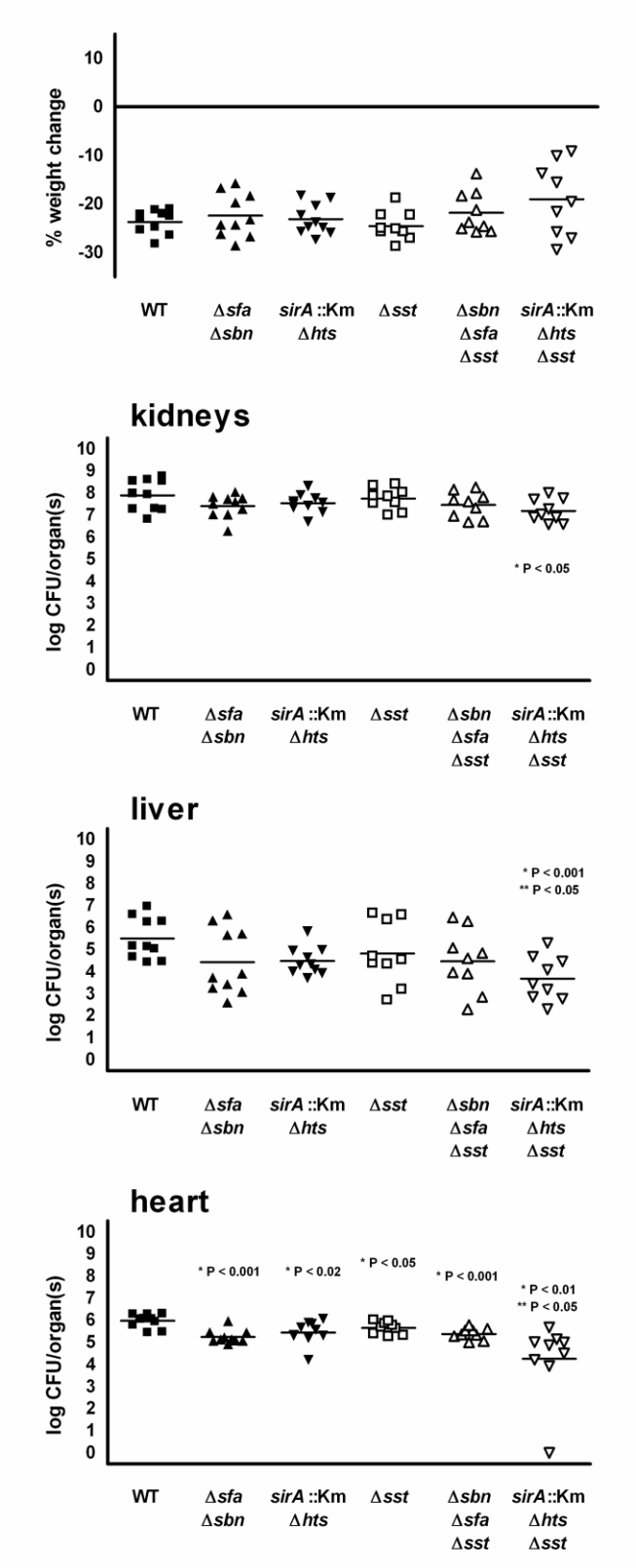
3.3.5 SstABCD contributes to virulence

A mouse systemic model of infection was used to evaluate the relative and combined contributions of siderophore biosynthesis, siderophore transport, and catecholamine iron acquisition genes *in vivo*. Mice were infected intravenously with approximately 5 to 10 x 10^6 bacteria and bacterial loads in target organs were enumerated 96 h postinjection. Trials were performed using both Charles River and Jackson Laboratories mice. Bacterial processes disrupted via mutation included catecholamine iron uptake (*sst*), siderophore biosynthesis (*sbn sfa*), and siderophore uptake (*sirA hts*). Furthermore, the effect of combined mutations for catechol iron uptake with staphyloferrin biosynthesis (*sbn sfa sst*) or staphyloferrin uptake (*sirA hts sst*) were tested. Disruption of any single process resulted in a mild but significant reduction in heart colonization in Charles River mice, and combined inactivation of all three transport systems resulted in attenuation phenotypes in kidneys, livers, and hearts for both Charles River and Jackson Laboratories mice (Figure 3-6, Appendix Figure A2).

Table 3-2: Dissociation constants (K_d) for SstD-ferric-catecholamine and SstD-ferric-catechol complexes

Ligand	K_d (μM)	Standard deviation
Norepinephrine	1.07	0.54
Epinephrine	1.65	0.08
Dopamine	0.49	0.22
L-DOPA	1.44	0.27
Bacillibactin	1.21	0.14
2,3-DHBA	1.62	0.27
Enterobactin	0.29	0.06
Petrobactin	1.62	0.25
Salmochelin S-4	0.35	0.20

Figure 3-6: Inactivation of siderophore and catecholamine transport attenuates *S. aureus* Newman in a mouse systemic model of infection. Approximately 5 to 10 x 10^6 CFU *S. aureus* Newman and isogenic iron transport mutant derivatives were injected intravenously to Charles River BALB/c immunocompetent mice. Percentage of mouse body mass lost over four days and CFU recovered from target organs are plotted. Statistical analysis was performed using the Student's unpaired *t*-test. *P* values denote significant differences from wild type (WT) (*) or from an isogenic siderophore biosynthesis/transport mutant parent (**). Infective doses were as follows (in millions of CFU/dose): 7.00 (WT), 5.50 (Δ*sbn* Δ*sfa*), 5.75 (*sirA*::Km Δ*hts*), 5.00 (Δ*sst*), 6.00 (Δ*sbn* Δ*sfa* Δ*sst*), 6.50 (*sirA*::Km Δ*hts* Δ*sst*).


% weight change
10
0
-10
-20
-30
WT
Δsfa Δsbn
sirA ::Km Δhts
Δsst
Δsbn Δsfa Δsst
sirA::Km Δhts Δsst
kidneys
log CFU/organ(s)
* P < 0.05
liver
* P < 0.001
** P < 0.05
heart
* P < 0.001
* P < 0.02
* P < 0.05
* P < 0.001
* P < 0.01
** P < 0.05

3.3.6 Augmentation of circulating epinephrine levels does not render mice more susceptible to *S. aureus* infection

Mice implanted with steady dose epinephrine dispensers were evaluated for enhanced susceptibility to *S. aureus* infection. In a pilot trial, it was observed that mice receiving surgical implants exhibited reduced external symptoms of infection compared to a surgery-free control group (less pronounced weight loss, fewer signs of septic arthritis, and generally healthy grooming and activity patterns), despite mice having been administered a typically infective dose of bacteria (~5.0 x 10^6 CFU). *Post mortem* analysis described atypically low bacterial colonization in target organs for implant-carrying mice (data not shown). It was thus hypothesized that the surgical procedure elicited an immunostimulatory change in mice prior to infection, so subsequent experiments were performed using a higher infective dose (~1.0 x 10^7 CFU).

In the first experiment, mice carrying epinephrine-dispensing pumps proved no more susceptible to infection with wild type *S. aureus* strain Newman than mice implanted with saline pumps (Figure 3-7). Both surgical groups exhibited decreased amounts of organ colonization than surgery-free control mice, although data did not achieve statistical significance; surgery-free mice, however, did experience significantly more weight loss, in agreement with the pilot study.

In the second experiment, mice carrying epinephrine-dispensing pumps proved no more susceptible to infection with wild type *S. aureus* Newman than its isogenic Δ*sst* mutant derivative (Figure 3-8). In agreement with data presented in Chapter 2, mice experienced reduced rates of colonization in the

Figure 3-7: Subcutaneous epinephrine pumps alter mouse immunity but epinephrine does not enhance virulence of *S. aureus*. Charles River BALB/c immunocompetent mice were fitted with subcutaneously implanted osmotic pumps dispensing 1 µL/h 1 mg/mL epinephrine (epi) or buffered saline (saline), or were not surgically treated (none). Weight loss and organ bacterial loads were calculated four days postinfection with *S. aureus* Newman (intravenous infective dose of 4×10^6 CFU). Statistical analysis was performed using the Student's unpaired *t*-test. *P* values denote significant differences from epinephrine (*) or from saline treatment (**).

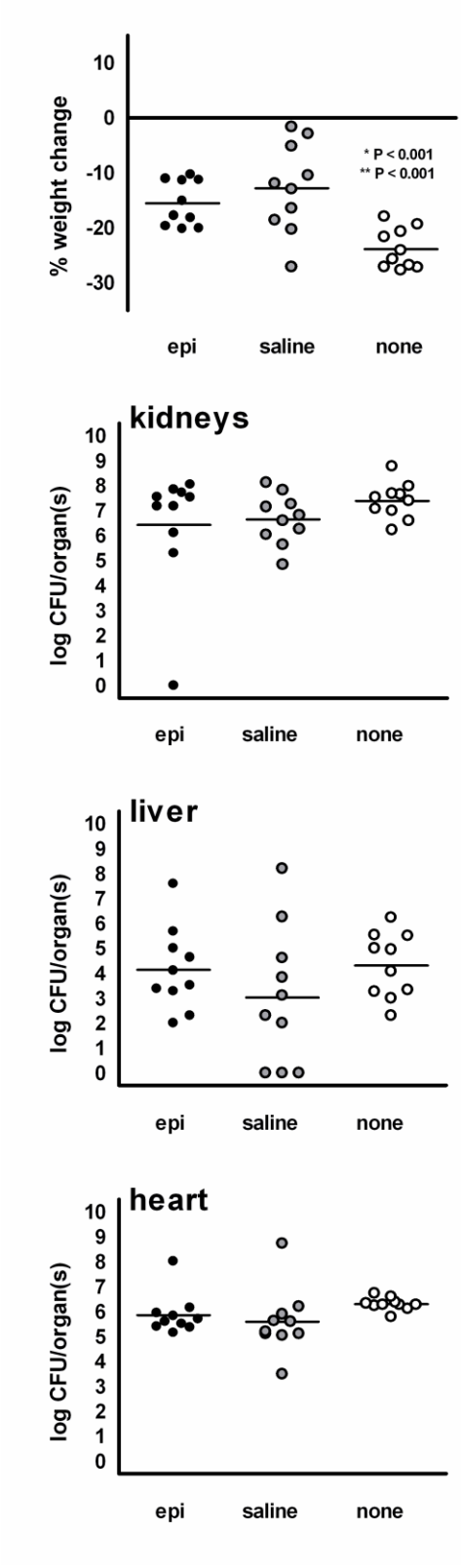
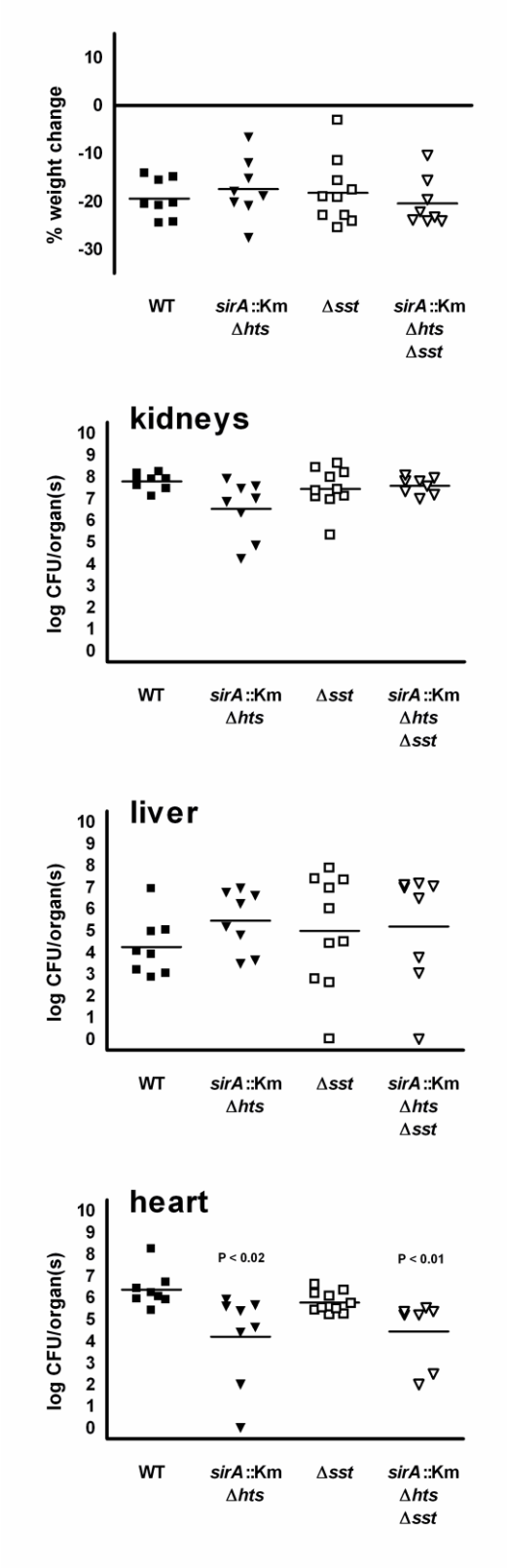

10
0
-10
-20
-30
% weight change
* P < 0.001
** P < 0.001
epi
saline
none
kidneys
log CFU/organ(s)
liver
heart

Figure 3-8: Subcutaneous epinephrine pumps do not enhance virulence of *S. aureus* expressing SstABCD. Mice were fitted with subcutaneously implanted osmotic pumps dispensing 1 µL/h 1 mg/mL epinephrine (epi). Weight loss and organ bacterial loads were calculated four days postinfection with *S. aureus* Newman. Intravenous infective doses were as follows (in millions of CFU/dose): 8.00 (WT), 8.50 (*sirA*::Km Δ*hts*), 9.00 (Δ*sst*), 9.75 (*sirA*::Km Δ*hts* Δ*sst*). Statistical analysis was performed using the Student's unpaired *t*-test. *P* values denote significant differences from wild type.

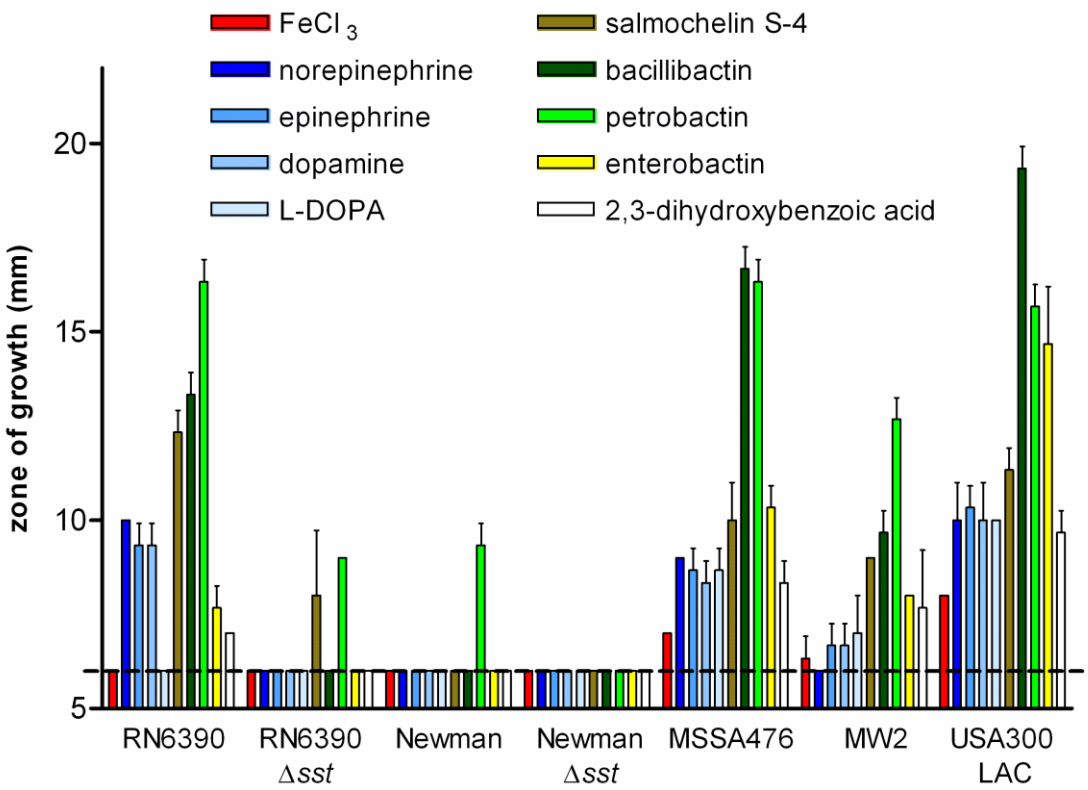

% weight change
10
0
-10
-20
-30
WT
sirA::Km Δhts
Δsst
sirA::Km Δhts Δsst
kidneys
log CFU/organ(s)
10
9
8
7
6
5
4
3
2
1
0
WT
sirA::Km Δhts
Δsst
sirA::Km Δhts Δsst
liver
log CFU/organ(s)
WT
sirA::Km Δhts
Δsst
sirA::Km Δhts Δsst
heart
log CFU/organ(s)
P < 0.02
P < 0.01
WT
sirA::Km Δhts
Δsst
sirA::Km Δhts Δsst

heart with *S. aureus sir*/*hts* mutants; this was not, however, enhanced by simultaneous inactivation of *sst*.

3.3.7 SstABCD contributes to growth on catecholate siderophores

It was previously reported that *S. aureus* strain RN6390 is capable of growing on ferric enterobactin, a tris-catecholate enteric siderophore, in a plate bioassay (32). Having established that SstABCD plays a role in uptake of catecholamine-liberated serum iron, it was of interest to investigate a possible role for the transporter in uptake of exogenous catechol siderophores. Curiously, when strain Newman and its isogenic Δ*sst* mutant derivative were examined for growth promotion on enterobactin, other catechol-coordinating siderophores, and catecholamines, following prescribed protocols (32), no enterobactin growth promotion was observed even for the wild type parent. The study was expanded to include RN6390, its Δ*sst* mutant, and the host of clinical isolates first mentioned in Chapter 2 (Tables 2-1, 3-1). Data are presented in Figure 3-9. Strains AD25 and UAMS-1 were discounted from the study as no amount of EDDHA chelation tested (up to 30 μM) was sufficient to eliminate substrate-independent background growth, making growth promotion around a disc unquantifiable; the mechanism for this phenomenon is unclear. Unlike for strain Newman, all isolates tested formed growth halos around three of the four catecholamines (L-DOPA excluded), and catecholate siderophores enterobactin, salmochelin S-4, bacillibactin, and petrobactin; Newman was responsive only to petrobactin, and as evidenced by the RN6390 Δ*sst* mutant, this siderophore is not exclusively reliant on SstABCD for transport. Strain Newman was

Figure 3-9: Plate bioassay growth promotion using ferrated catecholamines and catechol siderophores. A host of *S. aureus* strains seeded in TMS agar chelated with 10 µM EDDHA were evaluated for growth halo formation around a paper disc spotted with 20 µL ferrated catecholamine hormones (750 µM), 2,3-dihydroxybenzoic acid (750 µM), catechol siderophores (250 µM), or $FeCl_3$ (50 µM). Growth was measured after incubation at 37 ^{o}C for 36 h. Growth is represented as the diameter of the halo (in mm). The dashed line represents the diameter of the paper disc. Error bars represent standard deviation from the mean (n = 3).


FeCl 3
norepinephrine
epinephrine
dopamine
L-DOPA
salmochelin S-4
bacillibactin
petrobactin
enterobactin
2,3-dihydroxybenzoic acid
zone of growth (mm)
20
15
10
5
RN6390
RN6390 Δsst
Newman
Newman Δsst
MSSA476
MW2
USA300 LAC

subsequently demonstrated to be weakly responsive to enterobactin at millimolar concentrations (data not shown). $FeCl_3$ added at equimolar concentration was only weakly growth promoting, at best, for any strain examined.

3.4 Discussion

A key theme of the *S. aureus* iron uptake strategy is the blending of redundancy and complexity in targeting specific iron reservoirs. In the preceding chapter, the ability of *S. aureus* to capture iron from transferrin and bulk serum through two redundant siderophores, was demonstrated. Here, the molecular basis of an alternate mechanism for holotransferrin iron extraction has been elucidated. This second mechanism capitalizes on the liberation of holotransferrin iron following complexation with mammalian catecholamine stress hormones (31). Catecholamine iron was shown to promote the growth of *Bacillus* and *E. coli* on holotransferrin at concentrations comparable to those tested in this study (2, 20). Similar to this study, the role was characterized in strains mutated to inactivate production of the catecholate siderophores bacillibactin (*Bacillus*) and enterobactin (*E. coli*). Uptake was dependent on either organism's catechol siderophore ABC transporter. Due to the overshadowing contribution of staphyloferrins, characterizing the contribution of catecholamines toward iron uptake from human serum or transferrin was made possible only after constructing and characterizing two whole-locus siderophore biosynthesis knockout strains (Figures 3-1, 3-3). Catecholamine iron uptake was subsequently shown to be mediated by a distinct transporter, SstABCD

(Figures 3-2, 3-4), previously implicated in the *S. aureus* adaptive response to low iron environments (21).

A large reservoir of plasma catecholamines is found in the venous and arterial circuitry between mesenteric organs, kidneys, and the liver (8). While hormone concentrations tested in this study (50 to 200 µM) have been alluded to as physiologically or therapeutically relevant (10, 20), typical serum reference ranges are in the nanomolar range (28), and it is unlikely they approach micromolar concentrations in bulk plasma even after gastric surgery. In fact, a catecholamine concentration of 100 µM has been reported as neurotoxic *in vitro* (25). Nevertheless, in concert with siderophore activity, low concentrations of stress hormones may subvert the bacteriostatic effects of transferrin to promote sufficient proliferation in the bloodstream for evasion of phagocytic immune cells prior to colonization of organs. This highlights the necessity for precaution prior to therapeutic administration of catecholamines. Catecholamines might also be found in elevated levels in microenvironments surrounding wounds where nervous damage has occurred. Other research has noted increases in indigenous bacterial gut flora following localized destruction of noradrenergic neurons (16). In addition to providing a source of iron, these wounds provide an epithelial breach through which opportunistic bacteria may enter the bloodstream.

Previously, SstABCD was shown to make no significant contribution to bacterial survival in a rat intraperitoneal cage model of infection (21). In this study, *sst* inactivation significantly decreased colonization of the mouse heart or liver in two separate trials (Figure 3-6 A and B, respectively). This finding is

significant as *S. aureus* is a leading cause of infective endocarditis. Additionally, a mutant lacking both siderophore transport systems was consistently further attenuated in mouse kidneys, livers, and hearts by inactivation of *sst* (Figures 3-6A and B), demonstrating that highly coordinated extraction of iron from host sources other than hemoproteins contributes to the pathogenesis of *S. aureus*.

Elevation of a circulating catecholamine stress hormone, specifically epinephrine, did not render mice more susceptible to *S. aureus* infection (Figure 3-7), nor did it result in a difference in bacterial loads in mouse organs between wild type Newman and its isogenic *sst* mutant (Figure 3-8). These experiments proved to be extremely technically challenging and negative results could reflect inadequate protocol rather than a genuine lack of catecholamine-enhanced virulence. Owing to the extremely short half life of stress hormones in circulation (minutes) (34, 38), the only way to ensure steady dispensing of epinephrine within a mouse for the duration of the experiment (two days pre- and four days post-infection) was to use an implanted pump roughly equal in weight to 10% of the mouse's body mass. This invasive and intensive surgical procedure clearly affected the innate immunity of the mouse toward *S. aureus* by mechanisms unknown. While such a stress would intuitively render mice more susceptible to further medical insult, *i.e.* infection, the opposite was in fact observed; surgery enhanced the innate resistance of mice to *S. aureus* challenge, reflected in improved symptoms assessed externally and internally (Figures 3-6, 3-7). Future experiments to correlate increased levels of

circulating catecholamines to enhanced infectivity of *S. aureus* should involve larger animals with a more manageable body to implant mass ratio.

The opportunistic coopting of other organisms' siderophores may provide *S. aureus* with a competitive advantage in heterogeneously colonized host niches such as on skin or in the nares. Little is known about the dissemination of enteric catechol siderophores from the gut flora and it is possible that these may be found in sufficient quantities to contribute to growth of invasive *S. aureus*, confirmed here to be capable of growth on exogenous catecholate-type siderophores (Figure 3-9). Strangely, in bioassay conditions, there appear to be strain-specific differences in the ability to grow on these siderophores which did not correlate to strain-to-strain expression of SstD on the surface of iron-starved *S. aureus* cells (Figure 3-5 C). This may reflect inherent differences in cellular metabolic requirements between strains rather than an inability to transport iron ligands. Strain Newman was not responsive to ferrated catecholamines or catecholate siderophores, while the other strains tested in the plate bioassay exhibited varying degrees of growth around the supplied iron ligands. (It was subsequently determined that Newman would begin to grow on enterobactin spotted from a stock solution of 500 µM, while the data presented in Figure 3-9 represent growth on 250 µM.) Another interesting finding was that in the absence of SstABCD, siderophores salmochelin S-4 (glucosylated tris-catecholate) and petrobactin (bis-catecholate + carboxylate) promoted the growth of strain RN6390, albeit to a lesser degree than for the wild type parent. This could explain why strain Newman did form a growth halo around petrobactin; uptake efficiency was enhanced by redundancy in transport

systems. Salmochelin S-4 clearly enters *S. aureus* RN6390 through an alternate transporter to SstABCD as well.

SstD demonstrated affinity for ferrated catecholamines and catecholate siderophores; for all ligands, K_d values were in the μM range (Table 3-2). The K_d values resemble those determined for the *B. subtilis* FeuA protein and ferric-norepinephrine complex (1.6 μM) (20). Interestingly, of all ligands examined, SstD had the highest affinity for the enteric siderophores enterobactin and salmochelin S-4, suggesting that under certain favourable conditions, these ligands might be viable iron sources for *S. aureus in vivo*, in spite of the inconsistent ability of these siderophores to stimulate growth of *S. aureus* strains *in vitro*. The K_d values of SstD for its ligands, in the μM range, contrasts with the K_d values in the nM range that were determined for HtsA and SirA for their cognate ligands, staphyloferrin A and staphyloferrin B (12, 13). In line with the μM range of affinities of *E. coli* FhuD for several hydroxamate ligands (29), this might reflect a sacrifice in ligand affinity in lieu of greater ligand diversity.

Findings presented here demonstrate that inactivation of three transporters, namely Sir, Hts, and Sst, inhibits utilization of transferrin-iron. Combined with the lack of inhibitory feedback on siderophore production, this strain may enhance the bacteriostatic potential of its milieu through the secretion of nonviable iron chelators. This phenomenon may underlie the reduced fitness of the Δ*sst* Δ*hts sir*⁻ transporter mutant relative to the Δ*sst* Δ*sfa* Δ*sbn* mutant in the murine infection model used in this study. In combination, these lipoproteins may be worthy candidates for inclusion in a multivalent antistaphylococcal

vaccine, wherein the effectiveness of antibodies would rely upon inhibiting transporter function.

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Chapter 4

General Discussion

4.1 General overview

In this report, we have described two mechanisms through which *S. aureus* extracts iron from serum using endogenously derived molecules. These comprise the essentially redundant strategies of secreting a high affinity Fe^{3+} chelator, *i.e.* siderophore, produced by enzymes encoded from the *sfa* or *sbn* loci. Using mutants inactivated at those loci, the siderophores were shown to be staphyloferrins A and B (SA and SB), in agreement with previous studies that interrogated staphylococcal culture collections for siderophore production (20, 28, 32), and with concomitant biochemical studies characterizing the functions of *S. aureus* synthetases (9, 11). Furthermore, it was shown that SB is the same metabolite as the previously reported staphylobactin molecule (12). The staphyloferrins extract ferric iron from transferrin and facilitate its active uptake by the *S. aureus* cell through ABC transporters HtsABC and SirABC. Production of either of these siderophores was sufficient for maximal growth in human and animal sera, demonstrating that *S. aureus* overcomes the bacteriostatic nature of serum through secretion of SA and SB; thus, *hts*/*sfa* and *sir*/*sbn* could reasonably be described as virulence loci. In agreement with this statement, mutations impacting siderophore transport rendered *S. aureus* attenuated in a mouse sepsis model of infection.

We have further identified another mechanism by which *S. aureus* subverts host molecules for extraction of iron from serum. This mechanism exploits the propensity of catecholamine neurendocrine stress hormones to interfere with the iron scavenging capacity of transferrin. In addition to reductively liberating transferrin iron, these molecules possess 3,4-dihydroxylphenyl groups with

proposed Fe^{3+}-coordination capacities resembling that of the catecholate family of bacterial siderophores (15, 35, 45). *S. aureus* was shown to acquire transferrin iron via these ligands through the SstABCD transporter, which was also required for transport of catecholate xenosiderophores. Mutation of *sst* enhanced the attenuation phenotype of a staphyloferrin transport mutant.

4.2 Significance of this study

It has long been known that *S. aureus* is capable of growth in serum and on transferrin (29, 36, 41), and that catecholamine hormones may enhance this growth (39). This study is the first to employ a molecular genetics approach toward describing the genes associated with these processes, and to comprehensively assess their impact on virulence. Furthermore, this study complements biochemical characterization of the assembly of staphyloferrin siderophores using *sfa* and *sbn*-encoded NIS synthetases (9, 11), by establishing these genes encode proteins producing these metabolites in *S. aureus* itself.

In combination with the discoveries of other members of our research group (5, 9, 12, 13, 17, 18, 51), the findings of this study enable the drafting of a model describing a multifaceted, heme-independent serum iron acquisition strategy executed by *S. aureus*, presented in Fig. 4-1. This comprehensiveness suggests the lowly abundant but highly labile transferrin iron pool to be a vital reservoir supporting growth of invasive *S. aureus*. In comparison, published scientific reports describe only two transport systems involved in acquisition of heme iron by *S. aureus*. Furthermore, we have shown that one of these

transporters, HtsABC, is involved in the transferrin iron strategy, despite its earlier description as a heme transporter (48). The controversy over its true role will be addressed in greater detail shortly. The Hts debate notwithstanding, much has been said about the heme iron preference of *S. aureus* and its significance in virulence (30, 42, 48, 55), largely overshadowing contributions from other potentially significant iron reservoirs.

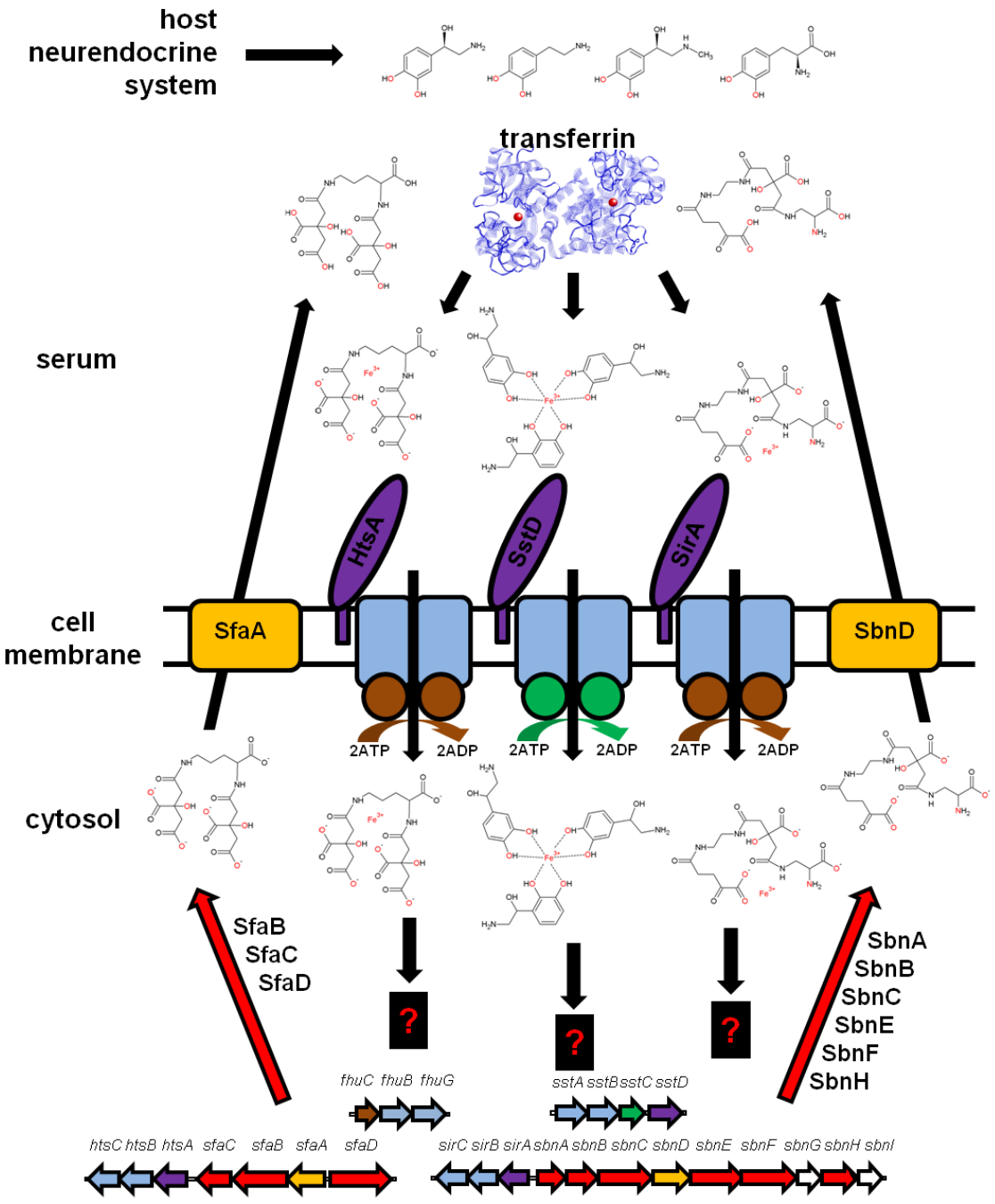
Despite involving less mechanistic redundancy (based on what is published to date), heme iron acquisition is certainly crucial for *S. aureus* infection. In murine sepsis models of infection, mutants deficient for production of either staphyloferrin and for acquisition of catecholamine-liberated transferrin iron were mildly attenuated relative to the wild type, but nevertheless quite capable of causing severe illness. Conversely, similar observations have been made for mutants of heme uptake system Isd (31, 55). There is a strong likelihood that alternate heme transporters are used by *S. aureus*; beyond characterizing these, it remains to be seen if combined inactivation of transferrin and heme iron acquisition mechanisms results in a severely attenuated mutant, or if alternate mechanisms permit *S. aureus* to continue to target these host iron reservoirs and to exploit alternate reservoirs.

4.3 Future investigative foci

4.3.1 What is the intracellular mechanism of Fe^{3+} liberation from siderophores?

As implied by the black boxes present in Fig. 4-1, the intracellular fate of Fe-siderophores in *S. aureus* is unknown. This information gap is remarkably

Figure 4-1: Model of transferrin iron acquisition by *S. aureus*. Secreted staphyloferrins (SA, left; SB, right) and host-derived catecholamine hormones (clockwise from top left: norepinephrine, dopamine, L-DOPA, epinephrine) extract iron from transferrin and are transported through the cell membrane through transporters HtsABCFhuC (right), SstABCD (centre, shown transporting Fe-tris-norepinephrine), and SirABCFhuC (left). The intracellular fate of ferrated ligands is still unknown.


host
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membrane
cytosol
HtsA
SstD
SirA
SfaA
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SbnE
SbnF
SbnH
fhuC fhuB fhuG
sstA sstB sstC sstD
htsC htsB htsA sfaC sfaB sfaA sfaD
sirC sirB sirA sbnA sbnB sbnC sbnD sbnE sbnF sbnG sbnH sbnI

frequent in the body of literature on microbial siderophores—much is known about their biosynthesis, target iron reservoirs, and transport, but very few studies describe their fate once returned, ferrated, to the bacterial cytosol. Is the mechanism for iron liberation reductive, permitting recycling of the deferrated siderophore, or destructive, requiring the enzymatic degradation of the molecule to a catabolite without iron coordination capacity?

It has been suggested that reductive mechanisms likely extract iron from SA and SB, given that both molecules incorporate amide bonds which are very resistant to hydrolysis (34). From the limited body of scientific reports, reduction appears to be the exclusive mechanism for cytosolic extraction of iron from hydroxamate and α-hydroxycarboxylate siderophores (34). Considering the range of hydroxamate and α-hydroxycarboxylate siderophores used by *S. aureus*, it is likely that multisubstrate reductases are responsible for siderophore iron extraction. No candidate reductase genes appear to be encoded on the *S. aureus* chromosome in the vicinity of any of the siderophore transport operons (*sir*, *hts*, *sst*, *fhu*). These might be identified by creating a transposon mutant library in a Δ*sfa* Δ*sbn* mutant strain and screening for impaired growth in low iron media supplanted with exogenously derived staphyloferrins. Impaired siderophore iron extraction might have similar effects to impaired siderophore transport, *i.e.* exacerbation of the iron deprivation response leading to enhanced secretion of unusable siderophore; thus, a siderophore reductase might make an attractive therapeutic target for small molecule drugs.

Destructive mechanisms of iron liberation are typically applied to high affinity siderophores assembled around bonds that can be efficiently attacked by water. The catecholate siderophores enterobactin and bacillibactin are consumed thusly, by iron-regulated esterases that partially or completely hydrolyze the ester bridges of the trilactone structure scaffolding the catecholate iron-coordinating groups (27, 33). Destructive liberation also circumvents the challenge posed to a reductase by the exquisitely high affinity for Fe^{3+} demonstrated by these siderophores. In this study, clinically relevant *S. aureus* strains were characterized growing on ferric bacillibactin and ferric enterobactin (Fig. 3-9), suggesting they produce the requisite esterase(s) required for destructive liberation of iron from these siderophores. *Bacillus* BesA is a broad specificity esterase capable of cleaving the tri-L-serine trilactone of enterobactin and the tri-threonine trilactone of bacillibactin; *E. coli* Fes is a more specific esterase mediating cleavage of only enterobactin (2). BLAST searches using these protein sequences failed to uncover any candidate homologues in *S. aureus* genomes. Identifying this esterase may provide insight into factors contributing to intestinal carriage of *S. aureus*, where iron uptake using coopted enteric siderophores could contribute to survival. Human intestinal carriage rates of may be as high as 20% (3), with obvious clinical implications; surprisingly, this reservoir of *S. aureus* has received very little scrutiny. Furthermore, there appeared to be great interstrain variability in responsiveness to application of these xenosiderophores (Fig. 3-9), which did not correlate to the amount of SstD expressed by these strains (Fig. 3-5); describing what

mechanisms dictate activity of this esterase could contribute to our understanding of niche adaptation by *S. aureus* strains.

4.3.2 Resolving the *hts* debate

There is currently an abundance of published evidence unambiguously describing a role for HtsABC in SA binding and uptake, crowned by the recently published crystal structure of HtsA liganded to SA (18). Nevertheless, this transporter is invariably cited as a heme uptake system (16, 30, 49, 50, 54) based on the findings of Skaar *et al*. (48), who characterized alterations to the acquisition profiles of isotopically labelled transferrin and hemin iron resulting from mutagenesis of putative iron transport systems. Mutating *htsB* and *htsC* biased the iron source preference of *S. aureus* toward transferrin. Strangely, this shift was not observed for an *isdDEF* mutant, despite the characterized function of IsdE as a heme-binding lipoprotein (37, 43); furthermore, no shifts toward a heme preference were associated with mutation of *sirABC* or *sstABCD*. Skaar *et al.* interpreted their findings to signify HtsABC is the primary ABC transporter of heme in *S. aureus*, and must work in conjunction with wall-anchored IsdABC for extraction of heme from host carrier proteins.

While we cannot empirically exclude a role for any Hts protein in heme transport, we feel it is extremely unlikely to contribute to this process, especially with regards to HtsA. The three dimensional folding of HtsA places it among the periplasmic binding protein family alternately referred to as class III (5) or cluster 8 (24). This family includes siderophore receptors FhuD (10) and SirA (17), but also heme receptor isdE (19). These demonstrate a conserved

bi-lobed architecture, the lobes of which are made up of central β-strands surrounded by several α-helices. The two lobes are bridged by a single long α-helix, which imparts relative inflexibility ensuring the protein undergoes minimal conformational change upon ligand binding and release (7, 26, 47). Beyond an overarching architectural relatedness to IsdE, the HtsA binding pocket is markedly different from that of its heme-binding counterpart. The binding groove is shallow and dominated by a large basic patch whose overall positive charge is the contribution of six arginine residues, conducive to the binding of an anionic compound such as a staphyloferrin but unlikely to coordinate a planar, hydrophobic heme molecule (5). The IsdE binding pocket is hydrophobic, deeper, nearly completely occludes the heme molecule, and is reliant on heme-iron-coordinating histidine and methionine residues (19). Importantly, the HtsA protein demonstrates a high affinity for Fe-SA (K_d < 15 nM) (18); in contrast, multisubstrate binding proteins (*e.g.* SstD) typically sacrifice affinity for variety.

With respect to HtsABC, we are at a loss to explain the findings of Skaar *et al*. (48). There remains the possibility that HtsBC forms a permease capable of interacting with multiple receptors, including HtsA for SA and an alternate lipoprotein. This phenomenon has been characterized in *Sinorhizobium meliloti*, whereby operon *hmuTUV* encodes an ABC transporter comprised of permease HmuUV which works in conjunction with heme receptor HmuT as well as a separately transcribed hydroxamate siderophore receptor, FhuP (38). A single gene ORF encoding a putative class III lipoprotein has been identified as iron-regulated *in vitro* (locus tag NWMN_0908) (4), and could be a candidate

heme receptor. In this case, however, it is unlikely HtsBC is the dominant heme permease in *S. aureus*, as we have repeatedly failed to demonstrate a heme uptake defect associated with the Δ*htsABC* mutation in wild type and *isdE*$^{-}$ strains. The heme transport function of HtsABC is an established paradigm that merits a courteous but assertive challenge and a more thorough investigation.

4.3.3 Therapeutic strategies targeting siderophore transport

In this study, it was shown that mutagenic inactivation of staphyloferrin transport did not abrogate staphyloferrin production; on the contrary, SA and SB secretion was enhanced, and transporter mutants accentuated the iron withholding capacity of their milieu (Fig. 2-17), presumably further exacerbating derepression of *sfa*/*sbn* and the remainder of the iron starvation regulon from Fur. In a collaborative study, it was shown that a *S. aureus* Δ*fur* mutant constitutively mounts an iron deprivation response regardless of iron availability, and furthermore demonstrates atypically coordinated expression of virulence factors despite a lack of direct influence of Fur on the genes involved (54). This response includes enhanced secretion of leukotoxins and hemolysins, with decreased expression of surface-associated immunomodulatory proteins. This disregulated mutant was severely attenuated in a mouse infection model. This suggests that therapeutics inhibitory to SirA and HtsA functioning might be especially efficacious in combating *S. aureus* by essentially coopting bacterial cells into administering chelation therapy within the infected host, enhancing an iron starvation response that ultimately compromises virulence.

In considering exploiting Sir and Hts to combat *S. aureus* infection, different strategic approaches can be considered. These include targeting receptor proteins SirA and HtsA with antibodies to promote bacterial clearance through opsonization; using said antibodies to block receptor/siderophore binding; blocking transport using competitive siderophore structural analogs; and exploiting transporters to deliver toxic siderophore structural analogs into the cell.

A vaccine-based approach to targeting staphyloferrin receptors intuitively seems unproductive. Gram-positive lipoproteins are membrane-tethered and seemingly under the protective canopy of a robust cell wall, which would shield them from anti-SirA and -HtsA antibodies. Surprisingly, lipoproteins have been shown to significantly enhance the antigenicity of *S. aureus* (8). Recently, a multivalent vaccine against a host of iron-regulated staphylococcal lipoproteins, including SirA, HtsA, and SstD, has been licensed by pharmaceutical conglomerate sanofi-aventis Group (1); reports on the vaccine's efficacy do not yet exist within the public domain. An independent study has corroborated that SirA, SstD, and FhuD2 are immunogenic in mammals, and that SstD can be detected on the surface of *S. aureus* (52); the protective value of vaccination with any combination of these components has not been described and merits further investigation.

With our recently improved understanding of the molecular interactions between HtsA/SirA and their staphyloferrin ligands (17, 18), and the biochemistry of staphyloferrin synthesis (9, 11), developing a drug-based strategy targeting siderophore uptake is also feasible. Siderophore transporters

could serve as conduits for the delivery of “Trojan horse” molecules to *S. aureus*. This principle is at play in the mechanism of albomycin killing of *E. coli*. Albomycin is a ferrichrome structural analog with a bound thioribosyl pyrimidine antibiotic (56). It is recognized by and transported through the *E. coli* Fhu transport pathway; in the cytosol, the antibiotic is cleaved off the Fe^{3+} carrier by a peptidase and exerts its killing effect (21). Few naturally-occurring “sideromycins” are known, but our understanding of the structural parameters defining receptor/staphyloferrin interaction, and our ability to synthesize staphyloferrins *in vitro*, could facilitate the design of antibacterial staphyloferrin analogs permissive to high affinity transport, or alternately, capable of irreversibly binding to SirA and HtsA.

4.3.4 Characterizing the contributions of staphyloferrins in other host niches

In this study, two different mouse models were used to characterize the contributions of siderophore biosynthesis and transport genes toward the ability of *S. aureus* to cause acute infection: i) the skin abscess model, and ii) the sepsis model, which essentially characterizes abscession or colonization of a limited organ set following bacteremia. Virulence was not detectably influenced by these genes in the first model, and significantly but modestly influenced in the second. The ecology and pathology of *S. aureus*, however, is more expansive than could be described by these two models, and siderophores may play a more prominent role in survival in other niches. Beyond colonization of nares, the rectum, and various regions of the skin, invasive *S. aureus* can target

virtually every organ of the body. Siderophores have been shown to contribute to virulence in other types of infections mediated by other pathogenic organisms, including pneumonia caused by *Klebsiella pneumonia* (25), *Burkholderia cenocepacia* (57), and *Pseudomonas aeruginosa* (53), and urinary tract infection caused by *Proteus mirabilis* (22). An acute pneumonia model might be especially pertinent in light of a recent report concluding heme uptake mechanisms are dispensible to *S. aureus* in this type of infection (31).

Staphylococcal species, chiefly *S. saprophyticus* and secondarily *S. aureus*, are leading causes of non-*E. coli* catheter-independent urinary tract infections (14, 23, 44, 58). Production of multiple siderophores in addition to enterobactin (notably salmochelin and aerobactin) is an epidemiological marker strongly associated with uropathogenic strains of *E. coli* (UPEC) (59). Additionally, urine is the primary means of catecholamine excretion from the body, and may contain micromolar concentrations of these molecules. Siderophore iron uptake thus might play a noteworthy role in staphylococcal urinary tract infections, yet nothing is currently known about the contributions of staphyloferrins, UPEC siderophores, and catecholamines.

Siderophores may also be important for asymptomatic colonization of the host. Siderophores are invariably overlooked in reviews on *S. aureus* factors enabling carriage among humans. Iron availability at colonized sites is low, however, in part due to lactoferrin, a normal component of mucous. Furthermore, *S. aureus* may have to compete for iron with other commensalist bacteria producing their own siderophores. In addition to expressing transporters for coopting siderophores produced by other species (Fhu and Sst),

it is tempting to speculate that production of a second staphyloferrin, SB, may offer a selective advantage to *S. aureus* over CNS species that are otherwise genetically homologous in terms of siderophore transport (Fhu, Sst, Hts) and biosynthesis (SA). It would be interesting to evaluate the fitness of *S. aureus* siderophore mutants in murine models for colonization (46), colonization/transmission (6), and colonization/competition (40).

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Appendix 1

Systemic infection trials with Jackson Laboratories mice

Figure A1. *S. aureus* Newman strains mutated in *sfa* and *sbn* loci are not attenuated in a mouse sepsis model of infection. Approximately 8 x 10^6 CFU **(A)** or 2 x 10^6 CFU **(B)** *S. aureus* Newman and an isogenic staphyloferrin biosynthetic mutant derivative were injected intravenously to Jackson Laboratories BALB/c immunocompetent mice. Percentage of mouse body mass lost over four days and CFU recovered from target organs are plotted. Statistical analysis was performed using the Student's unpaired *t*-test. No signficant treatment differences were noted. Calculated infective doses, in millions of CFU injected, were as follows: wild type (WT), 7.775 **(A)** and 2.100 **(B)**; Δ*sfa* Δ*sbn*, 10.050 **(A)** and 2.300 **(B)**.

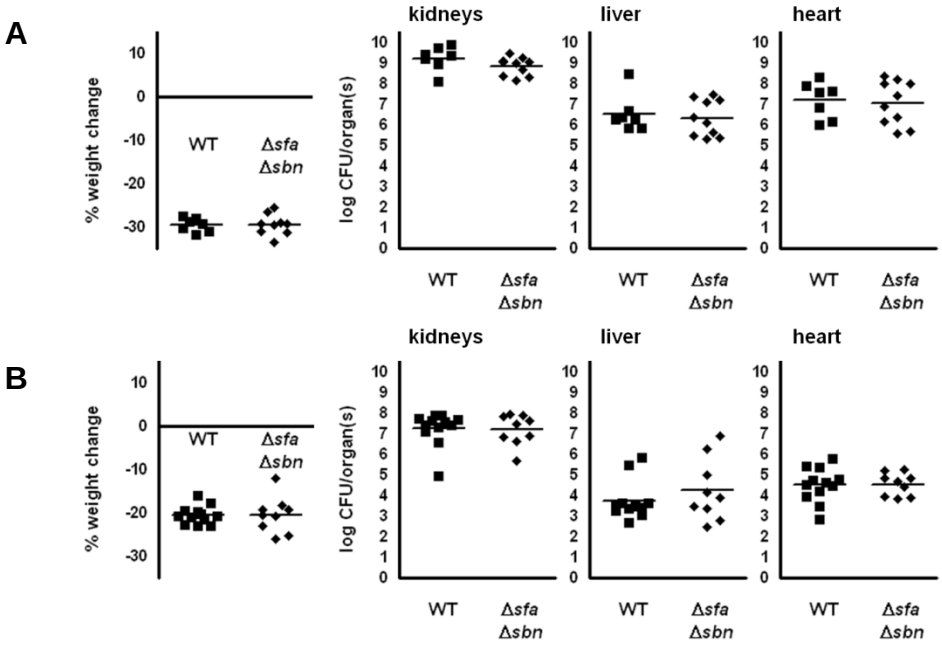
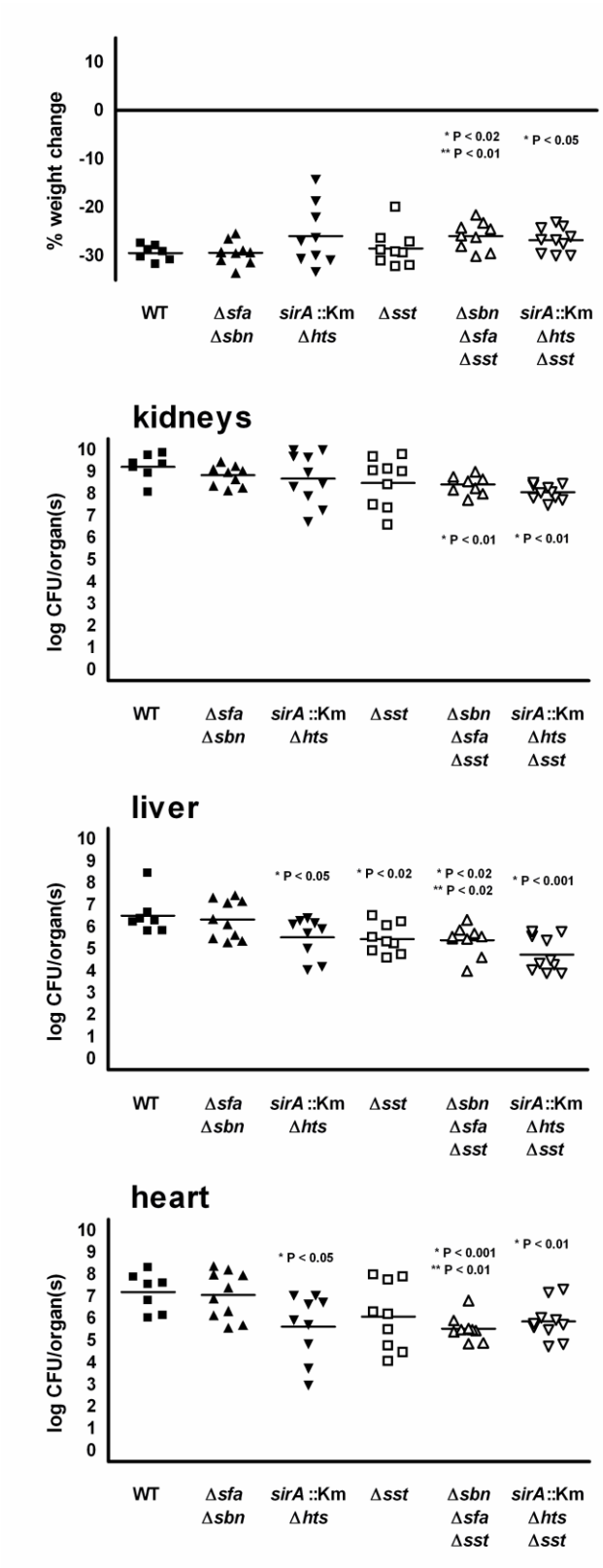

A
B
% weight change
log CFU/organ(s)
WT
Δsfa Δsbn
kidneys
liver
heart

Figure A2. Mutation of *S. aureus* Newman at *sst* enhances attenuation in a mouse sepsis model of infection. Approximately 1 x 10^7 CFU *S. aureus* Newman and isogenic iron transport mutant derivatives were injected intravenously to Jackson Laboratories BALB/c immunocompetent mice. Percentage of mouse body mass lost over four days and CFU recovered from target organs are plotted. Statistical analysis was performed using the Student's unpaired *t*-test. Asterisks denote significant differences ($P < 0.05$) from wild type (WT) (*) or from an isogenic *sst*$^+$ parent (**). Infective doses were as follows (in millions of CFU/dose): 7.75 (WT), 10.50 (Δ*sbn* Δ*sfa*), 8.50 (*sirA*::Km Δ*hts*), 13.25 (Δ*sst*), 11.00 (Δ*sbn* Δ*sfa* Δ*sst*), 9.25 (*sirA*::Km Δ*hts* Δ*sst*).


% weight change
10
0
-10
-20
-30
* P < 0.02
** P < 0.01
* P < 0.05
WT
Δsfa Δsbn
sirA ::Km Δhts
Δsst
Δsbn Δsfa Δsst
sirA::Km Δhts Δsst
kidneys
log CFU/organ(s)
* P < 0.01
* P < 0.01
liver
* P < 0.05
* P < 0.02
* P < 0.02
** P < 0.02
* P < 0.001
heart
* P < 0.05
* P < 0.001
** P < 0.01
* P < 0.01

Summary of this work:

Infection trials were performed as described in Chapters 2 and 3. Virulence data presented in those chapters were generated using mice purchased from Charles River. In those trials, siderophore biosynthesis mutants of *S. aureus* did not demonstrate robust attenuation phenotypes, conflicting with an earlier report describing a strong attenuation phenotype resulting from insertional inactivation of *sbnF* (1).

Animal vendors post online reports of infectious agent outbreaks in their breeding colonies. Upon examination of the Charles River reports, it was determined that the mice used for the experiments described in Chapters 2 and 3 may have had prior exposure to *S. aureus*, which could have altered their immunity to the pathogen for subsequent infection studies. It was therefore of interest to repeat the experiments using mice from breeding colonies with immaculate surveillance histories, as were available for purchase from Jackson Laboratories.

Data generated using Jackson Laboratories mice were in good agreement with those generated using Charles River mice. In summary, inactivation of siderophore biosynthesis (Δ*sfa* Δ*sbn*) did not reduce the virulence of *S. aureus*; inactivation of siderophore transport (Δ*hts sirA*::Km) reduced virulence in livers and hearts; and inactivation of catechol transport (Δ*sst*) in combination with inactivated siderophore transport or biosynthesis reduced virulence in kidneys, livers, and hearts.

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Appendix 2

sbnA and *sbnB* are required in generating L-2,3-diaminopropionic acid for staphyloferrin B biosynthesis

Summary of this work:

In addition to encoding synthetases SbnC, SbnE, and SbnF (1), the *sbn* operon includes genes for putative accessory enzymes that might play a role in substrate generation. The assembly of SB requires the input of two molecules of the rare, nonproteinogenic amino acid L-2,3-diaminopropionic acid (L-DAPA) (Figure 1-5B).

BLAST analyses revealed SbnA shares identity with an amidotransferase (VioB) and SbnB shares identity with an ornithine cyclodeaminase (VioK) encoded by the viomycin biosynthetic cluster of *Streptomyces vinaceus* (E values of $2e^{-45}$ and $5e^{-37}$, respectively). *S. vinaceus* produces viomycin from precursors including L-DAPA (3). It was hypothesized that the concerted roles of SbnA and SbnB might be to generate L-DAPA from L-ornithine and L-serine, whereby ammonia released from the cyclization of L-ornithine to L-proline by SbnB would be used as a nucleophile by amidotransferase SbnAB to aminate L-serine to L-DAPA (Figure A3).

To test the hypothesis that SbnA and SbnB are required to produce L-DAPA for siderophore biosynthesis, attempts were made to rescue low-iron growth defects of *S. aureus* Newman *sbnA* and *sbnB* insertional inactivation mutants (Table A1) using L-DAPA. Mutations were transduced into the Δ*sfa* background (Table 2-1) to eliminate the compensatory function of SA. On 10 µM human holotransferrin as a sole iron source, the growth defects of *sbnA*::Km and *sbnB*::Km mutants but not that of the Δ*sbnABCDEFGHI* (Δ*sbn*) mutant were rescued by application of 5 mM L-DAPA (Figure A3, A and B), but not its isomer D-DAPA (Figure A3, B) or the other predicted product of the

SbnA/SbnB reation, L-proline (Figure A3, C). Furthermore, L-DAPA restored the siderophore output of the *sbnA*/*sbnB* mutants, as measured using the CAS assay (Figure A3, E and F). The *sbnA*/*B* mutant phenotypes were also complementable *in trans* by expressing wild type copies of the genes from vector pALC2073 (Figure A3, D). A final noteworthy observation was that exogenous application of L-DAPA massively increased the siderophore output of a *sbn* wild type strain (Figure A3, F), suggesting that procurement of this molecule may be a limiting factor in SB production.

Materials and methods

The *sbnA*::Tc and *sbnB*::Tc mutant alleles and vectors for complementing these mutations *in trans* were generated using methodologies described in Chapters 2 and 3. Additional details are summarized briefly below:

To create an inactivation allele, *sbnA* was PCR amplified using primers with engineered *Sac*I and *Kpn*I restriction sites and cloned into vector pBC SK+ between those sites. A tetracycline resistance cassette was PCR amplified from vector pDG1513, digested with restriction enzymes *Nsi*I and *Pst*I, and ligated into a unique *Nsi*I restriction site in *sbnA* within pBC SK+; this allele was excised and ligated into temperature-sensitive suicide shuttle vector pAUL-A using restriction enzymes *Kpn*I and *Sac*I, then integrated via double homologous recombination into the *S. aureus* RN6390 chromosome, and transduced to other strains essentially as described in Chapters 2 and 3. To generate a complementation vector, *sbnA* was PCR amplified using primers with

engineered *Xho*I and *Eco*RI restriction sites and cloned directly to pALC2073, creating plasmid pFB5.

To create an inactivation allele, *sbnB* was amplified using primers with engineered *Bam*HI sites but cloned as a blunt-ended PCR product to vector pACYC184 digested with *Eco*RV. A tetracycline resistance cassette was excised from vector pDG1514 with restriction enzymes *Nsi*I and *Pst*I and ligated into a unique *Pst*I restriction site in *sbnB* within pACYC184; this allele was excised and ligated into temperature-sensitive suicide shuttle vector pAUL-A using restriction enzyme *Bam*HI, then integrated via double homologous recombination into the *S. aureus* RN6390 chromosome prior to transduction. To generate a complementation vector, *sbnB* was PCR amplified using primers with engineered *Eco*RI restriction sites and cloned directly to pALC2073, creating plasmid pSED52.

For growth assays, bacteria were cultured in C-TMS with human holotransferrin as described in Chapter 2. Amendments to the medium included 5 µg/mL chloramphenicol (Bioshop) (complementation assay only), 5 mM L- or D-2,3-diaminopropionic acid (Iris Biotech GmbH), or 5 mM L-proline (Sigma-Aldrich).

Figure A3. Proposed reactions for SbnA/SbnB-mediated generation of L-diaminopropionic acid from ornithine and serine.

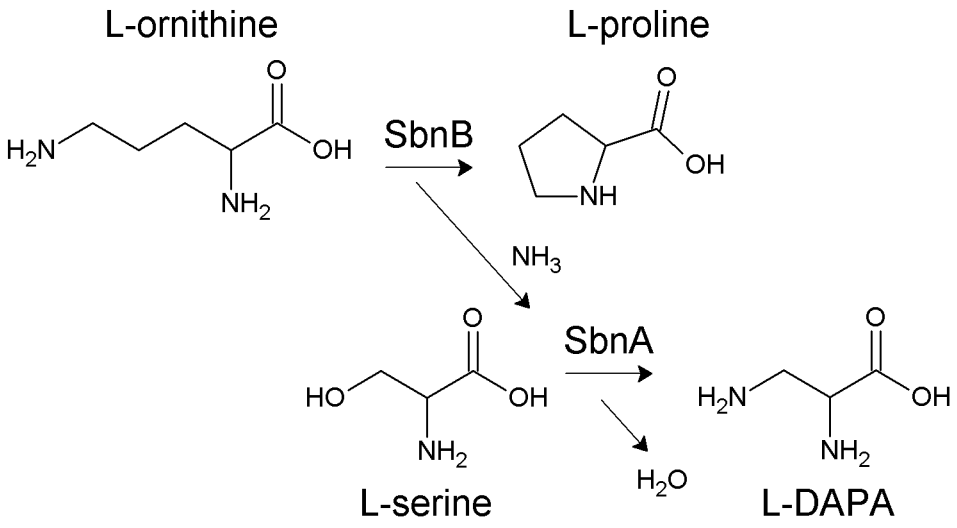
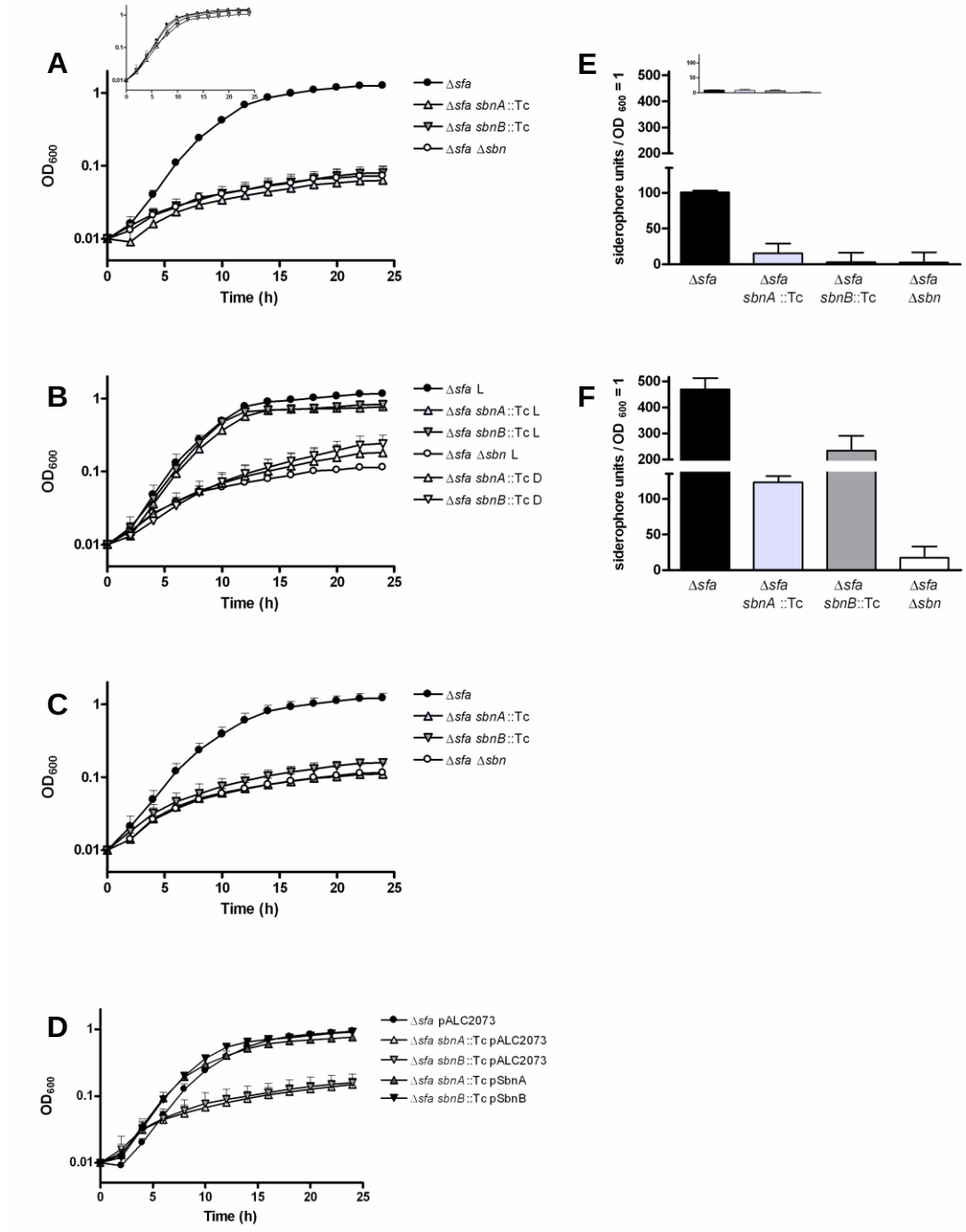

L-ornithine
L-proline
SbnB
NH3
SbnA
H2O
L-serine
L-DAPA

Table A3: Bacterial strains, plasmids, and oligonucleotides used in this study (see also Tables 2-1 and 3-1)

	Description	Ref.
***S. aureus* strains**		
H2131	Newman *sbnA*::Tc Δ*sfaABCsfaD*::Km	This study
H1718	Newman *sbnB*::Tc Δ*sfaABCsfaD*::Km	This study
Plasmids		
pACYC184	*E. coli* cloning vector; Cm^R	ATCC
pDG1514	pMTL23 derivative carrying tetracycline resistance cassette; Ap^R	(2)
pFB5	pALC2037 derivative carrying *sbnA*; Cm^R	This study
pSED52	pALC2037 derivative carrying *sbnB*; Cm^R	This study
Oligonucleotides*		
*sbnA*5'-*Sac*I	5' TGAGCTCGATTCTGTAGGGCAAACACC 3'	
*sbnA*3'-*Kpn*I	5' TTGGTACCTCTAAGTAACGATCGCCTCG 3'	
	Cloning of *sbnA* into pBC SK+	
Tet5'-*Nsi*I	5' TTGTATATGCATACGGATTTTATGACCGATGA 3'	
Tet3'	5' TGTGTGGAATTGTGAGCGGATAAC 3'	
	Amplification/cloning of a tetracycline resistance cassette from pDG1513	
*sbnA*5'-*Xho*I	5' TTTCTCGAGATTTTAAATTTGAGGAGGAA 3'	
*sbnA*3'-*Eco*RI	5' TTTGAATTCCCACATAAACTTGTGAATGATT 3'	
	Cloning of *sbnA* into pALC2073	
*sbnB*5'-*Bam*HI	5' TTGGATCCTAGTTTATTCAGATACATGG 3'	
*sbnB*3'-*Bam*HI	5' TTGGATCCTGTCCCAATATTTTGTTGTT 3'	
	Cloning of *sbnB* into pACYC184	
*sbnB*5'-*Eco*RI	5' TTGAATTCTCAAGTGATCCATGTAGATG 3'	
*sbnB*3'-*Eco*RI	5' TTGAATTCCAATTCCGGCTATATCTTCA 3'	
	Cloning of *sbnB* into pALC2073	

* underlined sequences in oligonucleotides denote restriction sites

Figure A4. Low iron growth defects resulting from inactivation of *sbnA* and *sbnB* can be rescued with L-DAPA. *S. aureus* Newman mutants (all Δ*sfa*) were grown in C-TMS containing 10 µM human holotransferrin (approximately 60% saturated). **(A; E)** Inactivation of *sbnA* or *sbnB*, or deletion of the entire *sbn* operon, resulted in a growth defect, which could be rescued by addition of 50 µM $FeCl_3$ (inset); this was due to loss of siderophore output, which could be repressed in the wild type by addition of $FeCl_3$ (inset). **(B; F)** For the *sbnA*/*B* mutants this defect could be rescued by application of L-DAPA (L) but not D-DAPA (D); L-DAPA restored siderophore production. **(C)** L-proline could not rescue growth defects. **(D)** *sbnA*/*B* mutant growth defects could be rescued by expression of wild type copies of those genes from a plasmid.


A
OD600
Time (h)
Δsfa
Δsfa sbnA::Tc
Δsfa sbnB::Tc
Δsfa Δsbn
B
Δsfa L
Δsfa sbnA::Tc L
Δsfa sbnB::Tc L
Δsfa Δsbn L
Δsfa sbnA::Tc D
Δsfa sbnB::Tc D
C
Δsfa pALC2073
Δsfa sbnA::Tc pALC2073
Δsfa sbnB::Tc pALC2073
Δsfa sbnA::Tc pSbnA
Δsfa sbnB::Tc pSbnB
D
E
siderophore units / OD 600 = 1
Δsfa
Δsfa sbnA ::Tc
Δsfa sbnB::Tc
Δsfa Δsbn
F

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CURRICULUM VITAE

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"Serum iron extraction and virulence in *Staphylococcus aureus*"

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- Supervisor: David Heinrichs, Ph.D.
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M.Sc., Agronomy, 2004
"Characterization of soil microbial responses to contamination with heavy metals and aromatic hydrocarbons"

- Purdue University, West Lafayette, Indiana, USA
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Hon. B.Sc., Botany, 2001

- University of Toronto, Toronto, Ontario, Canada
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Teaching

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Award	**Value**	**Date**
UWO Microbiology & Immunology John A. Thomas award	$1500	2009
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Ontario Graduate Scholarship in Research and Technology	$15000	2007
UWO Student travel award	$1000	2010, 2007
UWO Infection & Immunity Research Forum poster presentation award	$100	2009, 2007
UWO Microbiology and Immunology entrance award for academic achievement	$5000	2005
Dorothy Whiting Scholarship in botany (University of Toronto)	$688	2001
Victoria College Regents participation award (University of Toronto)	$1000	2001
NSERC undergraduate research award	$6000	2000
Victoria College Regents in-course scholarship (University of Toronto)	$1000	1999

Committee activity

Organization and position	**Date**
Graduate committee, student representative	2007 - 2009
UWO Infection & Immunity Research Forum organizing committee	2009
Department social committee	2009, 2007, 2006
Steward to teaching assistants' union	2005 - 2008
UWO Outdoors Club, Vice President	2007
Society for graduate students, council member	2006, 2005
Canadian Society of Microbiologists 56th annual general meeting, organizing committee	2006

Academic contributions

Refereed journal articles

Beasley, F.C., C.L. Marolda, J. Cheung, S. Buac, and D.E. Heinrichs. 2011. *Staphylococcus aureus* transporters Hts, Sir and Sst capture iron liberated from human transferrin by staphyloferrin A, staphyloferrin B and catecholamine stress hormones, respectively, and contribute to virulence. Infection and Immunity, *in press*

Wyatt, M.A., W. Wang, C.M. Roux, **F.C. Beasley**, D.E. Heinrichs, P.M. Dunman, and N.A. Magarvey. 2010. *Staphylococcus aureus* nonribosomal peptide secondary metabolites regulate virulence. Science, 329(5989), 294-296

Torres, V.J., A.S. Attia, W.J. Mason, M.I. Hood, B.D. Corbin, **F.C. Beasley**, K.L. Anderson, D.L. Stauff, W.H. McDonald, L.J. Zimmerman, D.B. Friedman, D.E. Heinrichs, P.M. Dunman, and E.P. Skaar. 2010. *Staphylococcus aureus fur* regulates the expression of virulence factors that contribute to the pathogenesis of pneumonia. Infection and Immunity, 78(4), 1618-1628

Beasley, F.C., and D.E. Heinrichs. 2010. Siderophore-mediated iron acquisition in the staphylococci (review). Journal of Inorganic Biochemistry, 104(3), 282-288

Cheung, J., **F.C. Beasley**, S. Liu, G.A. Lajoie, and Heinrichs, D. E. 2009. Molecular characterization of staphyloferrin B biosynthesis in *Staphylococcus aureus*. Molecular Microbiology, 74(3), 594-608

Beasley, F.C., E.D. Vinés, J.C. Grigg, Q. Zheng, S. Liu, G.E. Lajoie, M.E.P. Murphy, and D.E. Heinrichs. 2009. Characterization of staphyloferrin A biosynthetic and transport mutants in *Staphylococcus aureus*. Molecular Microbiology, 72(4), 947-963

McCully, M. L., T. Fairhead, C.S. Colmont, **F.C. Beasley**, D.E. Heinrichs, P.G. Blake, N. Topley, and J. Madrenas. 2008. Receptor-interacting protein-2 deficiency delays macrophage migration and increased intracellular infection during peritoneal dialysis-associated peritonitis. Journal of the American Society of Nephrology, 28(6), 879-889

Jerke, K., C.H. Nakatsu, **F. Beasley**, and A. Konopka. 2008. Comparative analysis of eight *Arthrobacter* plasmids. Plasmid, 59(2), 73-85

Nakatsu, C.H., N. Carmosini, B. Baldwin, **F. Beasley**, P. Kourtev, and A. Konopka. 2005. Soil microbial community responses to additions of organic carbon substrates and heavy metals (Pb and Cr). Applied and Environmental Microbiology, 71(12), 7679-7689

Conference presentations

Seminar: **Beasley, F.C.**, J. Cheung, C.L. Marolda, and D.E. Heinrichs. 2010. The invasive pathogen *Staphylococcus aureus* uses an array of coordinating ligands and cognate transport systems for extraction of iron from serum. UWO Infection and Immunity research forum, London, ON

Poster: **Beasley, F.C.**, J. Cheung, J.D. Cooper, C. Marolda, and D.E. Heinrichs. 2010. The invasive pathogen *Staphylococcus aureus* employs a multifaceted strategy for heme-independent extraction of iron from serum. Canadian Society for Microbiologists, 60th annual conference, Hamilton, ON

Poster: Cheung, J., **F.C. Beasley**, and D.E. Heinrichs. 2010. Genetics and biochemistry of staphyloferrin B biosynthesis in *Staphylococcus aureus*. Canadian Society for Microbiologists, 60th annual conference, Hamilton, ON

Poster: **Beasley, F.C.**, E.D. Vinés, S. Liu, G.A. Lajoie, and D.E. Heinrichs. 2009. Analysis of staphyloferrin A biosynthetic and transport mutants in *Staphylococcus aureus*. Gordon Research Conference on Staphylococcal Diseases, Waterville Valley Resort, NH

Seminar: Heinrichs, D.E., **F.C. Beasley**, J.D. Cooper, and J. Cheung. 2009. Staphyloferrin A synthesis and transport in the staphylococci. Georgian Bay International Conference on Bioinorganic Chemistry. Parry Sound, ON

Poster: Vinés, E.D., **F.C. Beasley**, and D.E. Heinrichs. 2008. Characterization of siderophore production and transport in *Staphylococcus aureus*. BioMetals conference, Santiago de Compostela, Spain

Poster: **Beasley, F.C.**, E.D. Vinés, C.L. Vermeiren, and D.E. Heinrichs. 2007. Evidence for production of a secondary endogenous siderophore by *Staphylococcus aureus*. Meeting on Microbial Pathogenesis & Host Response, Cold Spring Harbor, NY

Poster: **Beasley, F.C.**, C.L. Vermeiren, S.E. Dale, and D.E. Heinrichs. 2007. Inactivation of the *sbn* staphylobactin biosynthesis operon reduces siderophore activity and impairs growth of *Staphylococcus aureus* under iron restriction. American Society for Microbiology 107th annual general meeting, Toronto, ON

Poster: Henne, K.L., **F. Beasley**, M.D. Kane, C.H. Nakatsu, and A. Konopka. 2005. Chromium Resistance in *Arthrobacter* sp. FB24. American Society for Microbiology, 105th annual general meeting. Atlanta, GA

Poster: Vargha, M., **F. Beasley**, Z. Takáts, and C.H. Nakatsu. 2004. *Maldi MS* as a tool for differentiating *Arthrobacter* strains. American Society for Microbiology, 104th annual general meeting. New Orleans, LA

Poster: **Beasley, F.C.**, Nakatsu, C.H., Carmosini, N., and Konopka, A. 2003. Genotypic and phenotypic characterization of *Arthrobacter* isolates from chromate-contaminated soil. Annual Meeting of the American Society of Agronomy. Denver, CO

Poster: **Beasley, F.C.**, N. Carmosini, A. Konopka, and C.H. Nakatsu. 2002. Isolation and characterization of chromium resistant bacteria from mixed waste soil. Annual Meeting of the American Society of Agronomy. Indianapolis, IN

Poster: **Beasley, F.C.**, N. Carmosini, A. Konopka, and C.H. Nakatsu. 2002. Characterization of chromium resistance in soil bacteria. American Society for Microbiology 102nd annual general meeting, Salt Lake City, UT

Patents and copyrights

International application no. PCT/CA2009/001143, filed Aug. 14, 2009. "Siderophore-mediated iron uptake in bacterial infection". Inventors: D.E. Heinrichs, **F. Beasley**, E.D. Vinés, and J. Cheung