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Iron acquisition strategies employed by Staphylococcus lugdunensis

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Abstract

Iron is crucial for many cellular processes including DNA synthesis and respiration. The majority of iron in mammals is in heme within hemoproteins, inside cells, or transported through circulation by the glycoprotein transferrin, which constitutes the greatest iron source in serum. Limiting iron availability is an important facet of nutritional immunity to help prevent infection.

Staphylococcus lugdunensis is a human skin commensal and opportunistic pathogen capable of causing a variety of infections, including particularly aggressive endocarditis. It is an emerging pathogen with elevated virulence compared to other species of coagulase-negative staphylococci. The versatility of *S. lugdunensis* to infect multiple niches and cause aggressive infection indicates that it likely adapts its cellular physiology to overcome host defenses, including iron limitation.

In chapter 2, we demonstrate that, contrary to other staphylococci, *S. lugdunensis* does not produce a siderophore – small (<1 kDa) iron-chelating molecules that strip iron from host glycoproteins, including transferrin, and deliver it to microorganisms. As such, serum is growth-inhibitory to *S. lugdunensis*, unless it is supplemented with an iron source. We have identified and characterized several iron-compound transport processes through inactivation of genes required for acquisition of each respective compound. *S. lugdunensis* transports the staphylococcal carboxylate siderophores staphyloferrin A and staphyloferrin B through Hts and Sir, respectively, and is able to directly appropriate siderophores produced by *S. aureus* when in coculture, to support its growth. Heme and hemoglobin-iron is acquired via Isd.

In chapter 3, we demonstrate that hemolysis enhances growth in blood, in an Isd-dependent manner. An iron-regulated ATPase, FhuC, is required for import of several carboxylate and hydroxamate siderophores, whereas Sst1 transports catecholamine stress hormone-iron (ie. adrenaline, noradrenaline, dopamine). *fhuC* and *sst1* mutants are impaired for growth in absence of hydroxamates and catecholamines, indicating additional substrates acquired by these are vital to *S. lugdunensis*. Using a novel systemic model of *S. lugdunensis* infection, we show that a *isd fhuC sst* mutant is significantly impaired in its ability to colonize internal murine organs, and cause sickness. We have detailed several iron-acquisition systems in *S. lugdunensis* and are first to show specific transporters are important for pathogenesis in the host.

Keywords

Staphylococcus lugdunensis, iron, heme, hemoglobin, siderophores, staphyloferrin A, staphyloferrin B, hydroxamates, catecholamine stress hormones, hemolysis

Co-Authorship Statement

A portion of this work has appeared in a peer-reviewed, published manuscript. Work performed not exclusively by J.R. Brozyna is indicated below.

The majority of 'Chapter 2: Staphyloferrin siderophore and heme acquisition by *Staphylococcus lugdunensis*' has been published in:

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J.R.S. purified hemoglobin from fresh human blood. J.R.B. optimized and performed the bulk of experiments, and J.R.B. and D.E.H. conceived experimental. J.R.B. and D.E.H. crafted figures and wrote the manuscript.

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List of Abbreviations

°C	degrees Celsius
A	absorbance
ABC	ATP-binding cassette
ADP	adenosine diphosphate
Ap^R	ampicillin resistant
ATP	adenosine triphosphate
ATPase	ATP phosphatase
bp	base pairs
CA	community acquired
CAS	chrome azurol S
CFEM	common in several fungal extracellular membrane proteins
CFU	colony forming units
cm	centimeter
Cm^R	chloramphenicol resistant
CoNS	coagulase negative staphylococci
CNS	coagulase negative staphylococci
CPS	coagulase-positive staphylococci
CR	conserved region
C-TMS	chelex-treated Tris-buffered minimal succinate

Da	Dalton
DAP	diaminopropionic acid
DFO	desferrioxamine B
DHB	dihydroxybenzoic acid
DHBA	dihydroxybenzoic acid
DNA	deoxyribonucleic acid
EDDHA	ethylenediamine-di(*o*-hydroxyphenylacetic acid)
EDTA	ethylenediaminetetracetic acid
Em^R	erythromycin resistant
ESCRT	endosomal sorting complexes required for transport
FAD	flavin adenine dinucleotide
g	gram
g	gravitational force
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GST	glutathione-S-transferase
GTP	guanosine triphosphate
GTPase	GTP phosphatase
h	hour
Hb	hemoglobin
HFE	human hemochromatosis factor

His	histidine
Hm	hemin
Hp	haptoglobin
HpHbR	haptoglobin-hemoglobin receptor
Hx	hemopexin
hr	hour
IE	infective endocarditis
ID	identity
IPTG	isopropyl β–D-1-thiogalactopyranoside
IRE	iron-responsive element
IRP	iron-regulatory RNA-binding protein
K_D	dissociation constant
kDa	kilodalton
Km^R	kanamycin resistant
kb	kilobases
kbp	kilobase pairs
L	liter
LB	Luria-Bertani
L-DOPA	L-3,4-dihydroxyphenylalanine
Leu	leucine

LPS	lipopolysaccharide
μg	microgram
μL	microliter
μM	micromolar
μmol	micromole
M	molar
min	minute
Met	methionine
MFS	major facilitator superfamily
mg	milligram
mL	milliliter
mm	millimeter
mM	millimolar
N	normality (acid, base)
N	number of replicates
ng	nanogram
nm	nanometer
nM	nanomolar
NADPH	nicotinamide adenine dinucleotide phosphate
NEAT	near-iron transport

NGAL	neutrophil gelatinase-associated lipocalin
nm	nanometer
nM	nanomolar
NRPS	nonribosomal peptide synthesis/synthetase
OD	optical density
OMR	outer membrane receptor
ORF	open reading frame
P	probability
PBP	periplasmic binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PPi	pyrophosphate
PPIX	protoporphyrin IX
RBS	ribosome binding site
ROS	reactive oxygen species
RNA	ribonucleic acid
rpm	revolutions per minute
RPMIC	RPMI with 1% w/v casamino acids
SA	staphyloferrin A
SB	staphyloferrin B

SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLUSH	*Staphylococcus lugdunensis* synergistic hemolysins
sp.	species
spp.	species (plural)
SSTI	skin and soft tissue infection
Tc^R	tetracycline resistant
TCA	tricarboxylic acid
Tet^R	tetracycline resistant
TLR	toll-like receptor
TMS	Tris-buffered minimal succinate
TS	total similarity
TSA	tryptic soy agar
TSB	tryptic soy broth
v/v	volume per volume
Val	valine
vWF	von Willebrand factor
w/v	weight per volume

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Chapter 1

Literature Review

1.1 Iron

1.1.1 Iron chemistry

Iron is an essential nutrient for nearly all forms of life. It is crucial for vital cellular processes including DNA replication, amino acid synthesis, tricarboxylic acid cycle (TCA) activity and respiration. The functional versatility of iron is owed to its ability to readily interconvert between valence states, imparting single electron transfer chemistry to reactions that include almost the entire range of biologically significant redox potentials (1). Although able to achieve oxidation states between -2 and +6, iron is predominantly found in Fe^{2+} (ferrous) and Fe^{3+} (ferric) redox states. Ferric iron predominates in aerobic, oxidizing environments and forms insoluble hydroxides, with the predominant species at neutral pH being $Fe(OH)^{+}_{2}$ (2). This renders the solubility of iron in solution to be 10^{-8} to 10^{-9} M, well below the threshold to support microbial growth ($\sim 10^{-6}$ M) (3). Further restricting bioavailable iron is important to suppress infectious pathogen growth, and to keep it from undergoing reactions forming toxic products. Ferrous iron is able to catalyze the formation of free radicals, which are able to oxidize and damage cells (4).

1.1.2 Iron toxicity

Despite its low solubility in physiological conditions, the ability of residual free iron to readily interconvert between 2+ and 3+ redox states makes it a catalyst for production of harmful reactive species. Toxic reactive oxygen species (ROS) are produced through the Haber-Weiss reaction and Fenton chemistry (5, 6). Fe^{2+} reacts with hydrogen peroxide to form hydroxyl radicals (Equation 1), whereas Fe^{3+} reacts with hydrogen peroxide (Equation 2) or superoxide radical (Equation 3) to regenerate Fe^{2+}, shown in the three

equations below. Altogether, free iron is able to generate toxic species, of which the hydroxyl radical is extremely cytotoxic and capable to damage lipids, proteins and nucleic acids (7–10). Cellular life requires iron, and consequently must mitigate its toxicity. As such, biological systems tightly control iron mobility and storage with elaborate sequestration and transport methods.

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

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + H^+ + \bullet OOH \qquad \text{(Equation 2)}$$

$$Fe^{3+} + \bullet O_2^- \rightarrow Fe^{2+} + O_2 \qquad \text{(Equation 3)}$$

1.2 Iron processing in mammals

1.2.1 Iron absorption

Human iron metabolism is necessary to maintain homeostasis and important for promoting good health. Iron storage and trafficking within the human body has been extensively reviewed elsewhere (11–13). Briefly, iron from dietary sources is absorbed chiefly in the duodenum, with the absorbed amount dependent on sufficiency of stored iron in the body. The low pH of the intestinal lumen and ferric reductases in the apical brush border of duodenal enterocytes facilitate reduction of ferric iron to the more soluble ferrous form. Ferrous iron is transported into enterocytes through the divalent metal ion transporter DMT1 (Nramp2) (14). The internalized iron may be used for cellular processes, put into storage, or released into plasma via the basolateral membrane transporter ferroportin (15, 16). Macrophages recycle iron from senescent or dead cells,

including erythrocytes, and also elaborate ferroportin on their membrane. Nearly all iron in plasma is bound to the carrier protein transferrin, further described below.

A typical adult contains approximately 4-5 g of total iron in their body. Iron metabolism is regulated systemically by the peptide hormone hepcidin. A surplus of iron in the body stimulates hepcidin release from the liver. Hepcidin binds ferroportin on enterocytes and macrophages and triggers internalization and degradation, thereby decreasing iron export into circulation (17). Further absorbed dietary iron may be stored within intracellular ferritin protein, and excess iron may be shed from the body via enterocyte sloughing and feces production.

1.2.2 Iron storage

Ferritin is a primary means of iron storage for animals, microorganisms and plants alike. The mammalian ferritin is 450 kDa and made up of 24 heavy and light chains that together facilitate iron uptake into the hollow core of the large spherical protein (18, 19). Each ferritin structure may hold up to 4,500 iron atoms in the form of hydrous ferric oxides (19). Ferritin is abundant in liver hepatocytes, which essentially function as an iron storage and transport hub for the body (20). Additionally, ferritin release into plasma may aid in iron delivery to other tissues, including the brain and erythroid precursors (21–23). Furthermore, elevated serum ferritin is a nonspecific indication of infection or cancer, and can robustly predict cirrhosis development from hemochromatosis (24–26). Elevated serum ferritin is atypical of physiologically normal conditions and generally indicates an iron overload that may be associated with various disease states.

1.2.3 Iron for cellular processes: intracellular hemoproteins

The catalytic versatility of iron renders its usage in a number of cellular proteins including those that incorporate iron-sulfur clusters as redox cofactors, and in heme prosthetic groups within hemoproteins. Iron-sulfur proteins are predominantly involved in oxidoreductase, hydrogenase, dehydrogenase and hydratase reactions and are best known to offer redox capability for mitochondrial electron transport (27–30). The majority of bodily iron is complexed in heme within hemoproteins such as catalase, peroxidase, cytochromes, and foremost hemoglobin. Hemoglobin accounts for two-thirds of total iron within the body, the majority of which circulates within erythrocytes (31). The heme prosthetic group consists of a planar heterocyclic ring (porphyrin) able to coordinate a central iron atom with four nitrogen bonds. The central iron is able to form two additional coordinate bonds somewhat perpendicular to porphyrin and may interact with axial ligands including histidine, cysteine, tyrosine and methionine residues of binding proteins. Peroxidases and gas-carrying proteins such as hemoglobin contain heme B (protoporphyrin IX-iron), the most abundant type of heme. Other types of heme have various different constituents linked to the central porphyrin ring to differentiate compound chemistry for different purposes, including aspects of aerobic respiration and pathogen defense (32–34).

Each erythrocyte may contain more than 2.8×10^8 molecules of hemoglobin, and constitute the most abundant source of hemoglobin in the body (12). Hemoglobin is a globular heterotetramer in which each subunit coordinates a heme group with an imidazole nitrogen from a histidine. The sixth coordinate position of iron is left to bind O_2 and facilitate delivery from lungs to tissue. The average erythrocyte life span under

normal conditions is ~120 days, although these cells are more prone to oxidative damage due to their large amount of heme iron and great exposure to oxygen (35, 36). Erythrocyte lysis and hemoglobin damage lead to free heme and hemoglobin in serum. Extracellular hemoglobin tetramer is scavenged by the glycoprotein haptoglobin, which binds specifically and irreversibly (37). The haptoglobin-hemoglobin complex binds a receptor on the surface of macrophages for internalization and degradation (38). Iron is released from the porphyrin ring and recycled into transferrin or hemosiderin, a complex of ferritin and denatured ferritin (39, 40). Free heme in serum is primarily absorbed by the lipid core of the highly-abundant, fat-transport protein albumin (high or low density lipoprotein) (41). It is transferred to the glycoprotein hemopexin, which bears a very strong affinity for heme. The hemopexin-heme complex binds receptors on hepatocytes or macrophages for internalization and recycling in a similar manner to haptoglobin-hemoglobin employing lysosomal degradation (42, 43).

1.2.4 Iron transport: extracellular iron

Transferrins are glycoproteins of approximately 80 kDa that circulate in biological fluids, including serum, to facilitate iron transport between tissues (44). Although comprising less than 0.1% of total body iron (<5 mg), this reservoir constitutes an important means to limit free iron and control iron homeostasis (45, 46). A healthy adult has a serum transferrin concentration of ~25-30 μM (47, 48). Each transferrin glycoprotein has two high affinity Fe^{3+} binding sites, with stability constants of 4.7×10^{20} M^{-1} and 2.4×10^{19} M^{-1} at pH 7.4 (49). Iron-binding is random and noncooperative between the two domains and serum transferrin is typically ~30% saturated, while 20-50% saturation indicates a healthy state (50, 51). Synthesized by the liver, transferrin binds ferroportin-released

ferric iron (from intestinal enterocytes or other sources) and distributes bound iron to cells bearing transferrin receptor, which is ubiquitously expressed. Cells that require iron upregulate transferrin receptor expression. Holo-transferrin binds cell-surface exposed transferrin receptor and initiates receptor-mediated endocytosis for internalization and maturation into an endosome. A decrease in endosomal pH (below 5.5) triggers iron release from transferrin, which bears little affinity for ferrous iron. The released iron is mobilized for cellular processes or storage, whereas the endosome returns to the cell surface. The extracellular neutral pH allows apo-transferrin dissociation from transferrin receptor, such that apo-transferrin is released back into circulation to complete the transferrin cycle (52, 53). Additionally, the membrane protein HFE (human hemochromatosis factor) interacts with transferrin and transferrin receptor to detect saturation of transferrin and regulate hepcidin production to modulate iron absorption (54, 55). Transferrin receptor and ferritin expression is also regulated posttranscriptionally by iron-regulatory RNA-binding proteins (IRPs), which bind to iron-responsive elements (IREs) in mRNA to alter stability and translation efficiency (56).

Lactoferrin is a glycoprotein closely related to transferrin in sequence and structure, although it functions predominantly for scavenging iron rather than for transport (57). Found primarily in bodily secretions, lactoferrin releases bound iron at approximately pH 3 (compared to pH 5.5 for transferrin), and may act as a potent microbicidal agent by limiting available iron under various conditions (58). For this purpose, polymorphonuclear leukocytes release lactoferrin during degranulation upon phagocytosis (59). Released lactoferrin sequesters iron to prevent bacterial adherence and

promote bacteriostasis (60, 61). Lactoferrin-iron may also be used to catalyze free radical production via granule-phagosome fusion (62). Although having no significant role for iron transport, lactoferrin performs an important function for defense against pathogen infection by binding iron in bodily secretions and sites of infection.

1.2.5 Nutritional immunity: iron withholding

The human body takes advantage of the essentiality and toxicity of transition metals to protect itself against foreign invaders by restricting available nutrients, such as iron, to limit infection. Conversely, disease states associated with iron overload such as hemochromatosis, thalassemia, and hemoglobinopathies are associated with increased available serum iron and susceptibility to infection (63, 64). The inflammatory response results in further iron withholding in serum, including suppression of iron absorption from the diet and increased macrophage iron retention, creating an anemic environment (hyposideremia) (65). Hepcidin is upregulated by interleukin-1α, interleukin-1β, interleukin-6, and sustained H_2O_2 (66, 67). Increased hepcidin production by the liver and spleen during inflammation is responsible for triggering ferroportin degradation and reduced iron absorption (68).

Another facet of iron withholding is the rapid expulsion of iron-sequestering proteins. Hepatocytes secrete ferritin in response to interleukin-1β, whereas interleukin-6 stimulates haptoglobin and hemopexin secretion (69–71). Lactoferrin is produced by neutrophils at sites of infection to restrict iron availability (72). Neutrophil secretion of calprotectin (S100A8/A9) is induced in response to infection in order to restrict manganese, zinc and iron availability (73, 74). Calprotectin is a calcium-binding S100A8/A9 heterodimer or heterotetramer that exhibits broad spectrum growth inhibition

of infectious microbes through sequestration of the aforementioned nutrients (75). Each subunit binds two calcium ions, which help to facilitate greater binding affinity for two transition metal ions at the heterodimer interface (76, 77). In addition to calprotectin, extracellular S100A7 and S100A12 are present in high concentration in inflamed tissue, where they act as damage-associated molecular patterns and elicit immunomodulatory activity (78, 79). Serum transferrin concentration, on the contrary, is maintained during inflammation, however saturation remains low as this iron source is continued to be depleted by erythropoiesis (80–82). Iron sequestration as a method of nutritional immunity is a forefront function of the innate immune system to help combat infection.

1.3 Host-iron acquisition strategies of pathogenic eukaryotes

1.3.1 Iron acquisition by pathogenic fungi

Iron is an essential nutrient for nearly all species that infect humans, and pathogens must elaborate means to overcome host iron withholding to successfully colonize and cause disease. The essentiality of iron necessitates a diversity of mechanisms to acquire iron from a variety of host sources, to better ensure pathogen survival. Pathogenic fungi, like many other microorganisms, upregulate iron acquisition machinery upon sensing a low iron environment such as the mammalian host. Thus, lack of iron serves as a signal for transition from a commensal to a more invasive lifestyle. Aft1p and Aft2p transcription factors regulate iron acquisition systems by binding iron-responsive elements in target gene promoter regions to activate gene expression, and control the iron regulon in yeast (83, 84). Three predominant iron acquisition strategies have been described: iron

reduction and transport, ferric-siderophore uptake and heme acquisition (85). The various strategies are not exclusive and may occur simultaneously.

Opportunistic fungal pathogens use coupled iron-reduction and transport for iron acquisition from host chelating molecules, including transferrin and lactoferrin. Cell surface, non-specific metalloreductases Fre1 and Fre2 reduce iron at the cell surface (86, 87). Ferrous iron is subsequently oxidized by Fet3 and translocated across the membrane by the Ftr1 permease, with oxygen, copper, and heme as cofactors, serving as a high-affinity iron acquisition pathway (88–90). Reductive iron uptake is required for full virulence of fungal species, such as *Candida albicans* and *Cryptococcus neoformans*, in animals (91, 92).

High affinity non-reductive iron uptake is mediated by siderophores - low molecular weight (typically <1 kDa), high-affinity iron binding compounds that many microbes produce and are able to strip iron from host glycoproteins (such as transferrin and lactoferrin) (4, 93, 94). Most bacteria and fungi synthesize and secrete siderophores, which bind extracellular ferric iron and deliver it to microbes expressing a receptor for that specific siderophore. Fungi typically produce siderophores bearing hydroxamate moieties to coordinate iron, although many fungal species transport other types of siderophores from heterologous species as well (ie. catechol siderophores produced predominantly by bacteria) (85). This xenosiderophore ‘sharing’ is a cooperative behaviour in microbial communities where individual metabolic cost is outweighed by group benefit. Additionally, siderophore-iron may be reduced at the cell surface (as explained above) when siderophores are relatively abundant (95). At lower siderophore concentrations, siderophore-specific membrane transporters are required for uptake of

ferric siderophores from the external environment (95, 96). Fungal siderophore transport and specificity of receptors has been comprehensively reviewed elsewhere (88, 97). Siderophores are discussed in further detail in subsequent sections. Reliance on reductive iron uptake versus siderophore acquisition differs between species and one or both may be important to certain species of fungi for pathogenesis. Although *Aspergillus fumigatus* is capable of iron reduction at the cell surface, this activity is insufficient to fully compensate for the absence of siderophore biosynthesis and acquisition, which is furthermore essential for virulence in animals (98, 99). Moreover, intracellular siderophores help maintain iron storage and distribution, as well as promoting germination and resistance to oxidative stress (100).

The heme-dependent growth of several fungal species has been demonstrated (101–104). The most characterized of these is *C. albicans* iron acquisition from heme and hemoglobin. *C. albicans* binds erythrocytes using complement-receptor like molecules, and secretes a hemolytic mannoprotein to exhibit red cell lysis (105–107). Surface-exposed, cell wall-anchored CFEM domain proteins Rbt5 and Rbt51 bind hemoglobin and extract heme at the cell surface (108). CFEM domains are unique to fungi, contain eight cysteine residues of conserved spacing, and are important for pathogenicity (109, 110). Rbt5 and cell-wall associated Pga7 (not surface-exposed) exchange heme as a relay to the plasma membrane upon which heme is endocytosed in a Rbt5-dependant manner (111, 112). A type I myosin and the ESCRT system are required for endocytosis, whereas a vacuolar ATPase and a heme oxygenase are further required for heme-iron utilization (112, 113). The heme oxygenase Hmx1 is required for virulence in the mammalian host (114). Other pathogenic fungal species may use similar means for heme-iron

procurement. In *C. neoformans*, the ESCRT pathway is also important for heme-iron acquisition, and extracellular, heme-binding Cig1 is a potential hemophore that contributes to virulence (102, 115).

1.3.2 Iron acquisition by human parasites

Human parasites exhibit great diversity in terms of lifecycles and associated niches and as such elicit diverse strategies of obtaining host nutrients such as iron. *Trypanosoma brucei*, *Leishmania* spp., *Plasmodium* spp., and hookworms cause substantial disease burden in humans and will be the focus here.

T. brucei is a flagellated protist that uses an insect vector (tsetse fly) to transmit between mammalian hosts and is known to cause sleeping sickness in humans. Trypanosomes reside in the mammalian bloodstream, lymphatic system and interstitial spaces and may progress to the central nervous system in later stages of infection (116). They exploit host transferrin via receptor-mediated endocytosis at the flagellar pocket (invagination of the plasma membrane at the flagellum) (117). Structurally distinct from human transferrin receptor, the trypanosome counterpart is regulated by iron availability and encoded by ESAG6 and ESAG7 to form a heterodimer (118, 119). Upon endocytosis, acidification releases iron from the transferrin-receptor complex and is transported to the cytosol by a mucolipin-like protein (120, 121). Proteolytic cleavage of the transferrin receptor precedes endosomal cycling back to the cell surface (120). *T. brucei* may also bind lactoferrin in a similar manner to macrophages via glyceraldehyde-3-phosphate dehydrogenase (GAPDH), although this remains to be fully elucidated (122, 123). Trypanosomes are heme auxotrophs and acquire heme-iron mainly through a haptoglobin-hemoglobin receptor (HpHbR) exclusively expressed when in a mammalian

host environment (124, 125). The surface anchored glycoprotein localizes to the flagellar pocket region and undergoes endocytosis prior to heme-iron extraction (125). The free-heme transporter Hrg is exclusively expressed during insect vector-stage growth (125, 126). Trypanosome-induced macrophage hyper-activation leads to increased erythrophagocytosis and iron retention, leading to progressive anemia during chronic infection (127).

Leishmania protozoan parasites cause a spectrum of clinical manifestations ranging from cutaneous lesions to visceral infections, termed leishmaniasis (128). Motile, extracellular promastigotes multiply in the sand fly gut, whereas upon mammalian inoculation, infective parasites reside as non-motile amastigotes in the phagolysosomal system of macrophages (129). The *Leishmania*-carrying phagosome fuses with other vacuoles and endosomal compartments which carry nutrients, such as transferrin (as transferrin-receptor complex) as a source of iron for the parasite (130–132). Furthermore, the phagosomal acidity may release iron from transferrin, or transferrin may be degraded by secreted cysteine proteases (or released by lysed parasites) (131, 133). *Leishmania* plasma membrane-associated ferric reductase LFR1 reduces Fe^{3+} to Fe^{2+} using NADPH, FAD and heme as cofactors (134, 135). Fe^{2+} is transported to the cytosol by LIT1, serving as a non-specific, reductive iron transport mechanism (136). LFR1 and LIT1 are iron-regulated and detected on intracellular amastigotes and iron-starved promastigotes (135, 137). *Leishmania* may also be able to endocytose and degrade transferrin in cysteine protease-rich compartments, although this may be a non-specific interaction as lactoferrin and albumin also bind the receptor (134, 138). Only the last three enzymes of the heme biosynthetic pathway are present in *Leishmania* trypanosomatid parasites, thus

requiring external heme or protoporphyrin IX (PPIX) for growth (139). The *Leishmania* hemoglobin receptor localizes to the flagellar pocket and upon hemoglobin binding is endocytosed (140, 141). Endosomal maturation is mediated by Rab 5 and Rab 7, and lysosomal hemoglobin is degraded to release heme, which is translocated to the cytosol by the ATP-binding cassette protein LABCG5 (142–144). *Leishmania* heme utilization correlates with severity of patient anemia (145). Transmembrane LHR1 transports external or lysosomal heme directly into the cytosol and is important for causing pathogenesis in mammals (146, 147). Furthermore, LHR1 is proposed to be essential for promastigote viability, and orthologs are present in several *Trypanosoma* genomes as well (146, 148, 149).

At least five *Plasmodium* species are able to cause malaria in humans, with *P. falciparum* being the most deadly. Using a mosquito vector of transmission, the parasitic protist enters the human bloodstream and travels to the liver to infect hepatocytes. Infected hepatocytes eventually lyse to expel non-motile merozoites into the bloodstream to infect (and further re-infect) red blood cells (150). The intraerythrocytic stage of infection offers a gold mine of heme for the pathogen, however it actually uses hemoglobin primarily as a source of amino acids rather than iron, albeit still annihilating the host iron pool. Furthermore, iron deficiency is protective against malaria and iron supplementation increases risk of malaria (151). Initially, the parasite folds into itself in an actin-independent process to invacuate a large ‘gulp’ of host red cell cytoplasm as a food vacuole (152). Later on, host hemoglobin is endocytosed with other erythrocyte components into cytostomes, and further targeted for acidification and proteolytic degradation in an actin-dependent process regulated by Rab 5 (152–154). Together, *P.*

falciparum uses various means of internalizing large amounts of host hemoglobin, which are directed to digestive vacuoles for degradation as a source of amino acids (155). Hemoglobin is digested sequentially by aspartic proteases, cysteine proteases, metalloproteases and aminopeptidases (156, 157). The large amount of toxic free heme is polymerized into pigmented crystals termed hemozoin (158, 159). Heme groups dimerize via iron-carboxylate bonds, and dimers are bonded via proprionate hydrogen bonding (160, 161). Protease and hemozoin-formation inhibitors block parasite development and detoxifying capabilities, hinder viability and form potent antimalarial drugs (159, 162–164). This is exemplified by the potency of quinolone drugs for *P. falciparum* infection as they inhibit hemozoin synthesis and the parasites ability to detoxify heme, as it produces reactive oxygen species. Lysed protists release hemozoin to be ingested by phagocytes and accumulate in the reticuloendothelial system, which leads to a darkening of the liver and spleen after chronic or repeated infection (165, 166).

Human hookworm infection results in intestinal blood loss due to hematophagous feeding and causes substantial iron deficiency anemia in the developing world (167). Intensity of hookworm infection directly correlates to intestinal blood loss and host anemia. Hosts with lower iron stores, such as children and pregnant women, are more vulnerable to hookworm-induced anemia (168). *Necator* and *Ancylostoma* species cause the majority of hookworm disease in humans (169). The nematode helminths infect the mammalian host at the larval stage by oral ingestion or skin penetration (170). Larvae in circulation travel to the heart and lungs, penetrate the alveolar membrane and enter the trachea for migration to the gastrointestinal tract (171). The larvae mature in the gastrointestinal tract and attach to the duodenal mucosa, where they can reside for years

(172). Host capillaries are lacerated and secreted anticoagulant peptides help facilitate ingestion of blood (173). Ingested red cells are lysed in the hookworm gut by a pore-forming, membrane-bound hemolysin (174). Hemoglobin digestion is similar to that of *P. falciparum*, using aspartic, cystic and metalloproteases, albeit expressed in and released from the intestinal brush border of the helminth (175, 176). Glutathione-S-transferases are thought to play a role in heme detoxification as several of these bind heme with high affinity (177–180). Additionally, vaccination using recombinant hookworm glutathione-S-transferases elicited better protection against infection in hamsters and dogs (177, 181).

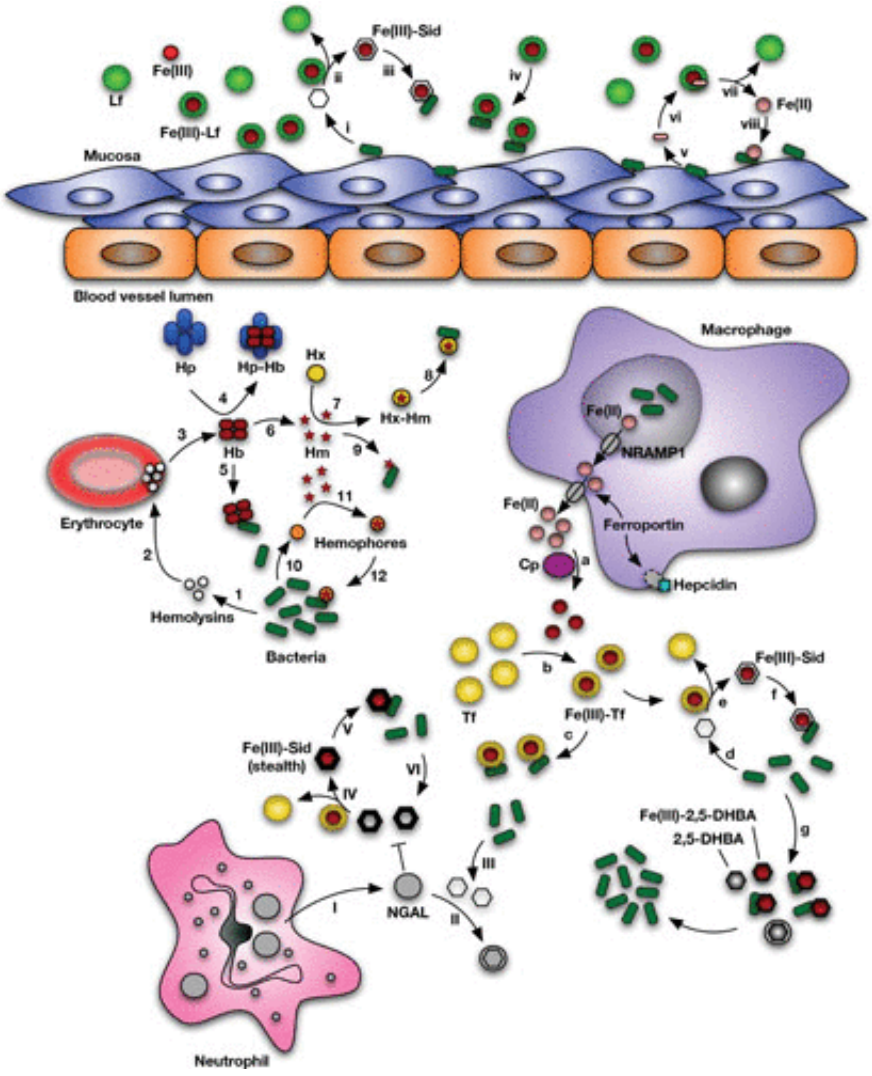
1.4 Host-iron acquisition strategies of pathogenic prokaryotes

1.4.1 Active transport across membranes

The host confines iron within proteins to mitigate the generation of damaging reactive species, and to help prevent infectious disease (nutritional immunity). As such the concentration of free iron in body fluids can be restricted up to 10^{-24} M, far below that required to support microbial growth (10^{-6} M) (3, 182). Invasive pathogens respond to this nutritional immunity by elaborating specialized, high-affinity iron transport mechanisms able to circumvent host immune effectors.

Bacterial iron acquisition strategies generally rely on free (un-liganded) ferrous iron, heme, and siderophore transport (Figure 1-1). All of these mechanisms rely on active transport across bacterial membranes, involving ATP-binding cassette (ABC) transporters. In Gram-negative bacteria, the presence of an outer membrane necessitates specific receptors for substrate recognition for larger compounds (>600 Da) including

Figure 1-1: Nutritional immunity and strategies of pathogens to overcome host iron limitation. Representative depiction of various strategies bacterial pathogens employ to attain host-iron. At the mucosa, lactoferrin (Lf) binds iron yet some bacteria are able to obtain this iron through secretion of siderophores (i – iii), binding Lf (iv), or secreting reductases (v – viii) to reduce iron to Fe(II). Bacteria may lyse cells through secreted hemolysins and obtain released heme/hemoglobin (Hm/Hb), as well as host-scavenged hemopexin-heme (Hx-Hm) and haptoglobin-hemoglobin (Hp-Hb) iron (1 – 12). Macrophages remove iron from phagosomes via natural resistance macrophage protein 1 (NRAMP1) and ferroportin. Membrane-bound ferroportin is degraded when bound by the iron homeostasis hormone hepcidin. Ceruloplasmin (Cp) oxidizes secreted Fe(II), and Fe(III) is bound by transferrin (Tf) in circulation (a, b). Transferrin-iron may be procured via direct transferrin-binding (c), by secreted siderophores (Sid) (d – f), or host-produced compounds such as 2,5-DHBA (g). Siderocalin (NGAL) produced by neutrophils sequesters several siderophores (I – III), although some 'stealth' siderophores (Sid stealth) are unrecognized by NGAL (IV – VI). Lf, lactoferrin; Hm, heme; Hb, hemoglobin; Hx-Hm, hemopexin-heme; Hp-Hb, haptoglobin-hemoglobin; Cp, ceruloplasmin; Tf, transferrin; Sid, siderophore; NGAL, siderocalin; Sid (stealth), stealth siderophore. Figure procured from (183).


Fe(III)-Sid
Fe(III)
Lf
Fe(III)-Lf
Fe(II)
Mucosa
Blood vessel lumen
Macrophage
Hp
Hp-Hb
Hx
Hx-Hm
Hb
Hm
Erythrocyte
Hemophores
Hemolysins
Bacteria
NRAMP1
Ferroportin
Cp
Hepcidin
Tf
Fe(III)-Tf
Fe(III)-Sid
(stealth)
Fe(III)-2,5-DHBA
2,5-DHBA
NGAL
Neutrophil

transferrin, lactoferrin, hemoglobin, and siderophores (184). The outer membrane receptors are β-barrel pores made of antiparallel β-sheets, with an N-terminal globular 'plug' that seals the pore until substrate-binding and energy transfer cause conformational changes to allow iron, heme or siderophore passage (185). The energy for this process is derived from the proton motive force across the inner membrane, channeled through the TonB, ExbB and ExbD complex (186, 187). The substrate is bound by a freely soluble, bilobal, periplasmic binding protein (PBP) with specificity for that ligand (188). The substrate-PBP complex is recognized by an inner membrane permease which translocates the substrate across the lipid bilayer using energy generated by an associated ATPase (185). The inner membrane permease is typically a homo or heterodimer spanning the membrane whereas the associated ATPase is a cytoplasmic homodimer (189, 190). Gram-negative bacterial active transport has been characterized and reviewed in detail elsewhere (184–190).

Gram-positive bacteria employ a similar means of active transport utilizing an integral membrane ABC transporter, although the PBP-functionally equivalent, high affinity receptor is linked to the cytoplasmic membrane by a lipidation motif. The Gram-positive cell wall is composed of a meshwork of peptidoglycan, polysaccharides, teichoic acid, and associated proteins of varying glycosylation. Although siderophores are able to pass through this meshwork, heme must be extracted from host proteins prior to cell wall transversion (191).

1.4.2 Free ferrous iron transport

Ferric iron predominates under oxidizing conditions and may become bioavailable via siderophores or reduction to ferrous iron. Ferrous iron predominates in anoxic, low pH

environments and can become an important iron source for bacterial pathogens in certain situations. The ferrous iron transport (Feo) system is present in many bacteria, although most extensively characterized in *Enterobacteriaceae* (192, 193). FeoB is a cytoplasmic membrane-spanning permease with a cytosolic GTPase domain driving active transport (194). Cytosolic FeoA interacts with the G-protein domain of FeoB and is required for FeoB-iron transport (195). FeoC is only present in γ-proteobacteria Feo systems and although initially thought to act as a transcription factor, more recent evidence indicates FeoC interaction with the FeoB N-terminal region for protection from FtsH-mediated proteolysis (196, 197). The putative FeoC-associated iron-sulfur (Fe-S) cluster may function as a redox/iron sensor as Fe-S cluster reduction renders it less susceptible to Lon-mediated proteolysis. In oxidizing environments, the FeoC Fe-S cluster is thought to become oxidized and degraded, facilitating FeoB-FeoC release and FeoB proteolysis as a means of post-translational regulation (198). Feo homologs exist in the genomes of many Gram-positive and Gram-negative bacteria.

ABC-type divalent metal transport has been described for many Gram-positive and Gram-negative species. SitABC (homologous transporters also termed MntABC or MtsABC) homologs across species vary in transport of ferrous iron, manganese or both. *Staphylococcus epidermidis* Sit is regulated by both iron and manganese availability through SirR, which represses transcription in presence of Mn^{2+} or Fe^{2+} (199–201). *S. epidermidis* Sit is highly upregulated in iron limited conditions such as those in vivo (202–204). The *S. aureus* homolog (MntABC) is a dedicated manganese transporter, repressed by MntR in the presence of Mn^{2+} (205). The streptococcal homolog (MtsABC) is regulated by Mn^{2+} and Fe^{2+} and can bind several divalent metals, with a preference for

ferrous iron over copper, manganese and zinc (206–208). *Salmonella enterica* SitABCD has high affinity for manganese over iron, although is regulated by both manganese and iron (209, 210). Sit systems in *Shigella* species and pathogenic *E. coli* are orthologous to YfeABCD in *Yersinia* and share overlapping functions with Feo and the manganese transporter MntH, although there are some variations in regulation between the metal acquisition systems which may play a role in response to oxidative stress (211–215).

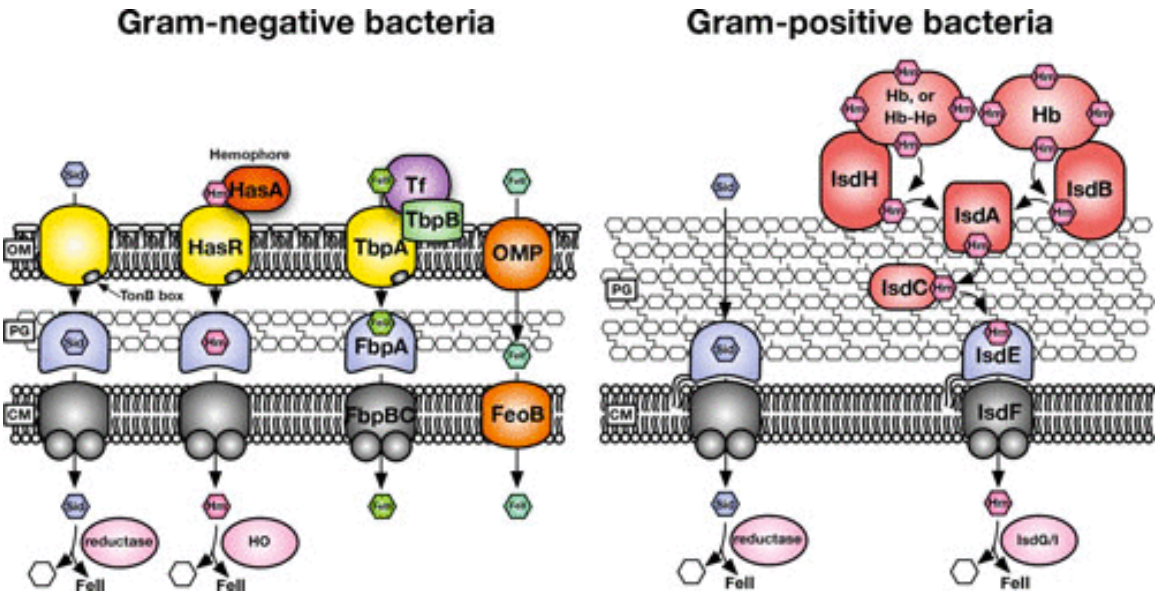
Although best characterized in eukaryotes, some bacteria may also possess ferric reductase activity as a mechanism of making iron bioavailable for acquisition. Extracellular and membrane-associated ferric reductase activity has been described for a variety of bacteria, and biochemical characterization of molecular determinants for this iron uptake strategy has been progressing (216–220). *E. coli* EfeUOB (YcdNOB) is upregulated in low iron and low pH conditions, and is involved in ferrous iron acquisition (221, 222). EfeU is homologous to the Ftr1 permease in yeast (221). EfeO is periplasmic with two potential metal binding sites and an N-terminal cupredoxin domain, which implies it functionally analogous to the Fet3 oxidase (223). EfeB is a periplasmic homodimer secreted by the twin-arginine translocation (Tat) system as a folded protein (224, 225). *Bacillus subtilis* also encodes *efeUOB* whereas orthologous genes are termed *fepCAB* in *Listeria monocytogenes* and *S. aureus* (226–228). Although EfeB and FepB have been implicated in heme deferrochelatase activity, removing iron from heme while keeping the tetrapyrrol ring intact, there is some contention to this proposed activity as PPIX is toxic and lacks a degradative pathway (224, 229, 230). Overexpression of YfeX (cytoplasmic EfeB homolog) results in intracellular porphyrin accumulation, likely from oxidation of endogenously synthesized porphyrinogen, and it may be that homologous

EfeB/FepB oxidize porphyrinogens to porphyrins as well (230). *L. monocytogenese* FepB exhibits ferric reductase activity, whereas *B. subtilis* EfeB oxidizes ferrous iron for uptake of ferric iron by EfeUO, indicating there may be functional variation with varied environmental conditions (226, 227). Natural substrates for EfeB/FepB-mediated oxidation or reduction have not been established and could entail iron-binding compounds in the host such as transferrin and siderophores (191). Additionally, the secretion of a fully folded EfeB/FepB with endogenous heme may be an oxidative stress response to counter damage from reactive oxygen/nitrogen species, such as would be encountered during an immune response (respiratory burst) (225).

1.4.3 Direct iron acquisition from host proteins

Several pathogenic bacteria express surface receptors to bind host iron-withholding glycoproteins and extract iron from them. *Neisseria* spp. preferentially acquire iron from host transferrin through the TbpAB outer membrane receptor, which is essential for virulence in human infection (Figure 1-2) (231, 232). Lipoprotein TbpB binds transferrin and facilitates interaction with transmembrane TbpA to extract iron by conformationally manipulating transferrin in a TonB-dependent manner (233, 234). LbpAB also utilize TonB/ExbB/ExbD and function in a similar manner to transport lactoferrin-iron (235–237). Periplasmic ferric iron is bound by FbpA and further shuttled through FbpBC into the cytoplasm (185, 238, 239). Homologous receptors that bind and extract iron directly from transferrin and lactoferrin also exist in *Pasteurellaceae* and *Moraxellaceae* (235, 240–242).

Figure 1-2: Schematic of iron-acquisition mechanisms in Gram-negative and Gram-positive bacteria. Depictions of cell envelope proteins mediating uptake of siderophores (Sid, unspecified transporter), heme (Hm, HasAR), transferrin (Tf, TbpAB) and ferrous iron (FeII, FeoB) In Gram-negative bacteria. Siderophore (unspecified transporter) and heme acquisition from hemoglobin (Hb) and hemoglobin-haptoglobin (Hb-Hp) via Isd proteins is depicted for Gram-positive bacteria. Further detail on transporters is within the main text. OM, outer membrane; PG, peptidoglycan; CM, cytoplasmic membrane; Sid, siderophore; Hm, heme; HO, heme oxygenase; Tf, transferrin; Hb, hemoglobin; Hb-Hp, hemoglobin-haptoglobin. Figure procured from (183).


Gram-negative bacteria
Gram-positive bacteria
Hemophore
HasA
HasR
Tf
TbpB
TbpA
OMP
FbpA
FbpBC
FeoB
OM
PG
CM
TonB box
reductase
HO
FeII
Hb, or
Hb-Hp
Hb
IsdH
IsdB
IsdA
IsdC
IsdE
IsdF
reductase
IsdG/I
FeII

1.4.4 Indirect iron acquisition from host proteins: siderophores

Siderophores are low molecular weight (~500 – 1500 Da), high affinity Fe^{3+} chelators produced by a large number of bacteria, fungi and plants to facilitate iron delivery in response to iron limitation (93). There is stunning diversity between siderophore structures and they may be classified as to their iron-coordinating moieties (hydroxamate, catecholate and hydroxycarboxylate), although certain siderophores may encompass more than one type of moiety to coordinate iron as a 'mixed type' siderophore. The small ferric chelators scavenge ferric iron and compete for it with other iron chelates. Siderophore-iron association constants can exceed 10^{-52} M, and as such they are able to remove iron from host glycoproteins including transferrin and lactoferrin (association constants of ~10^{-20} M) (49, 243, 244). The ferric-siderophore complex is recognized at the surface of cells expressing a cognate receptor for that specific substrate, engendering the organism with an iron-sourcing advantage (Figure 1-2).

1.4.5 Siderophore biosynthesis

Siderophore production occurs within the cell and may be through nonribosomal peptide synthetase(NRPS)-dependent or NRPS-independent synthesis. NRPS synthesis involves successive incorporation of non-proteinogenic amino acids and derivatives into an elongated chain, without the use of an RNA template (245). In addition to assembly of several common Gram-negative enteric siderophores including enterobactin, vibriobactin and yersiniabactin, NRPS mechanisms are also used to produce antimicrobial peptides such as penicillin and vancomycin (96, 246, 247). Independent of ribosomal peptide assembly, a multimodular NRP synthetase activates or modifies covalently linked reaction intermediates to control the iterative production of the compound via its catalytic

domains (248). Enterobactin synthesis is arguably the most extensively-characterized NRPS-dependent siderophore biosynthetic pathway. *entA-F* genes in *E. coli* encode subunits of the multienzyme complex that converts chorismate (intermediate in aromatic amino acid biosynthesis) to the catechol 2,3-dihydroxybenzoic acid (2,3-DHB). Three molecules of 2,3-DHB are linked to 3 molecules of L-serine via three amide and three ester bonds to produce the catecholamine siderophore enterobactin (249–252). The catecholamine siderophore petrobactin is a rare example of a product assembled by both NRPS-dependent and independent synthetases (253).

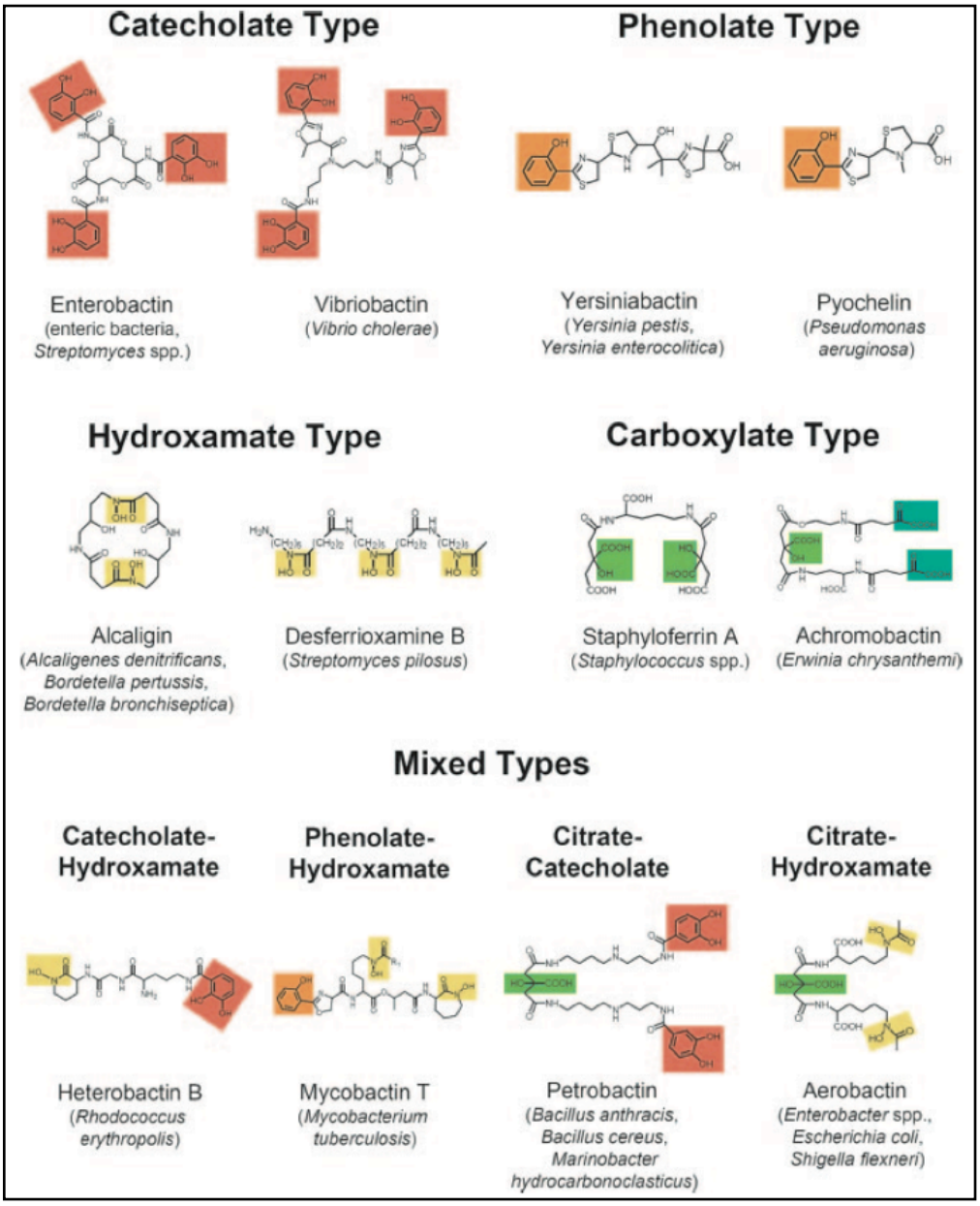
NRPS-independent synthesis, on the other hand, is performed by individual cytoplasmic synthetases that catalyze amide or ester bond formation between dicarboxylic acids (commonly citrate, succinate or α-ketoglutarate), diamines, alcohols or amino alcohols. The alternating siderophore subunits are linked by condensation reactions by the synthetases, which are not homologous to NRPS pathway counterparts (254). Aerobactin is produced by several pathogenic Gram-negative bacteria (including *E. coli*), contains hydroxamate and hydroxycarboxylate iron-coordinating moieties, and is the archetypal pathway for NRPS-independent siderophore biosynthesis. A four-gene cluster encodes IucA-D, which modify and condense two molecules of L-lysine with a citric acid linker in a presumably ATP-dependent process (255). A model has been proposed, based on phylogenetic analyses, to classify NRPS-independent synthetases based on substrate specificity (256). Type A enzymes such as IucA are specific for citrate and condense a prochiral carboxyl group of citrate with an alcohol or amine functional group of another substrate. Type B enzymes have specificity for α-ketoglutarate and substitute the C5 carboxyl group for a citrate carboxyl group. Type C enzymes, including

IucC, are specific for citrate or succinate derivatives and condense the monoamide/monoester with an amine or alcohol. The multitude and diversity of siderophore structures infers that these ‘guidelines,’ formed on the basis on sequence analysis, are simply a few of the more characterized biosynthesis mechanisms, and many exceptions exist. Microorganisms may have great variations in the ways they produce a certain NRPS-independent synthesized siderophore. Modifications to precursor molecules, such as oxidation, isomerization, or decarboxylation are catalyzed by enzymes encoded by genes adjacent to, or within siderophore biosynthetic loci. Many other siderophores have been suggested to be produced by NRPS-independent methods including vibrioferrin, rhizobactin, alcaligin, and desferrioxamine (257–260).

1.4.6 Siderophore structure

Although there is great diversity between siderophore structures, there are common features that make them very good iron acquisition mechanisms. They are small, electronegative compounds that coordinate Fe^{3+} with six donor coordination sites, at best. If less than six iron-coordinating donor atoms are present, surrogate electronegative bonds with solution molecules may facilitate further coordination. Higher order siderophore to iron stoichiometry than the typical 1:1 is possible when fewer coordination moieties exist within the siderophore. Examples include 2(iron):3 rhodoturolic acid, 1:3 cepabactin, and 1:1 or 2 pyochelin complexes (261–263). Mixed complexes of cepabactin and pyochelin with iron in 1:1:1 ratios have also been observed (262). Iron-coordination by siderophore and siderophore-like (pseudosiderophore) complexes is quite variable and dependent on the structure, concentration and protonation of compounds in the microenvironment.

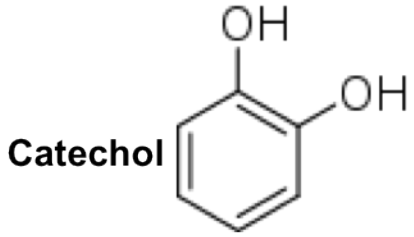
Figure 1-3: Molecular structure of representative siderophores. Structures of several siderophores including non-ribosomal peptide synthetase (NRPS)-dependent enterobactin, vibriobactin, yersiniabactin and pyochelin, as well as NRPS-independent aerobactin, desferrioxamine, vibrioferrin, staphyloferrin A, and staphyloferrin B, among others. Figure taken from (96).


Catecholate Type
Phenolate Type
Enterobactin
(enteric bacteria, Streptomyces spp.)
Vibriobactin
(Vibrio cholerae)
Yersiniabactin
(Yersinia pestis, Yersinia enterocolitica)
Pyochelin
(Pseudomonas aeruginosa)
Hydroxamate Type
Carboxylate Type
Alcaligin
(Alcaligenes denitrificans, Bordetella pertussis, Bordetella bronchiseptica)
Desferrioxamine B
(Streptomyces pilosus)
Staphyloferrin A
(Staphylococcus spp.)
Achromobactin
(Erwinia chrysanthemi)
Mixed Types
Catecholate-Hydroxamate
Phenolate-Hydroxamate
Citrate-Catecholate
Citrate-Hydroxamate
Heterobactin B
(Rhodococcus erythropolis)
Mycobactin T
(Mycobacterium tuberculosis)
Petrobactin
(Bacillus anthracis, Bacillus cereus, Marinobacter hydrocarbonoclasticus)
Aerobactin
(Enterobacter spp., Escherichia coli, Shigella flexneri)

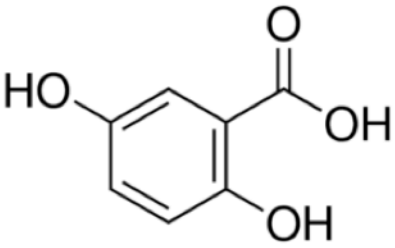
In general, three major iron-binding moieties act as coordination motifs, which are catecholates/phenolates, hydroxamates, and (α-hydroxy-)carboxylates (Figure 1-3). Predominantly bidentate coordination motifs employ hydroxyl groups separated by three covalent bonds. Enterobactin employs three catecholate (ortho-dihydroxybenzene) groups (2,3-DHB precursors) to coordinate iron with six bonds for an extremely stable structure (Figure 1-3). In fact, most catecholate siderophores use 2,3-DHB as a precursor for synthesis, and this compound is alone capable of coordinating iron in a 3:1 ratio. Some bacteria even secrete 2,3-DHB as a means to attain environmental iron (264, 265). Mammals encode an EntA (enterobactin synthesis enzyme) homolog responsible for synthesis of 2,5-DHB (266). The structural similarity between 2,3-DHB and 2,5-DHB affords microbial pathogens the ability to coopt 2,5-DHB as well as 2,3-DHB to attain iron through the same transporter (Figure 1-4). Therefore the mammalian host suppresses 2,5-DHB production upon TLR stimulation with LPS (except TLRs 5 and 7) (267). Mammalian catecholamine stress hormones are too structurally similar to 2,3-DHB and may also promote pathogen growth (Figure 1-4). Stress hormones such as epinephrine, norepinephrine and dopamine interact with transferrin and lactoferrin to reduce Fe^{3+} to Fe^{2+}, thereby liberating it (268). The hormones complex with the released iron in a 3:1 ratio for hexacoordinate symmetry and act as pseudosiderophores to facilitate iron acquisition by a number of pathogens (269–271).

Hydroxamate iron-coordination motifs use a carbonyl and adjacent aminohydroxyl group as a bidentate iron chelate. These types of siderophores are frequently produced by fungal species, including coprogen and ferrichrome, among others (272). Carboxylate motifs employ dual α-hydroxycarboxylates derived from citrate

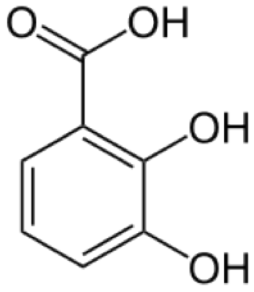
Figure 1-4: Molecular structure of iron-coordinating catecholate compounds. The bacterial siderophore precursor 2,3-dihydroxybenzoic acid (DHBA), and the other, mammalian-produced molecules coordinate iron with bidentate symmetry in a 3:1 catecholate compound to ferric iron ratio.



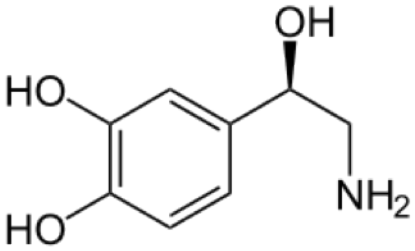
Catechol



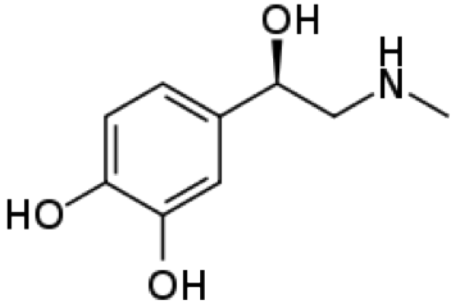
2,5-DHBA



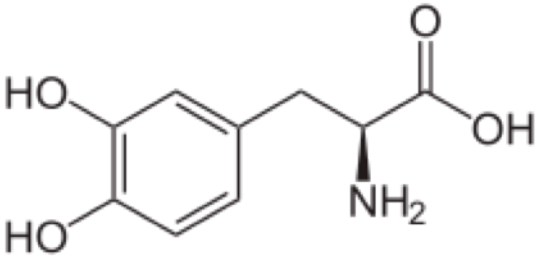
2,3-DHBA



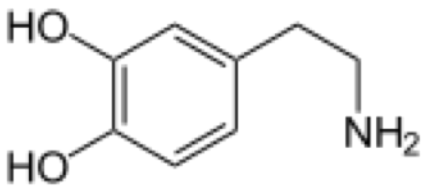
Norepinephrine
(Noradrenaline)



Epinephrine
(Adrenaline)



L-DOPA
(Levodopa)



Dopamine

or carboxylic acid moieties to coordinate iron in a similar manner, such as in staphyloferrin A and achromobactin (273, 274). Many siderophores contain a combination of the three predominant coordination motifs, including petrobactin and aerobactin (253, 275). Multiple bi- or tridentate entities form very stable iron-coordination complexes which may appropriate iron directly from host proteins such as transferrin, and constitute an important source of iron for invading pathogens (93, 276).

1.4.7 Siderophore transport

Systems for bacterial extracellular removal of iron from siderophores are not well known (as they are in eukaryotes), and prokaryotes primarily transport ferric siderophores to the cytoplasm prior to iron release. Gram-negative bacteria must import ferric siderophores across both membranes through outer membrane receptors, Ton-channeled energy, and PBP-permease-mediated translocation, as detailed previously. Gram-positive bacteria employ a lipoprotein-ABC transporter for ferric siderophore acquisition (Figure 1-2). Genetic loci for siderophore transporters are often proximal to the cognate siderophore biosynthetic locus in the genome. Siderophore-binding proteins may exhibit some degree of indiscrimination towards substrates. *E. coli* outer membrane FepA transports ferric-enterobactin, 2,3-DHB and 2,5-DHB, however cannot transport corynebactin, which is structurally similar to enterobactin but has opposite chirality, indicating a ferric-coordination chemistry is required for transport (267, 277). PBPs exhibit more plasticity than outer membrane receptors, including FhuD which binds several hydroxamate siderophores including ferrichrome, aerobactin, coprogen and ferrioxamine, which each have specific outer membrane receptors (278, 279). Fhu homologs exist in Gram-positive species as well, with the *S. aureus* Fhu transporter best characterized among them (280–

282). *S. aureus* actually elaborates dual FhuD1 and FhuD2 lipoproteins, which both interact with the FhuCBG transporter, although have variable substrate affinity and plasticity (283, 284).

1.4.8 Siderophore-iron removal

Once the siderophore enters the cytoplasm, both Gram-negative and Gram-positive bacteria employ similar means to extract the coordinated iron. Iron may be reduced and discharged via cellular ferric siderophore reductases, although most of these are nonspecific. Specialized enzymes are however produced to modify or degrade ferric siderophores such to release iron through a loss of complex stability, allowing other cellular iron-binding compounds access. Both reductive and degradative siderophore-iron removal processes occur in *E. coli*. Enterobactin, which binds iron more tightly than several hydroxamates, must be degraded by FesA prior to iron reduction and subsequent release, whereas hydroxamate siderophores are simply kept intact and recycled externally once iron is reduced by FhuF (285–287). An iron-sulfur cluster in FhuF mediates reduction, lending this protein to be a prime example of an iron-regulated reductase with specificity for a class of siderophores. Alternatively, YqjH contains a flavin adenine dinucleotide (FAD) binding domain, interacts with siderophores and is also involved in ferric iron reduction, with a much broader range of substrates (288). Somewhat similar systems exist in Gram-positive pathogens. Oxidoreductase IruO binds FAD and NADPH and helps to facilitate iron release from heme and desferrioaxamine (hydroxamate) in *S. aureus* (289, 290). Nitroreductase NtrA also aids in iron release from heme in addition to ferric-staphyloferrin A (290).

1.4.9 Host siderophore defense and stealth siderophores

The mammalian host responds to the production and usage of siderophores by infectious pathogens through siderophore sequestration, and limiting pathogen access to siderophore-iron (Figure 1-1). Another important tool in the arsenal of innate immunity is neutrophil secreted siderocalin (also referred to as lipocalin 2, 24p3, uterocalin and neutrophil gelatinase-associated lipocalin; NGAL), released by degranulation in response to TLR 2, TLR 4 or TLR 5 stimulation (291, 292). Siderocalin is part of the lipocalin superfamily which share an eight-stranded β-barrel conformation forming a central cavity for ligand-binding (293). Compared to other lipocalins, the siderocalin ligand-binding site is relatively broad, shallow, and lined with polar and positive residues to facilitate binding to a variety of catecholate and phenolate siderophores (294, 295). It is able to bind ferric enterobactin with similar affinity as the *E. coli* outer membrane transporter FepA ($K_D \sim 10^{-10}$ M) and therefore aptly competes for this iron source (293, 296). Siderocalin can also sequester siderophore-like molecules including DHB and catecholamine stress hormones (293, 297). It has been shown to be crucial for murine protection against enterobactin-producing *E.coli*-induced mortality (298).

Pathogenic bacteria have evolved to counter the host response to siderophores by enzymatic modification of these siderophores, rendering them unable to be recognized by siderocalin, or being able to produce multiple siderophores of different class. Siderophores able to bypass siderocalin entirely are termed stealth siderophores (Figure 1-1). The *iroA* gene cluster in *E. coli* is iron-regulated and encodes siderophore-modification machinery, specifically IroB, which decorates the DHB moieties in enterobactin with glucose residues (298, 299). The resultant glycosylated enterobactin

siderophore, salmochelin, is unable to be bound by siderocalin. The structure of salmochelin had previously been elucidated from cultures of *Salmonella enterica* (300). The production of another siderophore of a different class is another common strategy among pathogens looking to bypass siderocalin-mediated immunity. In addition to enterobactin, pathogenic *E. coli* strains also express the hydroxamate stealth siderophore aerobactin (301). Various siderophores are expressed under different conditions, which may be due to dissimilar stability of ferric-siderophore complexes in various conditions, or availability of precursor molecules for their synthesis (302). Based on protonation of iron-coordinating groups, ferric hydroxamates exhibit greater stability at more acidic pH than ferric catecholates, which favor a more neutral to alkaline pH for ferric complex stability. Carboxylates are the most efficient siderophores under low pH conditions in which hydroxamates and catecholates would be more protonated (96). Pathogenic *E. coli* that can produce up to four different siderophores have been isolated, and these are likely to present advantages to bacterial fitness in various environments (303).

Bacillus produce the catecholate bacillibactin and catecholate-carboxylate petrobactin as a similar means of avoiding siderocalin-mediated iron removal (304). Bacillibactin coordinates iron via three 2,3-DHB moieties, and much like enterobactin is also sequestered by siderocalin (293, 305). Petrobactin evades siderocalin by using unusual iron coordination chemistry via two 3,4-DHB moieties and a citrate carboxylate group (306, 307). While commensal and environmental isolates typically express bacillibactin, pathogenic *Bacillus* isolates express petrobactin (308). Furthermore, petrobactin expression contributes to *B. anthracis* growth in iron-limited media, survival and proliferation within macrophages, and subcutaneous murine infection (309).

Bacillibactin had little effect in these situations, however was found to play a role in iron acquisition from insect host ferritin, further highlighting that siderophore structural diversity contributes to fitness in diverse environments (310). Although catecholates bind iron with greater affinity than siderophores of other respective classes, the host has evolved to block this microbial iron acquisition mechanism using siderocalin, necessitating for pathogenic microbes to respond with counter measures to siderocalin.

1.4.10 Heme acquisition

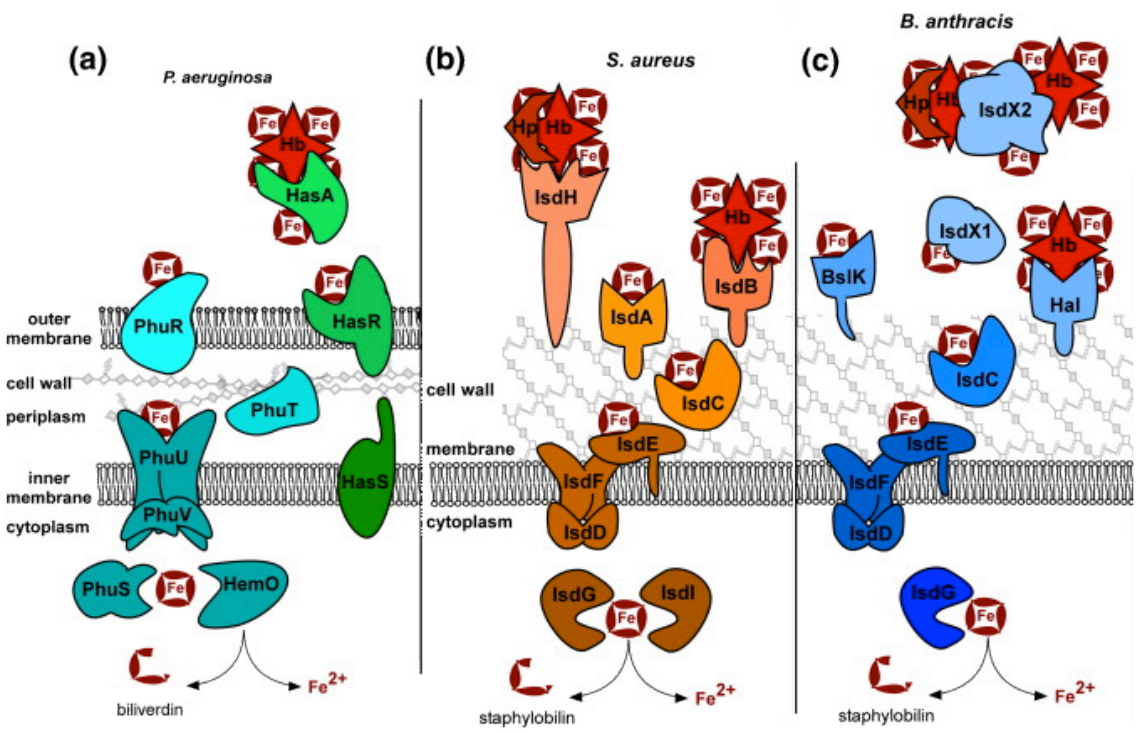
Heme represents the most abundant iron source within the mammalian host, with two-thirds of total bodily iron content in hemoglobin within circulating erythrocytes. Many pathogens have developed means to liberate hemoglobin from erythrocytes, predominantly through secretion of hemolysins. Hemolysins may be cytotoxic to not only erythrocytes but also cells of the immune system (ie. monocytes, lymphocytes, macrophages), and exert cytotoxicity through pore formation or membrane damage (311, 312). They are often secreted in response to iron depletion or a blood environment, and instrumental for pathogenesis (313, 314). Pathogens are able to appropriate heme-iron from a combination of extracellular heme, heme-hemopexin, hemoglobin and hemoglobin-haptoglobin using secreted hemophores, cell surface receptors, membrane transporters and cytoplasmic heme-degradation proteins. The intricate architecture and associations of heme-acquisition machinery underscores the significance of this substantial iron pool.

1.4.11 Heme uptake by Gram-negative bacteria

Neisseria meningitidis does not secrete hemophores, although has two outer membrane receptors for hemoproteins and is a simple starting model to describe heme acquisition by

Gram-negative pathogens. HmbR binds heme and hemoglobin at the cell surface and bipartite HpuAB binds hemoglobin and hemoglobin-haptoglobin (315, 316). HpuA serves as the coordinating lipoprotein and HpuB the β-barrel outer membrane protein, which translocate heme in a TonB-dependent manner (316). A PBP or inner membrane heme transporter are currently undefined, although cytoplasmic oxygenase HemO degrades heme to biliverdin to release iron (317). *Pseudomonas aeruginosa* is capable of both hemophore-mediated and independent heme acquisition (Figure 1-5). β-barrel, outer membrane PhuR binds heme and heme carrier proteins (hemoglobin, hemoglobin-haptoglobin, hemopexin) at the cell surface and transports heme across the membrane in a TonB-depedent manner (318). Periplasmic PhuT binds heme and directs it to the inner membrane PhuUV ABC transporter (319). Additionally, HasA is a secreted hemophore that binds hemoglobin and hemopexin and transfers heme passively to another TonB-dependent transporter, HasR (Figure 1-2) (320, 321). Inner membrane HasS interacts with HasR to release the transcription factor HasI, which activates transcription of *phu* and *has* genes (318). It has been proposed that Phu represents the principal heme acquisition method, whereas HasAR, also required for optimal heme uptake, acts more-so as an extracellular heme-sensor. Nonetheless, through either transporter heme may enter the cytoplasm via PhuTUV, where it is bound by PhuS and relayed to HemO for degradation (Figure 1-5) (322). It is worthy to mention *Haemophilus influenzae*, which is unable to itself synthesize heme and relies on exogenous heme scavenging for its entire supply. Like *P. aeruginosa*, *H. influenzae* is able to appropriate heme from a cohort of host hemoproteins. HxuAB are part of a two-partner secretion system in which outer membrane HxuB transports the HxuA hemophore to scavenge free heme and hemopexin-

Figure 1-5: Schematic of heme acquisition by bacterial pathogens. a) *Pseudomonas aeruginosa* hemophore-dependent and independent heme acquisition. Heme is imported through PhuR in a TonB-dependent manner and shuttled through PhuTUV into the cytoplasm. b) *Staphylococcus aureus* Isd-mediated heme acquisition from hemoglobin and hemoglobin-haptoglobin (Hb-Hp). c) The *Bacillus anthracis* heme acquisition machinery, including hemophores, Isd and other heme-binding proteins. Each system is discussed in the main text. Figure is adapted from (319).


(a)
P. aeruginosa
(b)
S. aureus
(c)
B. anthracis
Fe
Hb
HasA
Hp
IsdH
IsdB
IsdA
IsdC
IsdE
IsdF
IsdD
IsdG
IsdI
IsdX2
IsdX1
BslK
Hal
PhuR
HasR
PhuT
PhuU
PhuV
HasS
PhuS
HemO
outer membrane
cell wall
periplasm
inner membrane
cytoplasm
membrane
biliverdin
staphylobilin
Fe2+

heme (323, 324). HxuC transports heme from HxuA into the periplasm in a TonB-dependent manner (325). Lipoprotein HbpA acquires heme from hemopexin, hemoglobin and hemoglobin-haptoglobin (326). Additionally, HgpA, HgpC, and most importantly HgpB external receptors acquire heme from haptoglobin-complexed hemoglobin and myoglobin (327, 328). Many other proteins have been implicated for heme procurement in *H. influenzae*, although their function remains to be fully elucidated (329–333).

1.4.12 Heme uptake by Gram-positive bacteria

Heme acquisition by Gram-positive bacteria is arguably just as complex as that of Gram-negatives. The *S. aureus* iron-regulated surface determinant (Isd) pathway of heme acquisition from hemoglobin and hemoglobin-haptoglobin is the best characterized heme uptake system in Gram-positive pathogens (Figures 1-2 and 1-5). For optimal functionality, hemoprotein-binding Isd proteins must pass the thick peptidoglycan wall in order to bind larger ligands. This is accomplished through Sortase A (SrtA), which recognizes C-terminal LPXTG motifs and covalently attaches IsdA, IsdB and IsdH to peptidoglycan (334). Sortase B (SrtB) is transcribed with other Isd proteins and tethers IsdC to peptidoglycan through recognition of a NPQTN motif (335). Each sortase cleaves peptides at either respective C-terminal recognition sequence between TG and TN residues, and attaches the threonine C-terminal carboxyl to the peptidoglycan pentaglycine cross-bridge (334, 336, 337). At the cell surface, IsdH binds haptoglobin, IsdB binds hemoglobin, and IsdA binds free heme (338–340). The former two extract heme from hemoglobin by eliciting steric strain to destabilize hemoglobin and facilitate heme release (341, 342). Heme is passed down the Isd cascade via IsdA and IsdC cell wall anchored proteins, with IsdC being the conduit between the external environment

and the integral membrane heme transporter (343–345). Lipoprotein IsdE, in consort with integral membrane IsdDF, transfers heme from IsdC across the membrane (346, 347). Once internalized, cytosolic heme is incorportated into bacterial proteins or degraded. Heme oxygenases IsdG and IsdI degrade heme to staphylobilin and free iron (348–350). Although both oxygenases are upregulated in low iron conditions, IsdG undergoes proteolytic processing for degradation in low heme conditions, signifying a differential response to iron source availability (351, 352). The heme uptake system of *B. anthracis* shares many similarities to the *S. aureus* Isd pathway, however also uses two secreted hemophores (Figure 1-5). The two hemophores are the first to be described among Gram-positive organisms and may be absent from others that lack the complex surface architecture of Bacillus, including the antiphagocytic capsule and proteinaceous Surface (S)-layers (353, 354). IsdX1 (formerly IsdJ) and IsdX2 (formerly IsdK) are secreted to the external cellular milieu and extract heme from host hemoglobin and transfer it to bacterial cell wall-bound IsdC (355, 356). Alternatively, sortase A-anchored, cell wall-localized HaI can also extract heme from hemoglobin, and BslK (S-layer domain homology), which is non-covalently attached to the cell wall, transfers heme to IsdC (357–359). Much like in *S. aureus*, *B. anthracis* IsdC funnels heme through the cell wall towards IsdDEF, which translocates heme into the cell prior to IsdG-mediated heme degradation (360, 361). Although *B. anthracis* lacks IsdH, IsdB and IsdA, it makes up for these with its own set of heme-extraction factors (Figure 1-5). The IsdX2 hemophore has been proposed to also function as a heme-storage protein as it can bind multiple heme residues at once, essentially acting to slow bacterial heme acquisition, which may be important to mitigate toxicity.

Heme-binding proteins in the aforementioned Isd pathways have one or more conserved near iron transporter (NEAT) domains responsible for binding and coordinating the heme molecule (362). NEAT domains are composed of variable ~120 amino acids in domains with a highly conserved eight-stranded, antiparallel β-sandwich fold (363, 364). Heme is bound within a hydrophobic pocket and coordinated by tyrosine residues in an YXXXY motif (364, 365). IsdB and IsdH contain two and three NEAT domains, respectively, whereas IsdA and IsdC contain one (364, 366–368). NEAT domains may vary in functionality due to differences in primary sequence or overall domain structure such to facilitate hemoprotein-binding, heme extraction or intra- or inter-protein heme transfer. IsdB-NEAT1 (N1; N-terminal) binds hemoglobin or hemoglobin-haptoglobin whereas N2 binds heme (342). IsdH-N1 and N2 bind hemoglobin-haptoglobin and N3 binds heme (369). Hemoglobin-binding is mediated through a FYHYA motif by IsdB-N1 and IsdH-N2, or a YYHFF configuration for IsdH-N1 (367, 369, 370). On the other hand, *B. anthracis* IsdX1, HaI and BslK each have one NEAT domain and IsdX2 has five (191, 319). Interestingly, IsdX2-N2 is unable to bind heme due to a histidine replacing the second tyrosine in the heme-binding motif, yet this domain interacts with hemoglobin to facilitate heme capture by other NEAT domains (371, 372). The structural basis of heme extraction and transport between *B. anthracis* heme acquisition proteins is not fully detailed as of yet. NEAT-domain containing proteins have been characterized in a number of Gram-positive pathogens including *Streptococcus pyogenes, L. monocytogenes*, and *S. lugdunensis* (373–376). Bioinformatics analysis indicates that NEAT-domain containing proteins may be widely

distributed among Firmicutes and present in both pathogenic and non-pathogenic organisms, although these must be characterized to elucidate their functions (363).

Corynebacterium diphtheriae uses non-NEAT mediated mechanisms to acquire heme through HmuTUV, HtaABC and ChtABC/CirA. HmuTUV bear sequence homology to heme transporters in Gram-negative pathogens, including HmuTUV in *Yersinia pestis* (377–379). Lipoprotein HmuT is capable of binding heme and hemoglobin and thought to relay heme into the cell through the HmuU permease and HmuV ATPase (377). It was later discovered that genes proximal to *hmuTUV* are also involved in heme acquisition. *htaA* is encoded with *hmuTUV* and *htaBC* are encoded in an adjacent locus (380, 381). HtaA and HtaB both contain N-terminal secretion signals and C-terminal transmembrane domains. While HmuT and HtaB are predominantly found in the cytoplasmic membrane, the majority of HtaA is extracellular (380). HtaA is able to interact with hemoglobin and may function as an extracellular hemophore (382). The model includes heme extraction from hemoglobin by HtaA, and then relay to HtaB and further to HmuT prior to translocation via HmuUV (382). Binding of heme is facilitated by an N-terminal histidine and C-terminal tyrosine on HmuT, whereas HtaA and HtaB employ unique conserved regions (CR) of ~150 amino acids to bind heme or hemoproteins (382, 383). HtaA has two CR domains and CR2 is the predominant heme and hemoprotein-binding domain with conserved tyrosine and histidine residues critical for binding. HtaB has one CR domain with high affinity for heme, and can accept heme from HtaA (382). Complete deletion of the *hmu-hta* locus reduces, but does not completely abrogate growth with heme as a sole iron source, and *chtAB* and *cirA chtC* operons were further identified as important for heme acquisition. All four of the encoded

proteins are surface-exposed and ChtA, ChtB and ChtC each have one CR domain and are all capable of binding heme and hemoglobin (384). ChtA and ChtC, much like HtaA, are able to bind hemoglobin-haptoglobin, whereas ChtB appears to be functionally redundant to HtaB (384, 385). The interplay between these functionally redundant systems has not been investigated in detail, although a combination of HtaA, ChtA and ChtC may function to bind hemoglobin-haptoglobin, extract heme, and relay it to HtaB or ChtB and further to HmuTUV (385). Internalized heme is degraded by the canonical heme oxygenase HmuO (386). Factors affecting cell wall remodeling to facilitate surface exposure of the exposed proteins, such that they remain membrane-associated and also bind the larger substrates (ie. hemoglobin-haptoglobin) are unknown. The contributions of these heme acquisition mechanisms to survival and growth of *C. diphtheriae* in the host remain undefined.

1.4.13 Transcriptional regulation of iron acquisition mechanisms

Iron limitation serves as a sensory cue that triggers global changes in the protein expression profile of a pathogen to better adapt to starvation conditions that would be encountered within the host. Key nutrient acquisition mechanisms and virulence factors are expressed to better make use of the shifted environmental conditions for growth and evasion of host defense strategies. Transcriptional regulators are primary mediators of the observed changes, acting to promote or deter expression of specific genes. The ferric uptake regulator (Fur) is the classical, canonical iron responsive transcriptional regulator in prokaryotes. Several species have structurally and functionally analogous regulators, including DtxR in *C. diphtheriae* and MtsR in *S. pyogenes* (381, 387, 388). The homodimeric metalloproteins function predominantly as repressors of iron-responsive

genes. Each monomer contains a ~120 amino acid winged-helix DNA-binding domain and a regulatory metal-binding carboxyl moiety that enables dimerization upon corepressor (ferrous iron) binding (389–391). When cellular iron is replete and exceeds that required for proper functioning of iron-containing metalloproteins, ferrous iron associates with each subunit of the dimer and facilitates a conformation change to allow DNA binding (392). The target is a 19-bp sequence in the operator/promoter region of the target gene locus termed the Fur box. The recognition sequence is an AT-rich inverted palindromic repeat consisting of 9-bp with a 1-bp separation (389, 390, 393). Binding of Fur to the Fur box restricts spatial access of RNA polymerase to the promoter region, blocking transcription. Decrease of intracellular iron levels cause Fe^{2+} dissociation from Fur and release of DNA for derepression of genes including those for acquisition of various iron sources, in addition to many other virulence factors (394–396). Fur is also known to upregulate genes directly, and indirectly through repression of small silencing RNA molecules (397, 398).

The redox capability of iron confers on Fur a large role in pathogen defense against oxidative and nitrosative stress encountered in the host. PerR (or functionally equivalent OxyR or OhR) is a Fur paralog that plays a central role in the response to oxidative stress and sensing the Fe/Mn ratio in the bacterial cell (399). Both of these ions may be coordinated in the regulatory site of the zinc-containing metalloprotein (400). Iron and manganese compete for binding, and Fe^{2+}-PerR is greatly more sensitive to oxidation than Mn^{2+}-PerR and thought to be responsible for sensing oxidative stress (401). Like Fur, metal binding to PerR facilitates binding to the promoter region of genes in the PerR regulon for repression (402). Oxidation of Fe^{2+}-PerR leads to derepression of

target genes, whereas Mn^{2+}-PerR is insensitive to oxidation (403). Additionally, metal selectivity influences the subset of genes regulated, where Fe^{2+}-PerR only represses a subset of genes in the regulon most critical for response to oxidative stress (402). Furthermore, Fur is regulated by PerR and upregulated by oxidative stress, indicating crosstalk between iron and manganese homeostasis with stress response, which adds to the complexity of how dynamic environmental signals impact on the bacterial transcriptome through sensor proteins with varying specificity and sensitivity (402, 404–406).

1.5 *Staphylococcus* spp.

1.5.1 The genus *Staphylococcus*

The *Staphylococcus* genus includes 51 Gram-positive bacterial species. Most reside on the skin and mucous membranes of their mammalian hosts and remain harmless commensals. Several species are capable of opportunistic infection in humans and are of particular clinical consequence. *S. saprophyticus* is a leading cause of uncomplicated urinary tract infections (407). *S. epidermidis* is found ubiquitously on the skin of many mammals and may form dense biofilms on indwelling biomaterials (ie. catheters, prostheses), making it a common nosocomial pathogen associated with device-related infections (408, 409). *S. lugdunensis* is also a ubiquitous skin commensal although it is gaining notoriety for causing serious infections, including skin and soft tissue infections (SSTIs), osteomyelitis, pneumonia, meningitis, and particularly aggressive endocarditis (410–412). *S. aureus* is the most well known staphylococcal species, responsible for causing a broad spectrum of infections which include the clinical manifestations mentioned for *S. lugdunensis* (413). *S. aureus* is most commonly found in the anterior

nares, permanently in ~20% of the population and transiently in another ~60% (414). Although only present in a subset of the population, *S. aureus* is responsible for the majority of staphylococcal clinical manifestations requiring hospitalization.

S. aureus is distinguished from the former three (and other) staphylococci as coagulase-positive, in that it secretes abundant coagulase. Coagulase binds to and activates prothrombin in serum to convert fibrinogen to fibrin and cause clotting (415). *S. lugdunensis* does not secrete soluble coagulase, although does produce a membrane-bound coagulase (clumping factor) which elicits a similar positive result in a slide coagulation test (416). Although cases are rare, several common veterinary species of coagulase-positive staphylococci are also able to cause disease in humans, further reducing the specificity of coagulase analysis as a diagnostic tool (417–419). Despite somewhat inexact genotyping based on phenotype, *Staphylococcus* species are still (as of now) classified as coagulase-positive (CPS) or coagulase-negative (CNS).

1.5.2 *Staphylococcus aureus* pathogenesis

The large number of *S. aureus*-associated clinical manifestations has garnered the bacterium considerable attention among researchers, and it is the most widely studied *Staphylococcus* species. While traditionally thought of as a nosocomial pathogen, several strains have gained notoriety for causing disease in otherwise healthy individuals in community-acquired infections (420). The ability to colonize many bodily tissues endows the pathogen the ability to cause many various disease states, which may lead to host fatality (413). The success of *S. aureus* to colonize and cause pathogenesis in the host is, in part, attributed to its vast arsenal of virulence factors. Among the best studied include compounds involved in tissue attachment (host extracellular matrix-binding, clotting,

biofilm formation; clumping factors, coagulase, DNA and polysaccharide-laden biofilms), immune evasion (inhibition of complement, immune cell chemotaxis and phagocytosis; staphopains, aureolysin, protein A), causing host cell damage (numerous pore-forming toxins; Panton Valentine leukocidins, α-hemolysin), mitigation of destruction (resistance to lysozyme, antimicrobial peptides and oxidative damage; OatA, MprF, SodA and SodM) and overcoming nutritional immunity (vital nutrient acquisition; staphyloferrin siderophores, Isd, Mnt). For more comprehensive reviews of *S. aureus* virulence determinants, consult (191, 421–424).

1.5.3 *Staphylococcus aureus* response to iron withholding

The *S. aureus* metabolome changes quite drastically when the bacterium enters the host bloodstream. Changes in environmental nutrients govern these metabolic shifts. Glycolysis is upregulated, whereas tricarboxylic acid (TCA) cycle activity is downregulated through carbon catabolite repression as an iron-sparing response to take advantage of increased glucose, and redistribute iron from the TCA iron-sulfur cluster containing proteins (425–427). The iron-sparing response upregulates glycolytic and fermentative pathways, generating ATP independently of respiration, and accumulates acidic byproducts (lactate) in the extracellular milieu (425). The decreased pH may promote dissociation of iron from host proteins such as transferrin and lactoferrin.

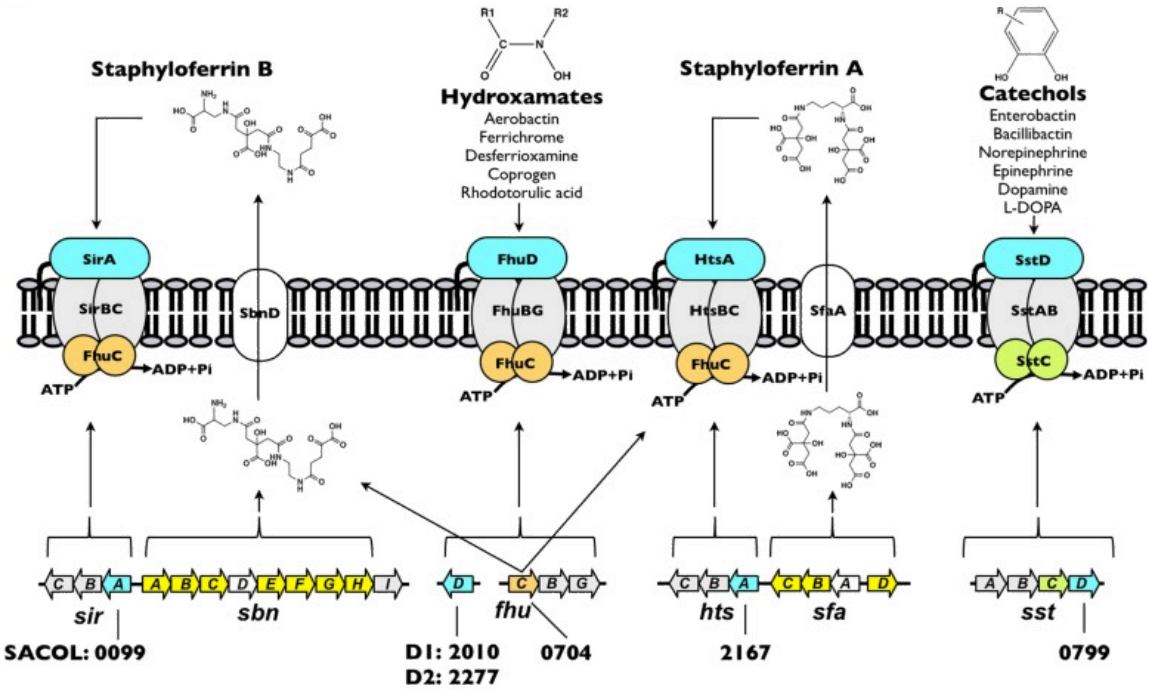
Iron depletion in the host confers a harsh environment and influences *S. aureus* pathogenesis via Fur. The iron-responsive regulator coordinates expression of many notable exoproteins including hemolysins, cytotoxins and immunomodulatory proteins, in addition to genes for iron procurement (313). *S. aureus* produces and secretes the stealth siderophores staphyloferrin A (SA) and staphyloferrin B (SB), which bind iron

specifically (428, 429). Staphylopine is a recently identified metallophore able to associate with several metal ions including iron, zinc, nickel, cobalt and copper. Staphylopine-metal binding differs among varying environmental conditions and bacterial growth status, and relevance for *in vivo* colonization and virulence is yet undetermined (430). *S. aureus* elaborates Isd proteins to acquire heme-iron from hemoglobin-haptoglobin, as well as transporters for acquisition of xenosiderophores with hydroxamate (Fhu) or catecholamine (Sst) moieties (271, 280, 340). Heme is the most abundant source of iron within the mammalian host and the preferred iron source of *S. aureus* (431, 432). A transcription factor (SbnI) encoded on the SB biosynthetic operon has been shown to modulate siderophore production and repress SB biosynthetic genes in the presence of heme (432). Interestingly, the staphylococcal siderophores are produced in distinct infection conditions, where staphyloferrin A is predominantly produced during subcutaneous infection whereas staphyloferrin B in glucose-rich environments such as in circulation (191, 433).

1.5.4 Staphyloferrin A and staphyloferrin B

The vast majority of *Staphylococcus* spp. produce SA (434). The biosynthetic operon consists of *sfaABC* and *sfaD*, transcribed divergently (Figure 1-6). The siderophore is produced by NRPS-independent synthetases. The SfaD synthetase condenses a citrate molecule with the δ-amine of D-ornithine as the rate limiting step, and SfaB further condenses another citrate molecule to the α-amine of the precursor (428). SfaC is a putative amino acid racemase that converts L-ornithine to D-ornithine, and SfaA is a major facilitator superfamily (MFS) efflux pump responsible for SA secretion (435, 436). SA coordinates Fe^{3+} using α-hydroxycarboxylate groups on the internal citrate moieties

Figure 1-6: *Staphylococcus aureus* siderophore transporters. Staphyloferrins A (SA) and B (SB) are synthesized by products of the *sfa* and *sbn* operons, respectively, and ferric-SA and ferric-SB are internalized through Hts and Sir transporters, respectively. Siderophores bearing hydroxamate or catechol moieties are transported by Fhu and Sst ABC transporters, respectively. Genes are colour-coded for distinction of protein products: yellow, siderophore synthetase; white/gray, integral membrane protein (for siderophore secretion or internalization); blue, lipoprotein receptor; peach/green, ATPase. Figure is adapted from (437).


Staphyloferrin B
Hydroxamates
Aerobactin
Ferrichrome
Desferrioxamine
Coprogen
Rhodotorulic acid
Staphyloferrin A
Catechols
Enterobactin
Bacillibactin
Norepinephrine
Epinephrine
Dopamine
L-DOPA
SirA
SirBC
FhuC
ATP
ADP+Pi
SbnD
FhuD
FhuBG
HtsA
HtsBC
SfaA
SstD
SstAB
SstC
sir
sbn
fhu
hts
sfa
sst
SACOL: 0099
D1: 2010
D2: 2277
0704
2167
0799

for 6 coordination sites (438). The ABC transporter for SA uptake is encoded on *hts* genes situated next to genes for SA biosynthesis (Figure 1-6) (273). HtsA is the lipoprotein component that facilitates SA-binding and transfer through the HtsBC permease for translocation via energy generated by the FhuC ATPase (273, 439). The HtsA SA-binding site is a charged pocket lined with basic arginine residues particularly adept to bind the anionic siderophore (438, 440).

SB (formerly known as staphylobactin) is produced by several staphylococcal species (429, 441, 442). The biosynthetic operon contains nine genes, sbnA-I, including several for NRPS-independent synthesis of SB (Figure 1-6). SbnA uses O-phospho-L-serine and L-glutamate to generate an intermediate product, which is hydrolyzed by SbnB to produce L-2,3-diaminopropionic acid (DAP) and α-ketoglutarate precursors for SB biosynthesis (443). SbnG is a novel citrate synthase that catalyzes citrate production from oxaloacetate and acetyl-CoA, thus compensating for the downregulation of CitZ (TCA cycle citrate synthase) during the iron-sparing response (433, 444). Citrate and DAP are condensed via SbnE, which is further processed by SbnH to decarboxylate a carboxyl group on the DAP end of intermediary product. SbnF catalyzes the condensation of another DAP molecule with the prochiral carbon of the citrate moiety on the precursor, and finally SbnC condenses the diaminoethane group on the precursor with an α-ketoglutarate (429). The complete SB molecule is secreted via the SbnD MFS efflux pump, or an alternate yet to be determined transporter (436). As mentioned previously, the final encoded protein, SbnI, is involved in regulation of siderophore production (432). Carboxylate SB coordinates Fe^{3+} in hexadentate fashion using a nitrogen and five oxygen atoms (445). Ferric-SB is transported through encoded products of the *sirABC* operon,

transcribed divergently from the *sbn* operon (Figure 1-6). Lipoprotein SirA binds to SB with unique coordination compared to SA-HtsA binding, with different conformational shifts upon substrate-binding and few conserved contacting residues between SirA and HtsA for coordinating their respective carboxylate siderophores (445). Ferric-SB is passed through SirBC and internalized via energy generated by the FhuC ATPase (282). *sfa, hts, sbn* and *sir* loci all have Fur boxes in their promoter regions and are indeed iron-regulated (273, 429, 437).

1.5.5 Xenosiderophore acquisition by *Staphylococcus aureus*

S. aureus cannot synthesize, yet is able to transport exogenously-produced hydroxamate siderophores (Figure 1-6). In the case of this transporter, either of two lipoproteins FhuD1 and FhuD2 are responsible for substrate recognition, binding of hydroxamate siderophores, with FhuD2 exhibiting both a broader substrate specificity and greater binding affinity for substrates (281, 283). These lipoproteins shuttle various hydroxamate substrates through the FhuBG permease, via energy derived from the associated FhuC ATPase (280). The FhuC ATPase required for hydroxamate transport is the same that is required for transport of SA and SB. The Fhu transporter in *S. aureus* is also relevant to medical practice, as chelation therapy for iron overload disorders (such as thalassemia and hemochromatosis) frequently use hydroxamate chelators as treatment, including Desferal™ (Novartis), which may exacerbate infection-favouring conditions (284).

S. aureus is also capable of using catechols for growth, although does not produce any catechol siderophore (Figure 1-6). The *sstABCD* operon has been described as a catechol transporter in *S. aureus.* The SstD lipoprotein functions in association with the SstAB membrane permease and SstC ATPase to transport host-produced catecholamine

stress hormones (ie. epinephrine, norepinephrine, dopamine, L-DOPA) as a source of iron, as these molecules are able to liberate iron from host proteins such as transferrin (271). The *sst* locus has a Fur box in the promoter region and has been shown to be iron-regulated (446). Further description of the method in which iron is released from SA, SB, hydroxamates and catecholamines is warranted, as well as any regulatory effects these compounds may have. It is plausible that since the Sst transporter uses a different ATPase than the other siderophore acquisition machinery, it may have other, yet to be determined functions in addition to iron acquisition.

1.5.6 *Staphylococcus aureus* heme-iron acquisition

Heme acquisition by *S. aureus* is mediated by the Isd system detailed in the earlier section 'Heme uptake by Gram-positive bacteria' (see Figure 1-5). The sortase-A anchored Isd proteins are transcribed separately from the core of the system. IsdH, IsdB and IsdA are each transcribed separately from the *isdCDEF srtB isdG* operon, with *isdA* and *isdB* transcribed divergently and separately from the other. *isdH* and *isdI* are dissociated in the genome from the other *isd* genes. The Isd system is a high-affinity heme acquisition mechanism that allows growth with low nanomolar concentrations of heme/hemoglobin typical of physiological circulatory conditions (447). At supraphysiological heme-iron concentrations (>50 nM), the Isd system becomes less relevant and another, yet unidentified heme-iron acquisition system permits growth of *isd* deletion mutants (447, 448). Non-Isd heme-iron acquisition is believed to play a larger role in prolonged infection states in which hemolysis/necrosis has taken place, although this notion remains to be validated. Nevertheless, Isd proteins are highly upregulated in

blood and serum and *S. aureus* single gene *isd* deletion mutants are attenuated for growth in mammalian infection models (339, 351, 447, 449, 450).

1.5.7 *Staphylococcus lugdunensis*

S. lugdunensis was first described in 1988, and named after where it was isolated, in Lyon, France (451). Despite being able to cause debilitating disease reminiscent of *S. aureus*, *S. lugdunensis* remains an underappreciated pathogen, likely due to its more recent emergence. The ubiquitous skin commensal largely inhabits the lower extremities, including the groin region and perineum (452). It is responsible for both nosocomial and community-acquired infections and while most clinical manifestations are SSTIs (55.4%), 17.4% of infections are of blood and vascular catheter origin (453). Strikingly, rates of required surgery for *S. lugdunensis*-associated infective endocarditis (IE) surpass that of *S. aureus* IE (70% vs. 36.9%), and the mortality rate of *S. lugdunensis*-associated IE (50%) is much greater than that of *S. aureus* (14.5%) and *S. epidermidis* (20%) (454, 455). Nearly half of infected patients have no comorbidities, indicating *S. lugdunensis* is able to cause disease in otherwise healthy individuals (411). In addition to skin trauma, many reports detail individuals who undergo surgical procedures including vasectomy and kidney transplant and acquire *S. lugdunensis* IE soon-after (456–459). Although *S. lugdunensis* infections typically occur at lower frequency than *S. aureus*, the true burden may be underestimated due to *S. lugdunensis* isolates producing clumping factor (membrane-bound coagulating protein), which elicits positive slide coagulase and/or latex agglutination results in the clinic, leading to misidentification.

The enigmatic *S. lugdunensis* exhibits elevated virulence compared to other CNS, yet genome sequence analysis indicates a lack of most of the virulence factors present in

S. aureus. How then is this odd, recently discovered CNS able to cause severe disease akin to or more virulent (for IE) than other staphylococci? Data on molecular determinants for *S. lugdunensis* pathogenesis is scarce, and CNS typically only have a few virulence determinants (460). In addition to fibrinogen binding protein (Fbl, clumping factor ClfA homolog), there is limited information on a yet uncharacterized von Willebrand-binding protein (vWbl), and *S. lugdunensis* synergistic hemolysins (SLUSH peptides) (461–466). SLUSH A, B and C are short β-type phenol soluble modulin-like peptides that function analogously to δ-toxin and exhibit cytotoxic effects on host cells (466, 467). SLUSH peptides also attract and activate neutrophils via formyl peptide receptor 2 (Fpr2) (468, 469). About half of *S. lugdunensis* clinical isolates lack SLUSH genes, whereas all isolates have genes for β-hemolysin and hemolysin III (ie. sphingomyelin phosphodiesterase) (470). Interestingly, 5% of isolates are non-hemolytic, and these may or may not contain SLUSH gene sequences (466, 470). The latter two mentioned hemolytic factors have yet to be investigated, and the reason for some isolates being non-hemolytic is perplexing given their genetic profiles to bear genes for expression of putative hemolytic proteins. Recent advancements in *S. lugdunensis* genetic manipulation has allowed for more detailed studies into the organisms biology. *S. lugdunensis* is the only other staphylococcal species to contain a region with similar sequence and organization to *S. aureus isd*, albeit with differences and most identity occurring within protein NEAT domains. *S. lugdunensis* IsdB has been shown to bind human hemoglobin and hemoglobin-haptoglobin, and *isd* genes are required for growth with hemoglobin as a sole iron source (376). IsdG has also been shown to degrade heme to release iron (471). In addition to the ability to accept heme from IsdB, IsdC is

important for biofilm formation under low-iron growth conditions (376, 472). Further detailing the *S. lugdunensis* Isd system is warranted, as well as other potential virulence determinants.

1.6 Objective and inquiries

S. lugdunensis is an emerging pathogen able to cause destructive infections with high rates of mortality even with therapeutic surgical intervention. There is a lack of described virulence factors for this staphylococcal species, fueling our interest to study this curious microbe. Prior to this study, genome sequences of two isolates had been recently published, and would aid us in ascribing biological functions to several genomic regions of interest (473, 474). During the course of this study, there were two publications that evaluated *S. lugdunensis* virulence in mammalian endocarditis models, and only Sortase A was shown as a virulence factor in the host (475, 476). Mechanisms of iron acquisition were largely undefined, and the Isd system, several components of which were well characterized, was not described completely. The objective of this study was to describe the host-iron acquisition strategies of *S. lugdunensis*. Experimental research was aimed to address the following inquiries:

1) Does *S. lugdunensis* produce a siderophore – likely one of the two common staphylococcal siderophores (SA and/or SB)?

2) What siderophores is *S. lugdunensis* capable to acquire, and can it acquire host-produced catecholamine stress hormones as sources of iron?

3) What is the impact of siderophore and/or heme acquisition for colonization and pathogenesis in the host?

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2

Chapter 2

Staphyloferrin siderophore and heme acquisition by *Staphylococcus lugdunensis*

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2.1 Introduction

In 1988, *Staphylococcus lugdunensis* was described as a new species of coagulase-negative staphylococcus (CoNS), isolated from a human clinical specimen (1). It is now widely considered to be an emerging pathogen with uncharacteristically elevated virulence in comparison with other members of the CoNS (2). In addition to being a skin commensal, *S. lugdunensis* is responsible for both nosocomial and community-acquired infections that may include skin and soft tissue infections (SSTIs), pneumonia, meningitis, and endocarditis (3–6). While the most common clinical manifestation of *S. lugdunensis* infection is SSTIs (55.4%), blood infections and those associated with vascular catheterization accounted for a notable 17.4% of diagnoses (7). Strikingly, the mortality rate of *S. lugdunensis* associated endocarditis may reach up to 50% (3). Despite that *S. lugdunensis* is gaining notoriety as an atypically virulent CoNS, the true burden of *S. lugdunensis* infection is likely underestimated. Since most *S. lugdunensis* isolates are hemolytic, and are capable of producing a membrane-bound clumping factor (coagulant), it is possible that many *S. lugdunensis* infections are misinterpreted as being caused by *S. aureus* (8, 9). Moreover, nearly half of patients infected with *S. lugdunensis* appear to have no comorbidities, indicating that this underappreciated pathogen is able to cause infection in the absence of overt susceptibility (5).

Iron is an essential nutrient for most pathogenic bacteria, including the Staphylococci, and represents a significant growth-limiting nutrient in the host (10). Virtually all host iron is bound to glycoproteins such as transferrin, ferritin, and lactoferrin (11), or is in complex with heme in hemoproteins. Hemoglobin iron accounts for up to 75% of total host iron, the vast majority of which is found within circulating

erythrocytes (12). To establish infection, pathogens must circumvent host iron sequestration strategies, and therefore, by extension, must possess elaborate iron acquisition mechanisms in order to obtain this limited nutrient. Frequently, these iron uptake strategies involve either the acquisition of heme contained in hemoglobin, or the removal of transferrin-bound iron through the secretion of siderophores (13). Siderophores are small molecules (commonly less than 1000 Da) capable of binding ferric iron with high affinity, and delivering iron back to the cell via surface localized and membrane-embedded receptor proteins.

Much of our molecular understanding of iron acquisition processes in the staphylococci comes from studies in *S. aureus*. The iron-regulated surface determinants (Isds) were first discovered in *S. aureus* (14, 15). The Isd system is now fairly-well characterized, and is constituted by a series of proteins that, together, are capable of extracting heme from hemoglobin at the bacterial cell surface, and relaying heme across the cell wall, through the cytoplasmic membrane, and into the cytoplasm where it is degraded to release iron for use in cellular processes (16, 17). *S. aureus* also produces two siderophores, staphyloferrin A (SA) and staphyloferrin B (SB), which are synthesized by gene products encoded from within the *sfa* and *sbn* genetic loci, respectively (18, 19). *S. aureus* internalizes ferric-SA and ferric-SB using the ABC-type transporters HtsABC and SirABC, respectively, encoded by genes found adjacent to the cognate biosynthetic loci (18, 20–22). *S. aureus* strains mutated for staphyloferrin production are severely restricted for growth in the presence of transferrin or animal serum (23, 24).

Investigations of the molecular mechanisms that contribute to the virulence of *S. lugdunensis* are in their infancy. Few mutants have, as of yet, been constructed and characterized, and even fewer tested in animal models. In one recent study it was demonstrated that, in *S. lugdunensis*, sortase A, responsible for the anchoring of LPXTG-containing proteins to the cell wall, was required for full virulence in a rat endocarditis model (25). Interestingly, the genome sequencing of two strains of *S. lugdunensis*, HKU09-01 and N920143, revealed that this species is unique among the CoNS in that it encodes an Isd system (26–29) and, moreover, in strain HKU09-01, the *isd* locus is tandemly duplicated. Work led by T. Foster's group has revealed that the *isd* system in strain N920143 is functional, contributing to the strain's use of hemoglobin as an iron source (29).

In this study, we investigate iron uptake mechanisms in *S. lugdunensis* through characterizing the role of the *S. lugdunensis* strain HKU09-01 *isd* locus in heme and hemoglobin utilization, as well as characterizing this species for the ability to produce and utilize staphyloferrins. We demonstrate that a mutant lacking *isd* is severely impaired for growth using heme and hemoglobin as a sole iron source, especially at nanomolar heme and hemoglobin concentrations. Moreover, we show that *S. lugdunensis* grows poorly in serum and in the presence of transferrin owing to a lack of detectable siderophore production. We further demonstrate that while *S. lugdunensis* cannot produce staphyloferrins, it encodes the transporters for their uptake and these transporters are functional, leading to the notion that *S. lugdunensis* may appropriate staphyloferrins from other staphylococcal species to augment its growth. In support of this, we show that

growth of iron-restricted *S. lugdunensis* is significantly enhanced in co-culture with staphyloferrin-producing *S. aureus*, in an *hts*- and *sir*-dependent manner.

2.2 Experimental procedures

2.2.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are summarized in Table 2-1. For all routine manipulations, *Escherichia coli* DH5α was grown in Difco Luria-Bertani broth (LB, BD Diagnostics) or on LB agar (LBA). *S. lugdunensis* and *S. aureus* strains were cultured in tryptic soy broth (TSB, BD Diagnostics) or on TSB agar (TSA). Antibiotics were used at the following concentrations: 100 μg mL^{-1} ampicillin for *E. coli* selection; 10 μg mL^{-1} chloramphenicol for *S. lugdunensis* selection. For subsequent experiments, *S. lugdunensis* and *S. aureus* were grown in several different iron-restricted media as detailed, (i) Tris-minimal succinate broth (TMS) (30); (ii) TMS treated with Chelex-100 resin (Bio-Rad) for 24 hours at 4°C (C-TMS); (iii) an 80:20 mixture of C-TMS to complement-inactivated horse serum (Sigma Aldrich); or (iv) RPMI media 1640 (Gibco) reconstituted from powder and supplemented with 1% w/v casamino acids (RPMIC, Difco) and 1 μM of the iron chelator ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA, LGC Standards GmbH). All bacteria were cultured at 37°C, shaking at 200 rpm, unless otherwise indicated. All media and solutions were prepared with water purified through a Milli-Q water purification system (Millipore).

2.2.2 Generation of *isd-sir* and *htsABC* mutants in *S. lugdunensis*

Primer sequences used for the construction and complementation of *S. lugdunensis* mutants can be found in Table 2-1. Allelic replacement was performed as previously

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

Bacterial strain, plasmid or oligonucleotide	**Description**[a]	**Source or reference**
Strains		
E. coli		
DH5α	φf80d*lacZ*ΔM15 *recA1 endA1 gyrAB thi-1 hsdR17*(r_K^- m_K^-) *supE44 relA1 deoR Δ(lacZYA-argF)U169*	Promega
S. lugdunensis		
HKU09-01	Human skin infection isolate	(28)
H2710	HKU09-01 Δ*isd-sir*	This study
H2773	HKU09-01 Δ*hts*	This study
H2774	HKU09-01 Δ*isd-sir* Δ*hts*	This study
S. aureus		
RN4220	Prophage-cured laboratory strain; r_K^- m_K^+; accepts foreign DNA	(31)
RN6390	Prophage-cured laboratory strain	(32)
H1324	RN6390 Δ*sbn*::Tet; Tet^R	(23)
H1661	RN6390 Δ*sfa*::Km; Km^R	(23)
H1649	RN6390 Δ*sbn*::Tet Δ*sfa*::Km; Tet^R Km^R	(23)

H306	RN6390 Δ*sirA*::Km; Km^R	(23)
H1448	RN6390 Δ*hts*::Tet; Tet^R	(23)
Plasmids		
pKOR1	*E. coli/Staphylococcus* shuttle vector allowing allelic replacement in staphylococci	(33)
pKOR1Δ*isd-sir*	pKOR1 plasmid for deletion of duplicated genetic region encompassing *isd* and *sir*	This study
pKOR1Δ*hts*	pKOR1 plasmid for in-frame deletion of *htsABC*	This study
pRMC2	*E. coli/Staphylococcus* shuttle vector: Ap^R Cm^R	(34)
pRMC2::*sir*	pRMC2 derivative for *sirABC* expression; Cm^R	This study
pRMC2::*hts*	pRMC2 derivative for *htsABC* expression; Cm^R	This study
Oligonucleotides[b,c]		

Purpose	Sequence (5'-3')
Primers for generating upstream and downstream recombinant regions for Δ*isd* Δ*sir* using pKOR1	**(*AttB1*)-isdUF:***GGGGACAAGTTTGTACAAAAAAGCAGGCT*CTACCACTGACAGCAACGGCAAT **isdUR:** CTCATTGCGATTCCTTCCTTCG **isdDF:**Phos/AGCACAGATAGGAGTTCATTTGCATGTA **(AttB2)-isdDR:***GGGGACCACTTTGTACAAGAAAGCTGGGT*AGATGCGCCTTGGATTTGACAC

Primers for generating upstream and downstream recombinant regions for Δ*hts* using pKOR1	**(*AttB1*)-htsUF:***GGGGACAAGTTTGTACAAAAAAGCAGGCT* ATTCCAGTATGTTGCCAC **htsUR:** AACAGTAGCCCAATGATAC **htsDF:** Phos/AGTAGGTGTCATAATAGC **(*AttB2*)-htsDR:***GGGGACCACTTTGTACAAGAAAGCTGGGT* TACTGGTAATGAGCACTC
Primers for cloning *sirABC* into pRMC2 for complementation	**XhoI-sirF:** GATCCTCGAGTACTGCTCCAAAATCCCC **EcoRI-sirR:** GATCGAATTCTTTAGCGTGCGGTATGTC
Primers for cloning *htsABC* into pRMC2 for complementation	**KpnI-htsF**: GATCGGTACCAAGCACTAACCCAGTCAATG **SacI-htsR**: GATCGAGCTCCTAAACAATCCCCATAAAGC

[a] Ap^R, Cm^R, Km^R and Tet^R; resistance to ampicillin, chloramphenicol, kanamycin and tetracycline, respectively.

[b] Restriction sites for cloning are underlined.
[c] Phos/ denotes a 5' phosphate on the primer.

described, using the plasmid pKOR1 (33). In brief, 500 - 1,000-bp DNA fragments were amplified from the genomic regions upstream and downstream of the tandem-duplicated *isd-sir* locus (Figure 2-1B) using the primers isdUF and isdUR, and isdDF and isdDR, respectively. The amplicons were cloned into pKOR1, generating pKOR1Δ*isd-sir*. Similarly, the plasmid pKOR1Δ*hts* for the in-frame deletion of *htsABC* (Figure 2-1A) was constructed by amplifying 500 - 1,000-bp DNA fragments flanking the start and stop codons of the operon using primers htsUF and htsUR, and htsDF and htsDR. The vectors were passaged through *S. aureus* RN4220 before introduction into *S. lugdunensis* HKU09-01 by electroporation. The strains HKU09-01 Δ*isd-sir* (H2710) and HKU09-01 Δ*hts* (H2773) were generated using the methodology for pKOR1 (29, 33), and introduction and recombination of pKOR1Δ*hts* with H2710 was used to produce the *isd, sir, hts*-deficient strain H2774. Chromosomal deletions were confirmed through sequencing of PCR amplicons generated from across the deleted regions.

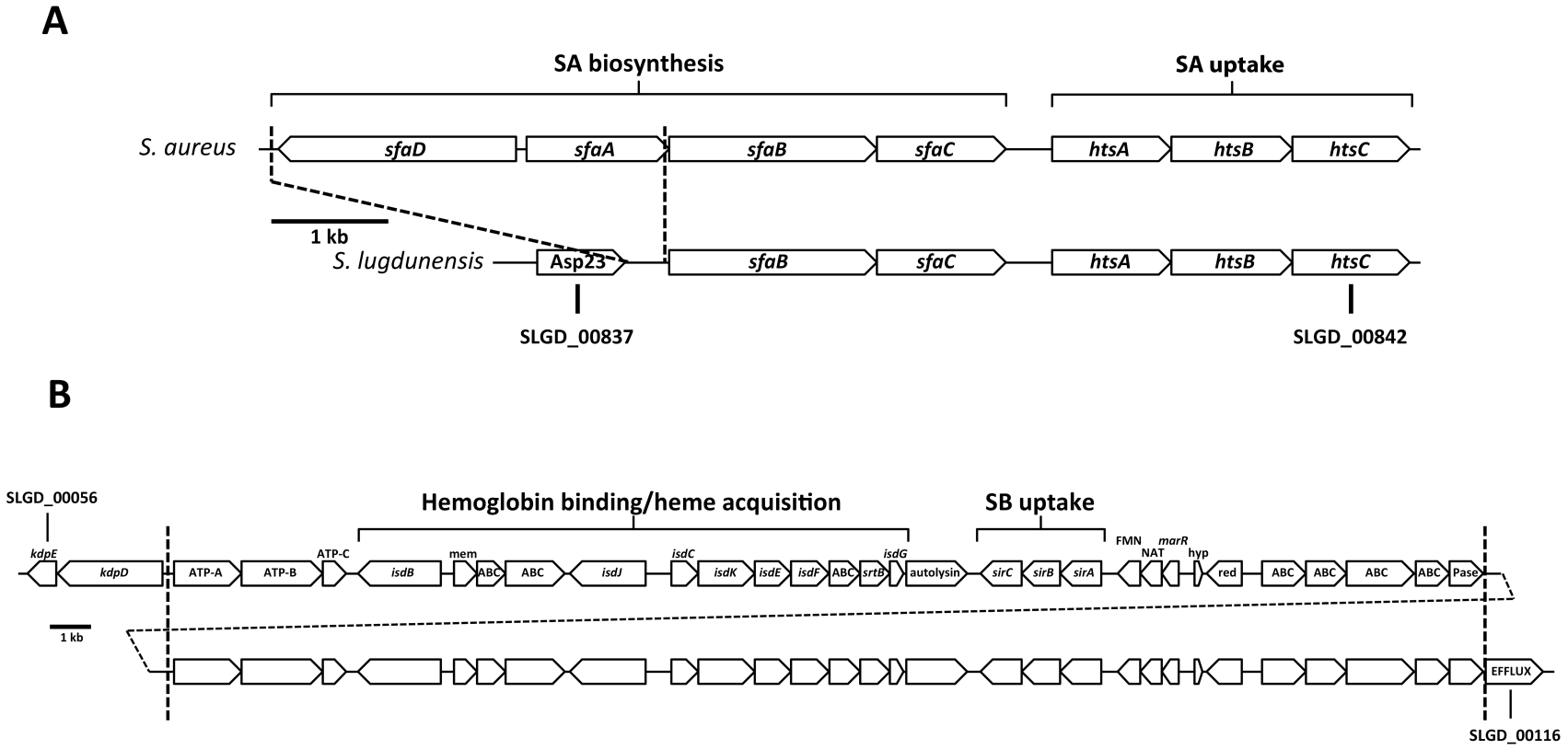
2.2.3 Complementation of *sir* and *hts* mutations

For complementation of the *S. lugdunensis sirABC* mutation, primers XhoI-SirF and EcoRI-SirR were used to amplify the wild-type *sirABC* operon, including its native promoter. The fragment was cloned into pRMC2 generating pRMC2::*sir*. The *htsABC* complementation vector pRMC2::*hts* was similarly created using the amplicon generated with XhoI-HtsF and KpnI-HtsR, again including the native promoter for *htsABC*.

2.2.4 Bacterial growth curves

Single, isolated *S. aureus* and *S. lugdunensis* colonies, taken from TSA plates after overnight incubation, were resuspended in 120 µL C-TMS and 100 µL of this suspension was used to inoculate 2 mL C-TMS. These cultures were then incubated at 37°C for at

Figure 2-1: Physical maps of the *sfa-hts* and *isd* loci in *Staphylococcus lugdunensis*. (A) Staphyloferrin A (SA) biosynthetic and uptake locus. Shown is the homologous locus in *S. aureus* versus that which is present in all sequenced genomes of *S. lugdunensis*. Note that the SA biosynthetic locus in *S. lugdunensis* carries a deletion that eliminates two genes completely (*sfaA* and *sfaD*), along with the promoter for the remaining two genes (*sfaB* and *sfaC*). The deleted region is indicated between the dashed lines. Asp23, alkaline shock protein 23. (B) Shown is the ~65-kb region of the *S. lugdunensis* strain HKU09-01 genome (spanning orfs SLGD_00056 to SLGD_00116) with the tandemly duplicated *isd-sir* locus. The duplicated region is shown between the dashed vertical lines. Abbreviations are as follows, with predicted or hypothetical functions: ABC, component of an ATP-binding cassette transporter; ATP-A,B,C, K+-ATPase components A, B, and C, respectively; mem, membrane protein; FMN, FMN binding protein; NAT, N-acetyltransferase; marR, MarR-type regulator; hyp, hypothetical; Red, reductase; Pase, phosphatase.


A
SA biosynthesis
SA uptake
S. aureus
sfaD
sfaA
sfaB
sfaC
htsA
htsB
htsC
1 kb
S. lugdunensis
Asp23
sfaB
sfaC
htsA
htsB
htsC
SLGD_00837
SLGD_00842
B
SLGD_00056
Hemoglobin binding/heme acquisition
SB uptake
kdpE
kdpD
ATP-A
ATP-B
ATP-C
isdB
mem
ABC
ABC
isdJ
isdC
isdK
isdE
isdF
ABC
srtB
isdG
autolysin
sirC
sirB
sirA
FMN
NAT
marR
hyp
red
ABC
ABC
ABC
ABC
Pase
1 kb
EFFLUX
SLGD_00116

least 4 hours until OD_{600} was approximately 1. The cultures were subsequently normalized to an OD_{600} of 1 and 1 µL was added to 200 µL aliquots of 80:20 C-TMS:horse serum growth medium. For iron replete conditions, 100 µM $FeCl_3$ was included. Chloramphenicol was added for strains harbouring pLI50, pRMC2 or their derivatives. Cultures were grown with constant shaking at medium amplitude in a Bioscreen C machine (Growth Curves) at 37^{o}C. OD_{600} was measured every 15 min, however for graphical clarity, measurements at 4-hour intervals are shown.

2.2.5 Siderophore preparations and plate bioassays

Concentrated culture supernatants enriched for staphyloferrin A (SA) and staphyloferrin B (SB) were prepared from *S. aureus* Δ*sbn* and Δ*sfa* mutants, respectively, as previously described (23). Concentrated culture supernatants from *S. lugdunensis* were similarly prepared. In brief, strains were grown in C-TMS with aeration, for 40 hours. Bacterial cells were removed by centrifugation and supernatants were lyophilized overnight and resuspended in methanol (one-fifth the original culture volume). Insoluble material was removed by centrifugation and the soluble fraction was rotary evaporated. Dried material was resuspended in water (one-tenth the original culture volume). The ability of concentrated culture supernatants to support staphylococcal growth was assessed using the plate bioassay technique as previously described (35). To assess growth promotion on *S. lugdunensis* cells, 1 x 10^4 cells mL^{-1} were incorporated into TMS-agar containing 5 µM EDDHA. Chloramphenicol was incorporated into media with strains harbouring pRMC2, or vector derivatives. Staphyloferrin A and staphyloferrin B were also synthesized enzymatically, using procedures that have been previously described (20,

36). Siderophores/supernatants were applied to sterile paper disks and placed onto the plates. Growth around disks was measured after 24 hours.

2.2.6 Chrome azurol S assay

Supernatants of iron-starved staphylococci were concentrated by lyophilization to 1/10 of their original volume and tested for iron-binding compounds using the chrome azurol S shuttle solution (37), as previously described (36).

2.2.7 Analysis of iron-regulated protein expression by Western blotting

Antisera against *S. aureus* HtsA and SirA used in this study were generated previously (23, 38). The antisera were used to assess the expression of homologous proteins in *S. lugdunensis,* as described below.

For analysis of iron-regulated protein expression in whole-cell lysates, cells were grown in C-TMS with or without 50 μM $FeCl_3$ for 24 hours, normalized to an OD_{600} of 1, and lysed. Proteins in lysates were resolved through SDS-polyacrylamide gel electrophoresis using a 12% acrylamide resolving gel. For Western immunoblots, proteins were transferred from gels to a 45-μm nitrocellulose membrane via standard protocols (39). Detection of transferred proteins was performed after blocking the membrane at 4^{o}C for 12 hours in PBS containing 10% w/v skim milk and 20% v/v horse serum. The membrane was washed and the primary antibody was applied at room temperature for 2 hours (1:3,000 dilution) in PBS with 0.05% Tween 20, and 5% horse serum. The membrane was washed again, and anti-rabbit IgG conjugated to IRDye 800 (Li-Cor Biosciences) was used as the secondary antibody, applied at room temperature

for 1 hour (1:20,000 dilution), in the same buffer as the primary antibody. Fluorescence was analyzed on a Li-Cor Odyssey infrared imager (Li-Cor Biosciences).

2.2.8 Staphylococcal growth in co-culture

Staphylococci were grown for 4 hours in C-TMS. Cells were washed three times in C-TMS and normalized to an OD_{600} of 1 (*S. lugdunensis*) or 0.1 (*S. aureus*). Staphylococci were inoculated into 2 mL of medium (80:20 C-TMS:horse serum) either in monoculture or in co-culture. For co-cultures, equal volumes of washed cells from each species were added. The 2-mL cultures were in 14-mL round-bottom polypropylene tubes and shaken at 200 rpm. Samples were taken at time 0, 12, and 24 hours for dilution and plating on TSA to obtain values for viable colony-forming units (CFUs). TSA plates were incubated at 37^{o}C for 24 hours and subsequently at room temperature for 2 days. Staphylococci were distinguished based on colony colour and morphology; *S. aureus* colonies were visibly larger in diameter and with lighter pigmentation, whereas *S. lugdunensis* colonies were distinguishably smaller (approximately ¼ to ½ the diameter of *S. aureus* colonies) and dark yellow under these culture conditions.

2.2.9 Preparation of hemin and hemoglobin

A solution of bovine hemin (Sigma Aldrich) was prepared as follows. A stock solution at 5 mM in 0.1 N NaOH is prepared and vigorous vortexing ensures the hemin is solubilized completely. The solution is filtered through 0.2 micron filter. The final concentration of hemin, post-filtration, was determined by making dilutions in 0.1 N NaOH and measuring the UV-Vis spectra, using the molar extinction coefficient for hemin in 0.1 N NaOH of 58400 cm^{-1} M^{-1} at 385 nm. Hemin stocks were stored at -20°C. For use in growth assays, hemin stocks are diluted in growth media immediately prior to use.

For hemoglobin purification, 25 mL of fresh, heparinized human blood was centrifuged at 1500 × *g* at 4°C for 10 minutes to pellet the erythrocytes. Erythrocytes were washed three times in three pellet volumes of ice-cold sterile saline and subsequently lysed through resuspension in two pellet volumes of 50 mM Tris pH 8.6, 2 mM EDTA. Erythrocyte lysis was allowed to proceed for 30 minutes at room temperature, mixing periodically by gentle inversion. Cell debris was removed by centrifugation at 11000 × *g* for 30 minutes at 4°C. The supernatant was transferred to a fresh tube and solid NaCl was added (50 mg/mL), with mixing by inversion. The stroma was then precipitated by centrifugation at 11000 × *g* for 30 minutes at 4°C. The hemoglobin-containing supernatant was dialyzed overnight at 4°C against 50 mM Tris pH 8.6, 1 mM EDTA (Buffer A). The dialyzed hemoglobin was passed once through a 0.4 μM syringe filter prior to purification via anion exchange on a Mono Q HR 16/10 column (GE Healthcare). Dialyzed hemoglobin solution was loaded in 4-6 mL batches on the column and a gradient run using 50 mM Tris pH 8.6, 1 mM EDTA, 0.5M NaCl as Buffer B; hemoglobin fraction eluted between 50 – 100 mM NaCl. The purified fractions were dialyzed into 50 mM Tris pH 8.0 and sterilized by passage through a 0.4 μM syringe filter. Purity was assessed using UV-Vis spectrometry. Briefly, a spectral scan was run between 200-800 nm, where a characteristic Soret peak at 415 nm, as well as distinct $\alpha_{576\ nm}$ and $\beta_{541\ nm}$ bands, and a peak at 345 nm, were indicative of intact ferrous oxyhemoglobin. Hemoglobin concentration was determined using published extinction coefficients at 560 and 577 nm (40) and was concentrated to 2 mM using Amicon Ultra-15 centrifugation units (30 kDa NMWL, Millipore). Small aliquots were flash frozen in a dry ice-ethanol bath and stored at -80°C.

2.2.10 Assessment of hemin and hemoglobin utilization by *S. lugdunensis*

In assessing hemin/hemoglobin utilization, single, isolated colonies of *S. lugdunensis* from overnight TSA plates were resuspended in 120 μL RPMIC. 100 μL of this suspension was used to inoculate 2 mL RPMIC with 0.1 μM EDDHA, which was grown for at least 4 hours until OD_{600} was approximately 1. Precultures were subcultured 1:400 into 2 mL aliquots of RPMIC with 1 μM EDDHA and either 1-500 nM human hemoglobin, purified as described above, 20-1000 nM hemin, prepared as described above, or 1 μM $FeSO_4$. Cultures were incubated with shaking at 200 rpm, in 14-mL round-bottom polypropylene tubes. OD_{600} was assessed at 12, 24, and 36 hours.

2.3 Results

2.3.1 Sequence analysis of key iron acquisition loci in *S. lugdunensis*

S. aureus contains several key iron acquisition loci that are well-characterized. These include the *isd* locus that promotes iron acquisition from heme and hemoglobin, the *sfa-hts* locus for synthesis and re-entry of staphyloferrin A (SA), and the *sbn-sir* locus for synthesis and uptake of staphyloferrin B (SB) (18, 19). The genome sequences of *S. lugdunensis* strains N920143 and HKU01-09 indicate that these strains possess *htsABC* genes downstream from an *sfa* locus (Figure 2-1A), with predicted products that are highly similar to the *S. aureus* HtsABC proteins (Table 2-2). However, in contrast to *S. aureus*, the *S. lugdunensis sfa* locus in both strains contains a deletion of approximately 3.3 kb that eliminates the *sfaA* and *sfaD* genes, as well as the promoter for the remaining *sfaBC* genes (Figure 2-1A). This suggests that *S. lugdunensis* does not synthesize SA, yet may be able to utilize it as an iron source, via HtsABC, if SA were provided

Table 2-2: Similarity of iron-regulated proteins between *S. aureus* and *S. lugdunensis*.

Protein	% ID / % TS
HtsA	70/82
HtsB	60/74
HtsC	71/87
SfaB	43/68
SfaC	57/79
SirA	85/92
SirB	84/94
SirC	74/84
IsdC	57/67
IsdE	75/85
IsdB	35/52
IsdA (Sa) vs IsdJ (Sl)	19/29
IsdA (Sa) vs IsdK (Sl)	14/31

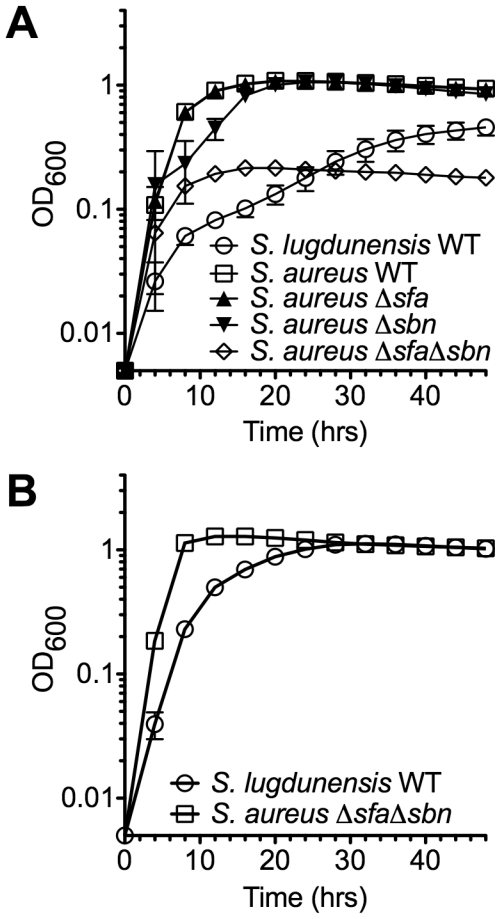
Abbreviations: ID, identity; TS, total similarity; Sa, *S. aureus*; Sl, *S. lugdunensis*

exogenously. In contrast to *S. aureus*, *S. lugdunensis* lacks the *sbn* operon, which encodes the products responsible for synthesizing SB. Interestingly, *S. lugdunensis* possesses homologs of the *S. aureus sirABC* genes (predicted products are highly similar to those from *S. aureus*, see Table 2-2), which encode a SB transporter, and these genes are situated immediately downstream of the *S. lugdunensis isd* locus (Figure 2-1B). In comparison to *S. lugdunensis* N920143, strain HKU01-09 contains an exact, tandem duplication of a large region of DNA encompassing the *isd-sir* locus (Figure 2-1B). Together, the sequence analysis of these loci suggests that *S. lugdunensis* should not be able to synthesize either SA or SB, in contrast to *S. aureus*, which synthesizes and secretes both staphyloferrin molecules. On the other hand, we would predict that *S. lugdunensis* should be able to transport iron via these siderophores, as well as acquire iron from heme/hemoglobin via Isd proteins.

2.3.2 *S. lugdunensis* grows poorly in iron-restricted media, owing to a lack of siderophore production

Our previous studies showed that, in comparison to wildtype *S. aureus*, mutants lacking the ability to synthesize the two staphyloferrin siderophores (i.e. *sfa sbn*) grow poorly in iron-restricted media containing transferrin or serum (as a source of transferrin) (23, 24). Given that *S. lugdunensis* genomic information suggests an inability to produce a staphyloferrin siderophore, we compared the growth of *S. lugdunensis* to that of *S. aureus* and its staphyloferrin-deficient mutants in iron-restricted media. In comparison to wildtype *S. aureus*, *S. lugdunensis* grew poorly in C-TMS with 20% serum (Figure 2-2A) or transferrin (data not shown) and, even after extended incubation periods, never reached a final biomass equivalent to that of *S. aureus*.

Figure 2-2: *Staphylococcus lugdunensis* grows poorly in iron-restricted growth media. (A) Growth kinetics comparing *S. lugdunensis* to that of *Staphylococcus aureus* WT and staphyloferrin A (*sfa*) and staphyloferrin B (*sbn*)-deficient mutants in C-TMS with 20% serum. (B) The growth deficiencies of *S. lugdunensis* and the *S. aureus* staphyloferrin-deficient mutant in iron-restricted media are complemented with addition of 100 μmol/L $FeCl_3$. All data points represent average values for at least three independent biological replicates, and error bars represent standard deviation from the mean.


A
1
0.1
0.01
OD600
S. lugdunensis WT
S. aureus WT
S. aureus Δsfa
S. aureus Δsbn
S. aureus ΔsfaΔsbn
0
10
20
30
40
Time (hrs)
B
1
0.1
0.01
OD600
S. lugdunensis WT
S. aureus ΔsfaΔsbn
0
10
20
30
40
Time (hrs)

Indeed, in these culture conditions, *S. lugdunensis* HKU01-09 (strain HKU01-09 was used throughout this study) grew at a slower rate than the *S. aureus sfa sbn* mutant. We demonstrated that the growth deficiency of *S. lugdunensis* and *S. aureus sfa sbn* was due to a deficiency in the ability to scavenge trace amounts of iron, since supplementation of the growth medium with 100 μM $FeCl_3$ promoted rapid growth of both (Figure 2-2B).

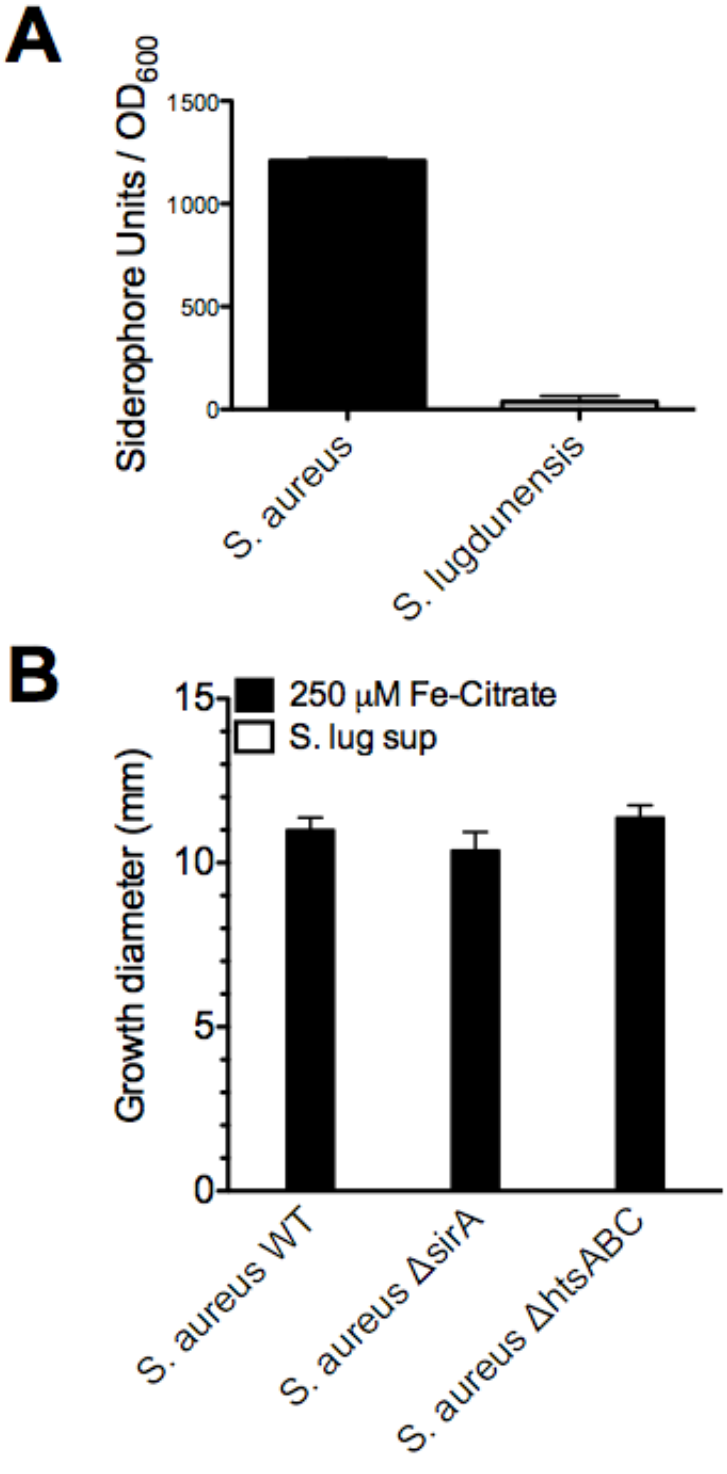
In further support of the bioinformatics analyses indicating that *S. lugdunensis* is incapable of staphyloferrin production, the culture supernatants of *S. lugdunensis*, grown in C-TMS, tested negative in the chrome azurol S assay (Figure 2-3A), indicating a lack of secreted iron-binding molecules. This is in contrast to the positive result obtained for *S. aureus* culture supernatants grown in the same media and under the same conditions.

In support of the result that we could not detect iron-binding molecules in the iron-restricted culture supernatant of *S. lugdunensis*, we demonstrated that concentrated culture supernatants of *S. lugdunensis* were incapable of enhancing iron-restricted growth of *S. aureus* (Figure 2-3B).

2.3.3 *S. lugdunensis* HtsABC and SirABC function as transporters for staphyloferrins A and B, respectively

Despite not synthesizing staphyloferrin A, *S. lugdunensis* has *htsABC* homologs that, in *S. aureus*, are required for utilization of ferric-SA complexes. Promoters containing putative Fur box sequences are found upstream of the *htsABC* and *sirABC* operons in *S. lugdunensis* (Figure 2-4A), suggesting that each operon is iron-regulated in a Fur-dependent fashion. Due to high amino acid sequence similarity of the *S. lugdunensis* HtsA and SirA with the *S. aureus* homologs (see Table 2-2), antibodies raised against *S. aureus* HtsA and SirA were used to successfully demonstrate iron-regulated expression

Figure 2-3: *Staphylococcus lugdunensis* does not secrete iron-binding compounds, and does not support *Staphylococcus aureus* growth in iron-restricted media. (A) Chrome azurol S (CAS) assay demonstrates that culture supernatants of *S. lugdunensis* lack siderophore activity. (B) Agar plate bioassays demonstrate that the culture supernatant of *S. lugdunensis* does not promote the growth of iron-restricted *S. aureus* strains. Ferric citrate was used as a positive control in the experiment. All data points represent average values for at least three independent biological replicates, and error bars represent standard deviation from the mean.


A
Siderophore Units / OD600
1500
1000
500
0
S. aureus
S. lugdunensis
B
250 μM Fe-Citrate
S. lug sup
Growth diameter (mm)
15
10
5
0
S. aureus WT
S. aureus ΔsirA
S. aureus ΔhtsABC

of the *S. lugdunensis* proteins (Figure 2-4B). We next deleted the *htsABC* genes from the *S. lugdunensis* genome and complemented the genes *in trans* by cloning the *htsABC* genes from *S. lugdunensis* back into the mutant strain, which restored expression of HtsA to the mutant strain (Figure 2-4B).

To generate a *S. lugdunensis* mutant lacking both copies of *sirABC*, we deleted the entire tandemly-duplicated region (Figure 2-1B) from the genome, a deletion of approximately 65-kbp. As expected, the mutant failed to express SirA (Figure 2-4B). Complementation of this *isd-sir* deletion strain with the *sirABC* genes from *S. lugdunensis* restored expression of SirA (Figure 2-4B).

With mutant and complemented strains in hand, we used them to test the ability of the strains to utilize the two staphyloferrin siderophores produced by *S. aureus*. As shown in Figure 2-5, the ability of *S. lugdunensis* strains to utilize SA and SB, whether provided in concentrated culture supernatants from *S. aureus*, or as enzymatically-synthesized molecules (data not shown), was absolutely dependent on the expression of *htsABC* and *sirABC*, respectively. Moreover, while the growth of *S. lugdunensis* was enhanced when the intact *S. aureus sfa* gene locus was introduced on a plasmid (Figure 2-3B), the same plasmid was incapable of complementing the iron-restricted growth defect of the *S. lugdunensis htsABC* mutant (data not shown). Together, these data prove that both the HtsABC and the SirABC transporters are functional in *S. lugdunensis*.

Figure 2-4: Expression of *Staphylococcus lugdunensis* HtsA and SirA homologues is iron-regulated. (A) Identification of Fur-boxes upstream of the *htsA*, *sirA* and *isdC* genes in *S. lugdunensis*. Numbers represent the number of identical bases between the 19-bp Fur boxes of *S. aureus* and *S. lugdunensis*. (B) Western blots demonstrating iron-regulated expression of HtsA and SirA, and confirmation of mutations and complementation, where pRMC2 is the vehicle control. Cultures were grown in C-TMS with (+Fe) or without (−Fe) addition of $FeCl_3$ (25 μmol/L). Mutant samples were all grown in C-TMS without addition of iron.

A

htsA TTTTCTCTATATAAATTTTAATAAAATTAGGTT**GACATTGATAATCGTTATC**AATACACTATATAATGTAAATACTATAATT**AAGGAG**TGACATGGA**ATG**

Fur box (17/19) **RBS** **Met**

sirA TTATAAAATTTTTGTTGACTTAGAAAACCTAATCATAGATACTAATCTT**GATAATGATTATCATTGTC**ATACATCATGA**AGGAGG**CTCATTTAGTCA**ATG**

Fur box (18/19) **RBS** **Met**

isdC AAATT**GACATTGATAATCATTTTC**AATTAATCTAATAAGTAGAACTAGAGTGAGCAAAATGATTATATTTTTATCGTGAAA**AGGAGG**CAAAATAAGC**GTG**

Fur box (16/19) **RBS** **Val**

B

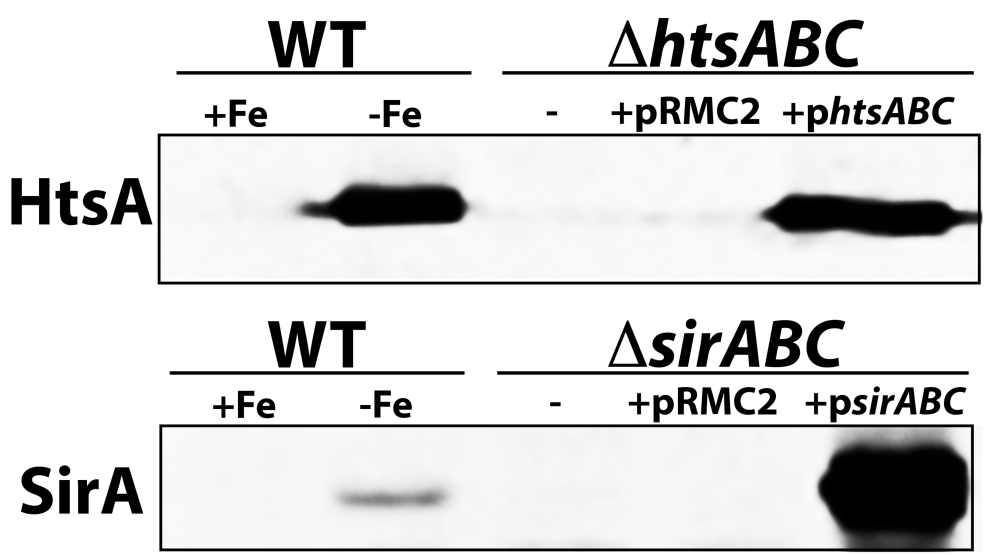
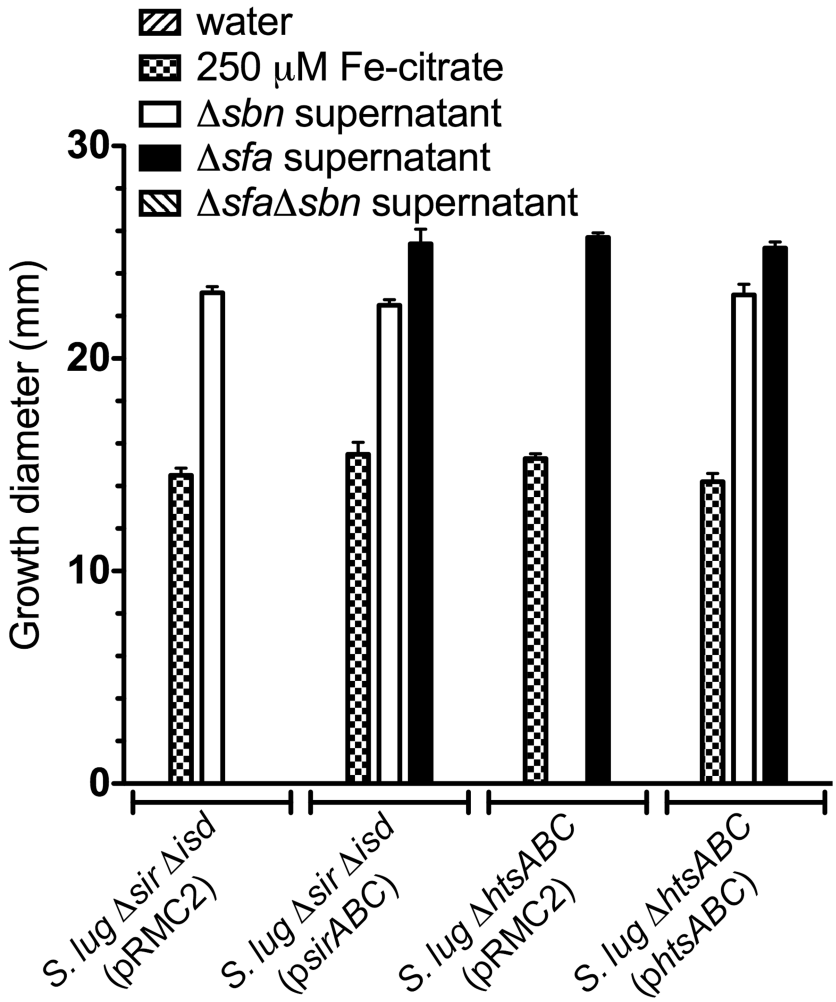


Figure 2-5: Plate bioassays demonstrate that *Staphylococcus lugdunensis* HtsABC and SirABC are required for uptake of staphyloferrin A and staphyloferrin B, respectively. Water and ferric citrate were used as negative and positive controls, respectively. Supernatant extracts supplied were those of *Staphylococcus aureus* mutants that secrete SA (*sbn* mutant), SB (*sfa* mutant) or neither SA or SB (*sfa sbn* mutant). All data points represent average values for at least three independent biological replicates, and error bars represent standard deviation from the mean.


water
250 μM Fe-citrate
Δsbn supernatant
Δsfa supernatant
ΔsfaΔsbn supernatant
Growth diameter (mm)
30
20
10
0
S. lug Δsir Δisd (pRMC2)
S. lug Δsir Δisd (psirABC)
S. lug ΔhtsABC (pRMC2)
S. lug ΔhtsABC (phtsABC)

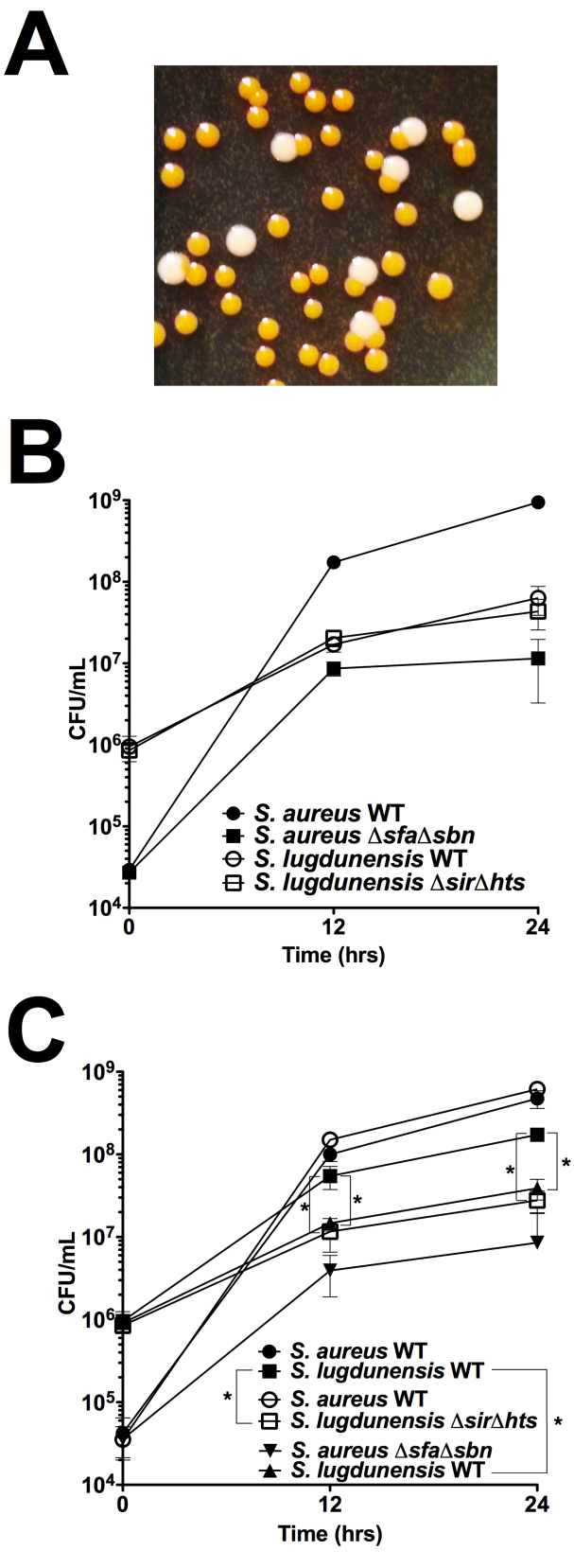
2.3.4 *S. aureus* enhances *S. lugdunensis* growth in a staphyloferrin-dependent manner

As shown above, we demonstrated that exogenously added staphyloferrins could promote the growth of *S. lugdunensis*. Knowing this, we next decided to test whether *S. aureus*, which secretes both SA and SB, could augment the growth of *S. lugdunensis* if they were cultured together in iron-restricted growth media. We first optimized culture conditions so as to be able to easily discern colonies of *S. aureus* RN6390 from those of *S. lugdunensis* HKU09-01 on TSA (Figure 2-6A) (see Experimental Procedures for details).

Figure 2-6B demonstrates that, when cultured in C-TMS containing 20% serum, wildtype *S. aureus* consistently grows from 2 x 10^4 CFU/mL to approximately 1 x 10^9 CFU/mL within 24 hours, whereas the isogenic staphyloferrin-deficient mutant grows to a density of only 1 x 10^7 CFU/mL over the same time frame. *S. lugdunensis*, on the other hand, inoculated at a higher cell density of 1 x 10^6 CFU/mL, only reaches a final cell density of less than 1 x 10^8 CFU/mL in 24 hours. The isogenic *S. lugdunensis isd-sir hts* mutant displays identical growth kinetics in these culture conditions.

For co-culture experiments, in pilot studies, we found that we needed to inoculate *S. lugdunensis* at much higher cell densities than *S. aureus* because *S. aureus* grows significantly faster than *S. lugdunensis* in these culture conditions. Data displayed in Figure 2-6C demonstrate that, when wildtype *S. aureus* is co-cultured with wildtype *S. lugdunensis*, *S. lugdunensis* grew to much higher density, approximately 2 x 10^8, than when cultured on its own (c.f. Figure 2-6B vs Figure 2-6C). We next demonstrated that this growth enhancement was due to the use of the *S. aureus*-produced staphyloferrins by *S. lugdunensis* by use of two complementary experiments (Figure 2-6C). First, wildtype

Figure 2-6: Coculture experiments demonstrate that *Staphylococcus aureus*-produced siderophores can enhance the iron-restricted growth of *Staphylococcus lugdunensis*. (A) Picture of colonies of *S. aureus* RN6390 (large and white) and *S. lugdunensis* (smaller and yellow) growing on a TSB plate after 24 h of incubation at 37°C, followed by 48 h of incubation at room temperature. (B) Growth of individual strains in C-TMS + 20% serum was monitored for CFU/mL at 12 and 24 h timepoints. (C) Growth of strains in cocultures with the pairs of strains grouped as indicated. All data points represent average values for at least three independent biological replicates, and error bars represent standard deviation from the mean. The Student's unpaired *t*-test was used to define statistical significance for the CFU values between strains as indicated by the brackets. $*P < 0.0001$.

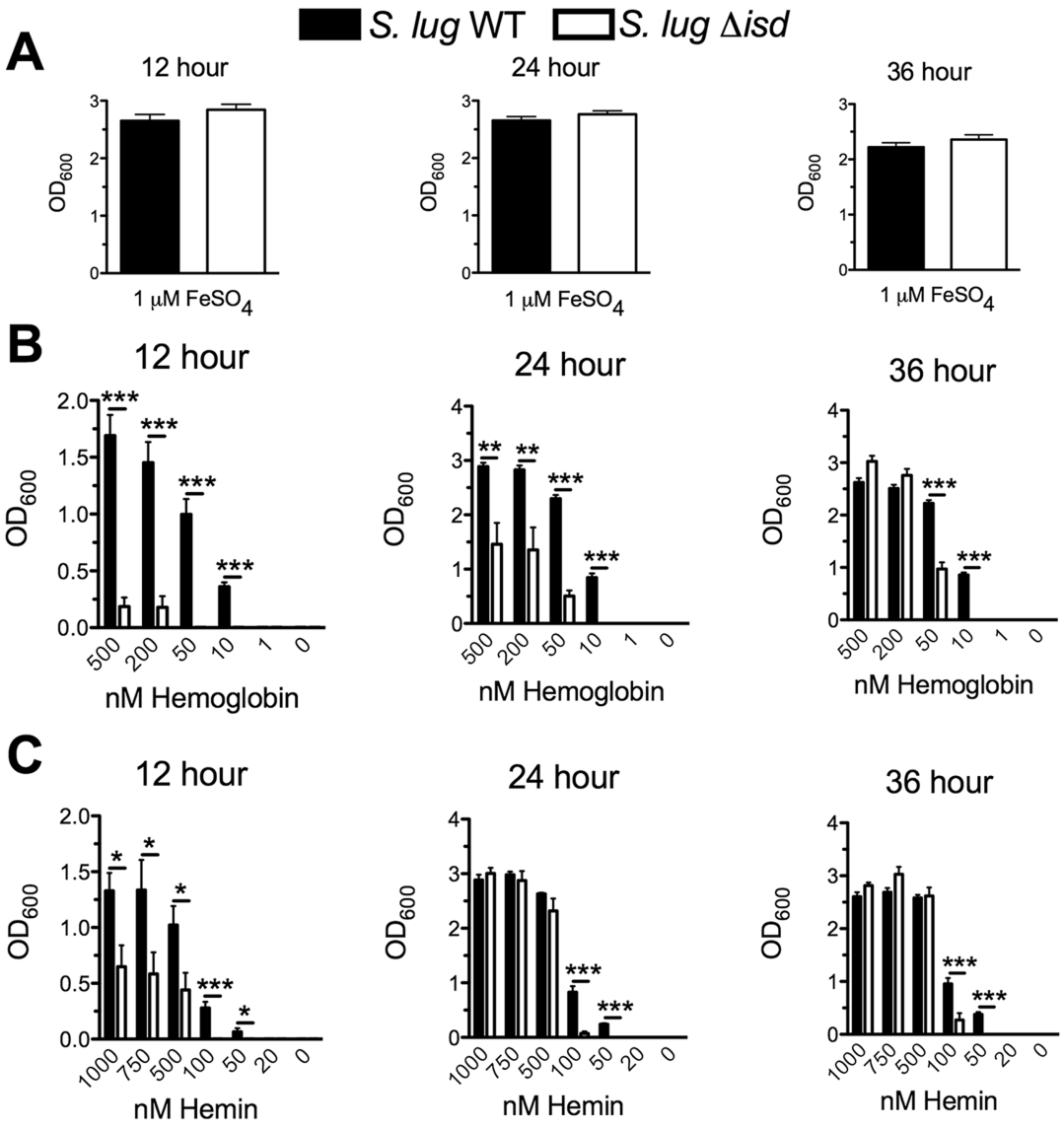

A
B
10^9
10^8
10^7
10^6
10^5
10^4
CFU/mL
0
12
24
Time (hrs)
S. aureus WT
S. aureus ΔsfaΔsbn
S. lugdunensis WT
S. lugdunensis ΔsirΔhts
C
10^9
10^8
10^7
10^6
10^5
10^4
CFU/mL
0
12
24
Time (hrs)
S. aureus WT
S. lugdunensis WT
S. aureus WT
S. lugdunensis ΔsirΔhts
S. aureus ΔsfaΔsbn
S. lugdunensis WT
*

S. aureus did not enhance the growth of the *S. lugdunensis isd-sir hts* mutant like it did wildtype *S. lugdunensis*. Second, staphyloferrin-deficient *S. aureus* had no effect on the growth of wildtype *S. lugdunensis*. Together, these data provide convincing evidence that *S. aureus*, through production of SA and SB, enhances the iron-restricted growth of *S. lugdunensis* in a HtsABC- and SirABC-dependent manner, respectively.

2.3.5 The *S. lugdunensis isd-sir* mutant is attenuated for utilization of heme and hemoglobin

Having constructed a complete *isd* locus deletion strain of *S. lugdunensis* HKU01-09, we next evaluated the mutant for its ability to utilize heme and hemoglobin as sole sources of iron. Zapotoczna *et al.* (29) have also recently generated *isdB* and *isd* locus deletion mutants in *S. lugdunensis* strain N920143 and shown that the mutants were impaired for growth on hemoglobin compared to wildtype. These experiments were performed using a single concentration of hemoglobin, and hemin as a sole iron source was not tested. We took a more comprehensive approach by examining the growth of the *isd-sir* deletion mutant in iron-starved media containing a range of hemin and hemoglobin concentrations, in media that contained enough of the non-metabolizable iron chelator EDDHA to completely restrict growth unless an iron source was added. Importantly, when $FeSO_4$ was provided as a source of free iron, *S. lugdunensis* and the isogenic *isd-sir* mutant grew equally well (Figure 2-7A). It is notable that the *isd-sir* mutant is attenuated for growth at all concentrations of hemoglobin tested (500 nM down to 10 nM; 1 nM hemoglobin is insufficient to promote growth of wildtype under these conditions), especially at 12 hours but also by the 24 hour timepoint (Figure 2-7B). By 36 hours it was

Figure 2-7: Growth of *Staphylococcus lugdunensis* WT and its isogenic Δ*isd* mutant using iron, hemoglobin or heme as a sole iron source. Growth of the Δ*isd-sir* mutant was compared to that of WT strain HKU09-01 at 12-, 24- and 36-h timepoints in RPMIC with 1 μM EDDHA, containing $FeSO_4$ (A) varying concentrations of human hemoglobin, (B) or varying concentrations of bovine hemin, (C) as the sole iron source. All data points represent average values for at least three independent biological replicates, and error bars represent standard deviation from the mean. Statistical significance was determined using the Student's unpaired t-test; *P < 0.05; **P < 0.01; ***P < 0.0001.


S. lug WT
S. lug Δisd
A
12 hour
24 hour
36 hour
OD600
1 μM FeSO4
B
12 hour
24 hour
36 hour
OD600
nM Hemoglobin
500
200
50
10
1
0
C
12 hour
24 hour
36 hour
OD600
nM Hemin
1000
750
500
100
50
20
0

apparent that hemoglobin was beginning to promote the growth of the mutant, especially at the higher hemoglobin concentrations. Notably, at the low nM concentrations of hemoglobin (i.e. below 50 nM), the *isd-sir* mutant is significantly attenuated for growth, compared to wildtype, even through 36 hours of incubation.

A similar growth pattern was observed for the *isd-sir* mutant when hemin was used as the sole iron source (Figure 2-7C). It is apparent that the *isd-sir* locus provides a significant growth advantage to *S. lugdunensis* at early stages of growth at all concentrations tested (up to 1 μM) and continues to provide a significant growth advantage to the cells through 36 hours of incubation in the presence of hemin at concentrations below 100 nM (Figure 2-7C).

2.4 Discussion

S. lugdunensis is a relatively recently recognized bacterium that is both a commensal, and an important human pathogen, capable of causing serious infections such as aggressive native valve infective endocarditis (IE) (8). That IE caused by *S. lugdunensis* can occur independent of indwelling medical devices differentiates *S. lugdunensis* from other CoNS, and makes it worthy of investigation. Molecular studies of *S. lugdunensis* are in their infancy and, thus, there is a paucity of information concerning important virulence factors that underpin the potential of this opportunistic pathogen to cause severe and invasive infections. The molecular mechanisms of iron acquisition that are key to the biology and infectivity of this bacterium are essentially unknown. Zapotoczna *et al*. (29) examined the role of the iron-regulated surface determinant system in the utilization of hemoglobin and found that, in strain N920143, the Isd-dependent heme/hemoglobin utilization system is functional by demonstrating that an *isd* deletion mutant and an *isdB*

mutant were both slightly debilitated for growth on hemoglobin as a sole iron source. Moreover, *S. lugdunensis* IsdG, like its *S. aureus* counterpart, degrades heme to staphylobilin and free iron (26). In this study, we furthered these findings by demonstrating that the tandemly-duplicated *S. lugdunensis isd* locus in strain HKU09-01 was required for promoting early and rapid growth on a wide range of hemoglobin and heme concentrations ranging from 10-500 nM and 50-1000 nM, respectively (Figure 2-7B and 2-7C), eventually leading to increased bacterial biomass that was sustained through 36 hours of incubation, especially at the lower concentrations. It is worth noting that these low concentrations of hemoglobin and heme are physiologically relevant, since these molecules are removed from circulation in the liver subsequent to being quickly bound by haptoglobin and hemopexin, respectively.

S. lugdunensis is severely attenuated for growth in iron-restricted media that do not contain a readily utilizable iron source such as hemin or hemoglobin. In media containing serum or transferrin, for example, *S. lugdunensis* is severely compromised for growth compared to *S. aureus*. We have shown that this growth defect is readily corrected with excess iron, indicating an inability to attain iron from the media. Based upon previous studies, it is known that the *sfa* gene cluster from *S. aureus* is both necessary, based on the phenotype of *sfa* mutants (23) and sufficient, based on heterologous cloning in *E. coli* (41) for staphyloferrin A synthesis. *S. lugdunensis* supernatant lacks siderophore activity (Figure 2-3A) and does not support iron-restricted *S. aureus* growth (Figure 2-3B), indicating a lack of staphyloferrins A and B.

That the *sfa* deletion is found in both *S. lugdunensis* strains where genome sequence is available suggests that this deletion may be common to the species. The

deletion (see Figure 2-1A) removes the promoter for each of the two transcripts *sfaD* and *sfaABC*, and also completely removes the *sfaA* and *sfaD* genes, encoding the putative SA efflux pump (SfaA) and one of the synthetases that joins a citrate molecule to the δ-amine of D-ornithine to form the first amide intermediate of staphyloferrin A (41). Studies in the early 1990s identified staphyloferrin A in culture supernatants of many species of staphylococci, whereas staphyloferrin B was found in supernatants of fewer species (42, 43). Of the species tested, which did not include a *S. lugdunensis* representative, only *S. sciuri* and *S. hominis* were found incapable of producing either siderophore, but only one strain was tested from each of these species and the result may not reflect the capabilities of the species overall. Based on available genomic data, on the other hand, *S. lugdunensis* (genome data exist for strains N920143 and HKU09-01) appears to be unique amongst the staphylococci in its inability to synthesize at least one of the two staphyloferrin molecules. This would imply that, if other *S. lugdunensis* strains also lack these loci, the species causes opportunistic infections independent of siderophore production, and would presumably rely on either heme acquisition or uptake of xenosiderophores to satisfy its iron requirements throughout the various stages of colonization and infection. Genomic information identifies that *S. lugdunensis* has homologs of *fhuCBG* and *sstABCD* where predicted products share high levels of similarity with those in *S. aureus* (24, 35). This would indicate that *S. lugdunensis* is capable of using hydroxamates (via FhuCBG) and catechols/catecholamines as means to acquire iron. The *S. lugdunensis* FhuC ATPase shares greater than 95% similarity with its *S. aureus* counterpart and we hypothesize that this ATPase is, like in *S. aureus* (44), important for providing the energy for not only the

uptake of hydroxamate siderophores, but also the staphyloferrins through HtsABC and SirABC.

Interestingly, despite its noted ability to cause serious infection in humans, *S. lugdunensis* N920143 was reported to cause only very mild infection in a rat model of endocarditis, much milder than that which would be caused by equivalent CFUs of *S. aureus* (25). In pilot experiments we, too, noted a relative lack of virulence of *S. lugdunensis* HKU09-01, using a mouse model of hematogenous spread. Mice challenged with up to of 1×10^8 CFUs showed no overt signs of illness, and continued to gain weight, despite detectable counts in the kidneys for at least 7 days following bacterial challenge via tail vein. This contrasts from the course of disease that would be caused by a much lower dose of *S. aureus*. As noted previously (8, 25, 27, 28), the *S. lugdunensis* genome indicates an absence of orthologues of well-characterized *S. aureus* toxin and immune evasion encoding genes, suggestive of a limited capacity to cause severe disease, at least in comparison to *S. aureus*.

That *S. lugdunensis* has retained the transport machinery for both staphyloferrin A and staphyloferrin B is interesting, despite an inability to synthesize either siderophore. This may represent a mechanism for scavenging these iron-binding molecules produced by other species of staphylococci, including *S. aureus*. Indeed, our data show that co-culture of the two bacteria in the same iron-limited growth media enhanced the growth of *S. lugdunensis* by at least one log. This growth enhancement was due to *S. lugdunensis*' ability to scavenge the *S. aureus* staphyloferrins in an Hts- and Sir-dependent manner. We speculate that this is a viable 'opportunistic' strategy used by *S. lugdunensis in vivo*. Although *S. lugdunensis* predominantly inhabits lower parts of the human body (i.e. the

perineum) (9) and *S. aureus* largely inhabits the nares, both species are present to some degree over the entire external surface of the body (45–47). Indeed, *S. lugdunensis* is recovered with other bacteria in approximately 60% of infections, including co-occurrence with *S. aureus* and other staphylococci (7). It may be that over time *S. lugdunensis* has evolved to simply steal siderophores produced by other species of bacteria, including *S. aureus*, and in these situations is more capable of causing opportunistic infections of humans.

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3

Chapter 3

Ferric hydroxamate and stress hormone acquisition by *Staphylococcus lugdunensis*

3.1 Introduction

Iron is an essential nutrient for nearly all forms of life as it is required for cellular processes including amino acid, DNA synthesis and respiration. In aerobic conditions, ferric iron (FeIII) is insoluble at neutral pH and the concentration of free iron is well below the requirement to support microbial growth (1). Ferrous iron (FeII) is able to catalyze the formation of free radicals which elicit macromolecular damage on cells (2). Iron within mammals is sequestered in order to alleviate toxicity and purpose it for cellular processes. The majority of iron within the host is bound within heme in hemoglobin, predominantly found within circulating erythrocytes (3). Alternatively, iron may be bound within the intracellular iron-storage protein ferritin, or sequestered by extracellular glycoproteins such as transferrin and lactoferrin (1, 4). Transferrin has a high binding affinity for ferric iron (K_D of approximately 10^{-22} M) and is the primary iron-sequestration factor in vertebrate serum (5). As such, the circulating concentration of free iron is well below the requirement to support invading pathogen growth, and iron sequestration by the host remains an important form of nutritional immunity (1).

Staphylococcus lugdunensis, like several other coagulase-negative staphylococci (CoNS), is a human skin commensal. However, it is also regarded as an emerging pathogen with elevated virulence compared to other species of CoNS. Infections are reminiscent of those caused by *S. aureus* and when host external protective barriers are breached, *S. lugdunensis* is able to cause an array of distinct infections including skin and soft tissue infections (SSTIs), bacteremia, pneumonia, and osteomyelitis (6–9). Although most commonly associated with SSTIs, *S. lugdunensis* is best known for causing aggressive infective endocarditis, which can bear a mortality rate of up to 50% (10, 11).

Additionally, many reports detail *S. lugdunensis* endocarditis following skin trauma or surgical procedures including vasectomy and kidney transplant (9, 12–14). The versatility of this pathogen to colonize different host niches likely requires *S. lugdunensis* to adapt its cellular physiology to overcome challenges presented in these diverse environments, including iron limitation. As such, the ability to acquire host iron as an essential nutrient must contribute to growth promotion of *S. lugdunensis* in various host niches.

Many pathogens overcome host iron limitation through the ability to acquire heme from hemoglobin (15, 16). The iron-regulated surface determinant (Isd) system was first identified in *S. aureus* and acts as a multiprotein transport pathway to exploit host hemoglobin to acquire heme-iron (17, 18). The Isd pathway is able to extract heme from hemoglobin at the bacterial cell surface and transport it across the cell wall and through the cytoplasmic membrane into the cytoplasm, using a consort of proteins (19–22). Function of the Isd system in *S. aureus* is critical to its pathogenesis, and *S. lugdunensis* is unique among CoNS to encode a similar pathway (23–26). The *S. lugdunensis* Isd system is not nearly as well characterized, however studies have shown that it does function to acquire heme and hemoglobin-iron at nanomolar concentrations, for growth under iron-limitation (27–29).

Another strategy of iron acquisition is the removal of iron from glycoproteins, such as transferrin, via secreted siderophores (low molecular weight, high affinity FeIII chelators) (30). *S. aureus* elaborates the siderophores staphyloferrin A (SA) and staphyloferrin B (SB), of which biosynthetic proteins are encoded by *sfa* and *sbn* loci, respectively (31, 32). SA is transported into the cell by HtsABC and SB is transported through SirABC, each of which are encoded by loci adjacent to their respective cognate

siderophore biosynthetic genes (33, 34). Although both SA and SB are required for optimal growth of *S. aureus* in human serum, SB is thought to be the more predominant of the two during systemic infection, whereas SA has been found to contribute to the formation of subcutaneous lesions in *S. aureus*-infected mice (16, 35). Contrary to other staphylococci, *S. lugdunensis* is unable to produce either SA or SB, due to gene deletions, contributing to its inability to grow in animal serum, although it has retained the transport pathways for both of these siderophores (28).

In addition to SA and SB, *S. aureus* can also capture xenosiderophores bearing hydroxamate moieties through the use of the ferric hydroxamate uptake (Fhu) pathway (31, 36, 37). Furthermore, the iron-withholding capability of serum may be compromised by host-produced stress hormones (catecholamines), which are able to reduce FeIII within transferrin to FeII, thereby releasing it (38–42). Catecholamine stress hormones are able to form 2:1 or 3:1 complexes with FeIII and may provide iron as a 'pseudosiderophore' to bacteria expressing catechol transport systems. It has been shown that *S. aureus* employs the Sst transporter to acquire catecholamine-iron for growth in low-iron environments (41). There exists a paucity of information regarding the mechanisms of iron acquisition in *S. lugdunensis* and their role in virulence.

In this study we investigate iron procurement strategies of *S. lugdunensis* by characterizing siderophore and host stress hormone acquisition strategies. We demonstrate that *fhuC* is required for acquisition of hydroxamate and polycarboxylate (SA and SB) siderophores. Furthermore, we examine the role of the inexact-duplicated Sst gene sets in *S. lugdunensis*, finding differences in ligand-binding affinity and expression pattern between the two. Additionally, we utilize a novel *S. lugdunensis*

systemic murine infection model to demonstrate that a *isd fhuC sst* mutant is impaired for growth in kidneys, and for inducing weight loss.

3.2 Experimental procedures

3.2.1 Bacterial strains and media

Bacterial strains and vectors employed in this study are summarized in Table 3-1. *Escherichia coli* strains were grown in Luria-Bertani broth (LB, BD Diagnostics) or on LB agar. For routine culture and genetic manipulation, *S. lugdunensis* and *S. aureus* strains were cultured in tryptic soy broth/agar (TSB/TSA; BD Diagnostics) or on TSB agar. For growth experiments, *S. lugdunensis* and *S. aureus* were cultured in: (i) RPMI 1640 (Life Technologies) supplemented with 1% w/v casamino acids (BD Diagnostics) (RPMIC) and ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA; LGC Standards GmbH); (ii) Tris-minimal succinate (TMS) broth/agar (43); (iii) TMS treated with 5% (w/v) Chelex-100 resin (Bio-Rad) at 4°C for 24 hours (C-TMS); or (iv) an 80:20 mixture of C-TMS and complement-inactivated horse serum (Sigma Aldrich). Bacteria were cultured at 37°C with shaking at 220 rpm unless otherwise indicated. For *E.* coli selection, 100 μg mL^{-1} ampicillin or 30 μg mL^{-1} kanamycin were used. For *S. lugdunensis* and *S. aureus* selection, 10 μg mL^{-1} chloramphenicol was used.

3.2.2 Real-time PCR

Quantitative real-time PCR was performed as previously described (35). Briefly, *S. lugdunensis* HKU09-01 RNA was prepared from triplicate 3 mL cultures grown in C-TMS or C-TMS with 100 μM $FeCl_3$. Cultures were harvested to an OD_{600} of 3.0 and RNA was extracted using the Aurum Total RNA Mini Kit (BioRad). 500 ng extracted

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

Bacterial strain, plasmid or oligonucleotide	**Description**[a]	**Source or reference**
Strains		
E. coli		
DH5α	Φf80d*lacZ*ΔM15 *recA1 endA1 gyrAB thi-1 hsdR17*(r_K^- m_K^-) *supE44 relA1 deoR Δ(lacZYA-argF)U169*	Promega
BL21λ(DE3)	F^-*ompThsdS*$_B$(r_B^- m_B^-) *dcmgal* λ(DE3)	Novagen
H3320	BL21λ(DE3) pET28::*sst1D*; Km^R	This study
H3321	BL21λ(DE3) pET28::*sst2D*; Km^R	This study
S. lugdunensis		
HKU09-01	Human skin infection isolate	(25)
H2710	HKU09-01 Δ*isd-sir*	(28)
H2970	HKU09-01 Δ*fhuC*	This study
H3016	HKU09-01 Δ*sst*	This study
H3325	HKU09-01 Δ*isd-sir* Δ*fhuC* Δ*sst*	This study

H3316	HKU09-01 Δ*fhuC* pRMC empty vector control; Cm^R	This study
H3317	HKU09-01 Δ*fhuC* pRMC::*fhuC* (pfhuC); Cm^R	This study
S. aureus		
RN4220	Prophage-cured laboratory strain; r_K^- m_K^+; accepts foreign DNA	(44)
H1666	Newman Δ*sbnABCDEFGHI*::Tc Δ*sfaABCsfaD*::Km; Tc^R Km^R	(41)
H2224	Newman Δ*sstABCD*::Em Δ*sbnABCDEFGHI*::Tc Δ*sfaABCsfaD*::Km; Em^R Tc^R Km^R	(41)
H3311	H1666 pRMC empty vector control; Tc^R Km^R Cm^R	This study
H3312	H2224 pRMC empty vector control; Em^R Tc^R Km^R Cm^R	This study
H3313	H2224 pRMC::*sst1; sst* mutant complemented with *S.lugdunensis sst1ABCD*; Em^R Tc^R Km^R Cm^R	This study
H3314	H2224 pRMC::*sst2; sst* mutant complemented with *S.lugdunensis sst2ABCD*; Em^R Tc^R Km^R Cm^R	This study

Plasmids		
pKOR1	*E. coli/Staphylococcus* shuttle vector allowing allelic replacement in staphylococci; Ap^R Cm^R	(45)
pKOR1Δ*fhuC*	pKOR1 plasmid for in-frame deletion of *fhuC*; Ap^R Cm^R	This study
pKOR1Δ*sst*	pKOR1 plasmid for deletion of genetic region encompassing duplicated *sstABCD*; Ap^R Cm^R	This study
pRMC2	*E. coli/Staphylococcus* shuttle vector: Ap^R Cm^R	(46)
pRMC2::*fhuC*	pRMC2 derivative for *fhuC* expression; Ap^R Cm^R	This study
pRMC2::*sst1*	pRMC2 derivative for *sst1ABCD* expression; Ap^R Cm^R	This study
pRMC2::*sst2*	pRMC2 derivative for *sst2ABCD* expression; Ap^R Cm^R	This study
pET28a(+)	*E. coli* vector for overexpression of recombinant hexahistidine-tagged proteins; Km^R	Novagen
pET28::*sst1D*	pET28a(+) derivative encoding N-terminally hexahistidine-tagged soluble portion of Sst1D; Km^R	This study

pET28::*sst2D*	pET28a(+) derivative encoding N-terminally hexahistidine-tagged soluble portion of Sst2D; Km^R	This study

Oligonucleotides[b,c]	
Purpose	Sequence (5'-3')
Primers for generating upstream and downstream recombinant regions for Δ*fhuC* using pKOR1	**(*AttB1*)-fhuCUF:***GGGGACAAGTTTGTACAAAAAAGCAGGCT* CTTGGTATTGGGATAATCG **fhuCUR:** GTTGTCCATTCAAGCGAC **fhuCDF:**Phos/CAGGCAAACCATTATTAGTTACC **(AttB2)-fhuCDR:***GGGGACCACTTTGTACAAGAAAGCTGGGT* TGTCAATGGCAATACTTTAG
Primers for generating upstream and downstream recombinant regions for Δ*sst* using pKOR1	**(*AttB1*)-sstUF:***GGGGACAAGTTTGTACAAAAAAGCAGGCT* TATTGCTCGGGTATCAAG **sstUR:** GCCAACAAACAATGAAATG **sstDF:** Phos/AAATCATCAGCCAAACAGG **(*AttB2*)-sstDR:***GGGGACCACTTTGTACAAGAAAGCTGGGT* AAACACGCTGGCTTTATG
Primers for cloning *fhuC* into pRMC2 for complementation	**KpnI-fhuCF:** GATCGGTACCAAGACGCAAGTGTCAAGAG **SacI-fhuCR:**

	GATCGAGCTCACAGCACCTAAATCTCTTGG
Primers for cloning *sst1* into pRMC2 for complementation	**KpnI-sst1F**: GATCGGTACCTGCCTTAGACACAACGAC **SacI-sst1R**: GATCGAGCTCGACTCGTAAGAAAGCAAACC
Primers for cloning *sst2* into pRMC2 for complementation	**EcoRI-sst2F**: GATCGAATTCAGGTTCTGTTGTTGGTGG **EcoRI-sst2R**: GATCGAATTCTAAATGTTGTCCCGCTCC
Primers for cloning *sst1D* into pET28a(+) for overexpression	**NdeI-sst1DF:** GATCCATATGGAAACAAAGAGTGGCGAATCA **SacI-sst1DR:** GATCGAGCTCGGAATGATATCCCCACTTCA
Primers for cloning *sst2D* into pET28a(+) for overexpression	**NdeI-sst2DF:** GATCCATATGAGCTCAGATGCTAAGTCATCA **SacI-sst2DR:** GATCGAGCTCGCTAAACAAGATGTCTTGAAAT
Primers for RT-PCR of *rpoB*	**F:** AGAGAAAGACGGCACTGAAAACAC **R:** ATAACGACCCACGCTTGCTAAG
Primers for RT-PCR of *fhuC*	**F:** TGGACCAAATGGATGTGG **R:** GCTACTTCTGGAGATTGTGG
Primers for RT-PCR	**F:** CTCGTTTGCTTTCCTCAAG

of *sst1A*	**R:** TGCCACCCAACATAATACC
Primers for RT-PCR of *sst2A*	**F:** GGCATTATGTTAGGTGGTATTG **R:** CGTCCACTTGTAATAATGGC
Primers to confirm deletion of *sfaDA*	**F:** TGATAGTTTCTCTAATGCGTTCTC **R:** CAGAATACATCAAATCTTGCG
Primers to confirm presence of *isdJK*	**F:** GAATGATTTCCACCAGTCAG **R:** GTTTCCCTTGAATCGTGAC
Primers to confirm presence of *sirABC*	**F:** TACTGCTCCAAAATCCCC **R:** TTTAGCGTGCGGTATGTC
Primers to confirm duplicated *sstABCD*	**F:** GAGTTATCAACATTCGGC **R:** AAGGGTCACTAACACATAG

[a] Ap^R, Cm^R, Km^R, Em^R and Tc^R; resistance to ampicillin, chloramphenicol, kanamycin, erythromycin and tetracycline, respectively.

[b] Restriction sites for cloning are underlined.

[c] Phos/ denotes a 5' phosphate on the primer.

RNA was reverse-transcribed and PCR-amplified using iScript™ One-Step RT-PCR Kit with SYBR Green (Bio-Rad) and primers outlined in Table 3-1. Data were normalized relative to expression of the *rpoB* housekeeping gene.

3.2.3 Blood cultures

The growth of several *S. lugdunensis* strains was assessed in iron-restricted media with blood as a sole iron source. Single, isolated colonies were resuspended in 2 mL RPMIC with 0.1 μM EDDHA and incubated for at least 4 hours until OD_{600} was about 2. Cultures were normalized to an OD_{600} of 1 and 5 μL was subcultured into 2 mL culture medium for a starting bacterial density of 2-5 x 10^6 CFU/mL. Culture medium consisted of 2 mL RPMIC with 1 μM EDDHA and 0.001% fresh, whole human blood from healthy volunteer donors, with or without addition of 50 μM $FeCl_3$. Cultures were grown at 37°C on a rotisserie for 20 hours prior to dilution and spot plating on TSA for bacterial enumeration.

3.2.4 Molecular genetic methods

For in-frame deletion of *fhuC* and deletion of the tandem-duplicated *sstABCD* regions (Δ*sst*) in *S. lugdunensis*, allelic replacement using the pKOR1 vector was performed as described previously (45). Briefly, 500 – 1,000-bp DNA fragments flanking regions of interest were amplified using the primers found in Table 3-1. Upstream and downstream flanking amplicons were cloned into pKOR1. Knockout vectors were passaged through *S. aureus* RN4220 before introduction into *S. lugdunensis* by electroporation of 4μg DNA (47, 48). Plasmids were integrated onto the genome at 43°C in the presence of chloramphenicol prior to counter-selection at 30°C in the presence of anhydrotetracycline (200 ng mL^{-1}). Chloramphenicol-sensitive colonies were chosen for screening by PCR

across the deleted region in the chromosome, which was further confirmed by sequencing (45). The same process was used over to generate multiple deletions in one strain.

For complementation, *S. lugdunensis fhuC, sstA1B1C1D1* and *sstA2B2C2D2* were cloned with each respective native promoter, using primers described in Table 3-1. Amplicons were cloned into pRMC2 for creation of p*fhuC*, p*sst1* and p*sst2*, further electroporated into target strains. For *sstA2B2C2D2* cloning into pRMC2, alkaline phosphatase treatment (Antarctic phosphatase; New England BioLabs) was performed post-digestion to prevent self-ligation of the insert (both ends bear EcoRI cut sites).

3.2.5 Siderophore preparation and plate bioassays

S. aureus concentrated culture supernatants were prepared from Δ*sbn,* Δ*sfa,* and Δ*sbn*Δ*sfa* mutants, respectively, as described previously (31). Strains were grown in C-TMS with aeration for 36 hours prior to removal of cells. Supernatants were lyophilized and insoluble matter was removed by methanol extraction (one-fifth original culture volume). Methanol was removed by rotary evaporation, and dried material was resuspended in water to one-tenth culture volume to provide culture extracts. Staphyloferrin B was prepared *in-vitro* enzymatically, as described previously (32, 34, 49). Enzymes were removed from the reaction mixture using an Amicon Ultra-0.5 10k filter column (Millipore) and the Staphyloferrin B reaction mixture was normalized to Deferoxamine (DFO, London Health Sciences Center) equivalents as determined using the chrome azurol S (CAS) siderophore detection assay (50). Staphyloferrin A was commercially prepared by Indus BioSciences (India). Ferric –enterobactin, -salmochelin S4, -aerobactin and coprogen were purchased from EMC Microcollections. Ferrichrome was purchased from Sigma, whereas citrate was purchased from Fisher Scientific.

The ability of culture supernatants and purified siderophores to support *S. lugdunensis* iron-restricted growth was assessed with agar plates using plate-based disk diffusion bioassays (28, 51). Briefly, 1 x 10^4 *S. lugdunensis* cells were incorporated into TMS-agar containing 5 μM ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA, LGC Standards GmbH). Siderophores/supernatants applied to sterile paper disks were placed onto the agar, and growth around disks was measured after 24 hours at 37°C.

3.2.6 Iron-regulated protein expression by *S. lugdunensis*

Antisera against *S. aureus* SstD, used in this study, was previously prepared (41). This antisera was used for analysis of iron-regulated SstD expression in *S. lugdunensis*. Cells were grown in C-TMS with or without 100μM $FeCl_3$ for 24 hours, normalized and lysed with lysostaphin (Sigma). Whole cell lysates were normalized to 8 μg total protein and resolved by SDS-polyacrylamide gel electrophoresis. Western blotting was performed as previously described (28). The membrane was blocked in phosphate buffered saline (PBS) with 10% (w/v) skim milk, 0.05% Tween 20 and 20% (v/v) horse serum. Antiserum was applied at a 1:5,000 dilution in PBS with 0.05% Tween 20 and 5% horse serum, prior to addition of anti-rabbit IgG conjugated to IRDye-800 (1:20,000 dilution; Li-Cor Biosciences). The membrane was washed between each step. Fluorescence imaging was performed using a Li-Cor Odyssey infrared imager (Li-Cor Biosciences).

3.2.7 Growth curves

Growth of *S. lugdunensis* and *S. aureus* strains was assessed in C-TMS with serum. Single, isolated colonies were resuspended in 2 mL C-TMS and grown for over 4 hours until OD_{600} was above 1. Each culture was normalized to an OD_{600} of 1 and subcultured 1:200 in C-TMS:horse serum. Wildtype *S. lugdunensis* as well as *S. aureus* strains

bearing Δ*sbn and* Δ*sfa* mutations, are impaired for growth in this media compared to siderophore-producing strains (28, 31). Human stress hormones were added to the media for a final concentration of 50 μM to assess for catecholamine-iron acquisition for growth enhancement. Dopamine hydrochloride, L-3,4-dihydroxyphenylalanine (L-DOPA), DL-norepinephrine hydrochloride, (-)-epinephrine, 2,3-dihydroxybenzoic acid (DHBA) and 2,5-DHBA were purchased from Sigma. Chloramphenicol was also included for strains harbouring pRMC2 or derivatives. Cultures were grown in a Bioscreen C plate reader (Growth Curves USA) at 37°C with constant shaking at medium amplitude. OD_{600} was assessed at 15 minute intervals however, for graphical clarity, 4 hour intervals are shown.

3.2.8 Protein overexpression and purification

Recombinant *S. aureus* SstD was purified as previously described (41). Regions of the genes encoding the soluble portions of *S. lugdunensis* SstD1 and SstD2 (excluding lipobox motifs) were amplified and cloned into pET28(a)+ (Novagen) using primers listed in Table 3-1. *E. coli* BL21 bearing pET28::*sstD1* or pET28::*sstD2* were grown to mid-log phase at 37°C in LB with kanamycin, prior to induction with 0.4 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). After addition of IPTG, cultures were grown at 25°C overnight. Cells were collected by centrifugation, resuspended in 20 mM Tris, pH 8.0, 500 mM NaCl, 10 mM imidazole (binding buffer), and ruptured in a cell disruptor (Constant Systems Ltd). Insoluble matter and debris were removed by centrifugation at 3,000 x *g* for 15 minutes, followed by 150,000 x *g* for 60 minutes, sonicating samples in between. Soluble material was filtered and applied to a nickel-loaded 1 mL HisTrap column (GE Healthcare) equilibrated with binding buffer. His_6-tagged proteins were eluted in 1 mL fractions from the column over a 0-80% gradient of 20 mM Tris, pH 8.0,

500 mM NaCl, 500 mM imidazole (elution buffer). Fractions bearing pure SstD1 and SstD2 (analyzed via SDS-PAGE) were pooled and dialyzed into 10 mM Tris, pH 8.0, 100 mM NaCl (working buffer) at 4°C. Protein concentrations (Bio-Rad protein assay) were normalized to equality and aliquots were frozen at −80°C.

3.2.9 Protein-ligand binding

Intrinsic tryptophan fluorescence quenching was used to assess protein-ligand binding affinity for *S. lugdunensis* SstD1, SstD2 and *S. aureus* SstD as previously described (41). Proteins were adjusted to 0.5 μM in 3 mL working buffer and ligands were added at 2-fold concentration increments. Dopamine, L-DOPA, epinephrine, norepinephrine, DFO and salmochelin S4 were used as ligands. Ligands were incubated in 3:1 (catecholamine hormones) or 1:1 (siderophores) molar ratios to $FeCl_3$ for 5 minutes at room temperature prior to use. Bovine serum albumin (Sigma) was used as a protein negative control. Fluorescence was measured at room temperature in a Fluorolog instrument (Horiba Group), with excitation at 280 nm and emission detection at 345 nm. An excitation slit width of 5 nm and an emission slit width of 5 nm were used. Changes in fluorescence due to ligand additions and sample volume increase were corrected for (52). Fluorescence intensity data analysis and K_D determination were performed as previously described (41).

3.2.10 Western blots

Recombinant proteins were analyzed for purity and immunogenicity towards αSstD (*S. aureus*) antisera. *S. lugdunensis* SstD1, SstD2 and *S. aureus* SstD purified protein volumes were normalized to contain 3 μg total protein and resolved by SDS-PAGE. Western blotting was performed as described above (iron-regulated protein expression by

S. lugdunensis) with modifications. After blocking, αSstD antisera were applied at a 1:20,000 dilution, and αHis antibody was applied 1:10,000. Anti-rabbit IgG conjugated to IRDye-800 (1:20,000 dilution) was secondary to αSstD antisera, whereas anti-mouse Alexa Fluor 680 (Life Technologies) was secondary to αHis (1:20,000 dilution). Antibodies/antisera were applied in PBS with 0.05% Tween 20 and 5% horse serum, washing in between applications.

3.2.11 Spot dilution plate assays

Overnight TSB cultures of *S. lugdunensis* strains were pelleted, resuspended in saline and normalized to an OD_{600} of 1.1. Normalized bacterial suspensions were ten-fold serially diluted and 10 µL aliquots of 10^{-4} to 10^{-7} dilutions were spot plated onto TSA and TMS agar. Images of TSB plates were taken after 17, 24 and 28 hours of incubation, whereas images of TMS plates were taken after 24, 28 and 42 hours of incubation.

3.2.12 Murine model of systemic *S. lugdunensis* infection

All protocols for murine infection were reviewed and approved by the University of Western Ontario's Animal Use Subcommittee, a subcommittee of the University Council on Animal Care. Six-week-old, female, BALB/c mice were obtained from Charles River Laboratories and housed in microisolator cages. *S. lugdunensis* strains were grown to mid-exponential phase (OD_{600} 2 - 2.5) in TSB, washed twice with PBS, and resuspended in PBS to an OD_{600} of 0.50. 100 µL of bacterial suspension, equivalent to 2-3 x 10^7 CFU, was injected into each mouse via tail-vein. Mice were weighed at time of challenge and every 24 hours after, where infection was allowed to proceed for 3-6 days before mice were euthanized via intraperitoneal injection of pentobarbital. Organs were aseptically harvested into PBS with 0.1% (v/v) Triton X-100, homogenized, diluted and plated onto

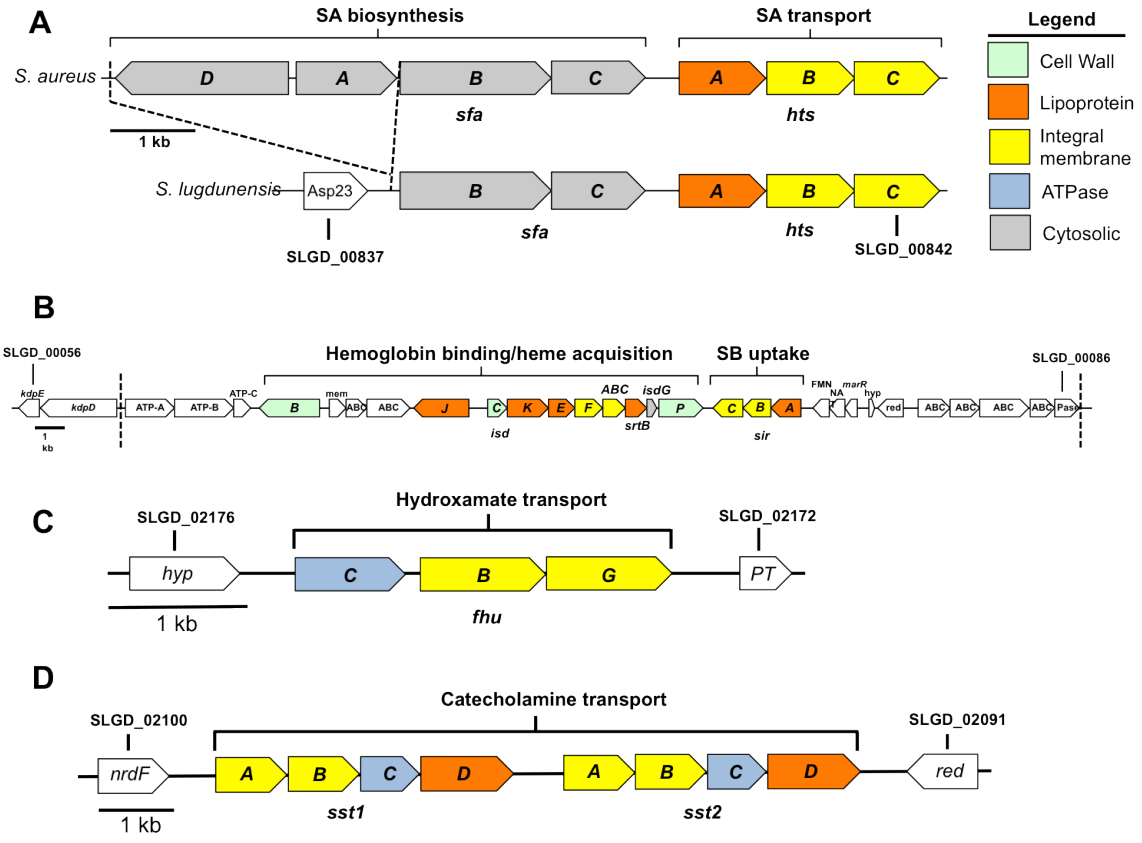
TSA to enumerate bacterial burden. Weight data are presented as the difference in percentage from mouse weight at time of challenge. Recovered bacterial load from kidneys is presented as $\log_{10}$ CFU per kidney pair.

3.3 Results

3.3.1 Bioinformatic analysis of sequences implicated for iron acquisition by *S. lugdunensis*

There are several known loci important for *S. aureus* iron acquisition. Isd proteins acquire heme-iron from hemoglobin, *sfa-hts* and *sbn-sir* loci are required for biosynthesis and transport of SA and SB, respectively, the *fhu* locus for hydroxamate siderophore acquisition, and the *sst* locus for catecholamine uptake (41, 53, 54). The study of iron acquisition mechanisms in *S. lugdunensis*, on the other hand, is still in its infancy. Several genomic regions of interest were identified in *S. lugdunensis* based on sequence comparison. Genome sequences of strains N920143 and HKU09-01 indicate a deletion in the *sfa* locus such that *sfaAD* and the promoter region for *sfaBC* have been removed (Figure 3-1A), yet downstream *hts* genes are intact and have been determined to be functional for SA acquisition (28). No genes for SB synthesis are present in the *S. lugdunensis* genome, and the bacterium is indeed inhibited for growth in serum compared to *S. aureus* strains competent for either SA or SB production (28). Genes for SB acquisition are proximal to genes for heme acquisition in the *S. lugdunensis* genome, and the *isd-sir* region is in exact tandem duplication in strain HKU09-01 (Figure 3-1B). *sir* and *isd* genes are required for SB acquisition and high-affinity heme acquisition from hemoglobin, respectively, for this species (27, 28). *S. lugdunensis* possesses genes homologous to *S. aureus fhuCBG* required for ferric-hydroxamate acquisition

Figure 3-1: Physical maps of genetic loci implicated in *Staphylococcus lugdunensis* iron transport. (A) Comparison of the *S. aureus* staphyloferrin A (SA) biosynthetic and transport locus with the homologous gene set in *S. lugdunensis*. All sequenced *S. lugdunensis* strains carry the depicted deletion, between the dashed lines, of *sfaAD* and the promoter region for the remaining two biosynthetic genes. (B) The *isd-sir* locus in *S. lugdunensis* is depicted. The region between dashed lines is duplicated in tandem in strain HKU09-01, with an identical region upstream of *sir* genes (ATP-A duplicate at SLGD_00087). (C) Ferric-hydroxamate transport locus in *S. lugdunensis*. (D) Ferric-catecholamine uptake loci in *S. lugdunensis*. The tandemly duplicated loci encode proteins that share ~70% identity. Abbreviations as follows: Asp23; Alkaline shock protein 23, ATP-A,B,C; K+-ATPase components A, B, and C, respectively, mem; membrane protein, ABC; ATP-binding cassette transporter component, FMN; FMN binding protein, NAT; *N*-acetyltransferase, marR; MarR-type regulator, hyp; hypothetical protein, red; reductase, Pase; phosphatase, PT; phosphotransferase.


A
SA biosynthesis
SA transport
Legend
S. aureus
D
A
B
C
A
B
C
sfa
hts
1 kb
S. lugdunensis
Asp23
B
C
A
B
C
sfa
hts
SLGD_00837
SLGD_00842
Cell Wall
Lipoprotein
Integral membrane
ATPase
Cytosolic
B
SLGD_00056
Hemoglobin binding/heme acquisition
SB uptake
SLGD_00086
kdpE
kdpD
ATP-A
ATP-B
ATP-C
B
mem
ABC
ABC
J
C
K
E
F
ABC
isdG
P
C
B
A
FMN
NA
marR
hyp
red
ABC
ABC
ABC
ABC
Pase
isd
srtB
sir
1 kb
C
SLGD_02176
Hydroxamate transport
SLGD_02172
hyp
C
B
G
PT
fhu
1 kb
D
SLGD_02100
Catecholamine transport
SLGD_02091
nrdF
A
B
C
D
A
B
C
D
red
sst1
sst2
1 kb

(Figure 3-1C, Table 3-2). The *S. aureus fhuC* ATPase is somewhat promiscuous as it is able to provide energy for transport of not only hydroxamates but also carboxylate siderophores SA and SB (31, 36, 37). The functionality and promiscuity of FhuC in *S. lugdunensis* is not yet known. Additionally, *S. lugdunensis* has gene sets homologous to *S. aureus sst* (Figure 3-1D). Interestingly, the *sst* gene sets are tandemly duplicated but are not identical at the sequence level (Table 3-2). All sequenced *S. lugdunensis* strains have these tandemly duplicated *sst* gene sets, which we predict to transport ferric-catechols and catecholamines. Together, based on genome sequence data, we predict that *S. lugdunensis* should be able to transport hydroxamate siderophores through the Fhu transporter, and catecholamines through Sst1 and Sst2 transporters.

3.3.2 Prevalence of iron acquisition genes in *S. lugdunensis* clinical isolates

Based on the genome sequences of strains HKU09-01 and N920143, *S. lugdunensis* appears to harbor several key iron acquisition loci. To assess whether these strains are indeed representative of all or the majority of clinical isolates, we PCR amplified iron acquisition loci in a number of *S. lugdunensis* clinical isolates obtained from London Health Sciences Centre - Victoria Hospital (London, Ontario, Canada) (see Table 3-1 for primers used). Including prototype HKU09-01 strain, all isolates (27/27) have a deletion in the *sfa* locus, and thus would not be able to produce SA and should not be able to grow well in serum due to the inability to produce this siderophore common to staphylococcal species (Table 3-3). Additionally, all isolates were PCR-positive for *isd* genes (PCR across *isdJ* and *isdK*), indicating a presumptive ability to acquire heme-iron from hemoglobin. All isolates also possess *sir* genes for SB acquisition (Table 3-3). Using

Table 3-2: Similarity between iron-regulated proteins of *Staphylococcus aureus* and *Staphylococcus lugdunensis*.

Protein	% ID/% TS	Function
FhuC	86/95	ATPase; required for transport of hydroxamates, ferric-staphyloferrin A, ferric-staphyloferrin B
FhuB	72/85	Permease; specific for hydroxamates
FhuG	70/88	Permease; specific for hydroxamates
SA SstA vs. SL SstA1	80/92	Permease; specific for catechols
SA SstB vs. SL SstB1	72/88	Permease; specific for catechols
SA SstC vs. SL SstC1	65/83	ATPase; required for transport of catechols
SA SstD vs. SL SstD1	68/81	Lipoprotein; receptor for catechols
SA SstA vs. SL SstA2	74/88	
SA SstB vs. SL SstB2	67/86	
SA SstC vs. SL SstC2	65/83	
SA SstD vs. SL SstD2	62/77	
SL SstA1 vs. SL SstA2	80/91	
SL SstB1 vs. SL SstB2	72/87	
SL SstC1 vs. SL SstC2	71/83	
SL SstD1 vs. SL SstD2	71/84	

Table 3-3: Genes for iron acquisition in *Staphylococcus lugdunensis* clinical isolates. *sfa* – genes for production of staphyloferrin A. *isd* – genes for heme acquisition from hemoglobin. *sir* – genes for staphyloferrin B uptake. *sst* – genes for catecholamine transport. - not hemolytic; + mild hemolysis 0.5-1.5mm; ++ moderate hemolysis 2.0-2.5mm, +++ more hemolysis >2.5mm after 24 hours at 37°C on sheep blood agar (BD Diagnostics).

***S. lugdunensis* strain**	**Description**	**Infection**	**Hemolysis[a]**	**Confirmed deletion in *sfa* locus[b]**	**Growth in serum[c]**	**Confirmed *isd* genes[b]**	**Confirmed *sir* genes[b]**	**Confirmed duplication of *sst* loci[b]**	**Source or reference**
HKU09-01	Human skin infection isolate	Skin infection	++	+	Poor	+	+	+	(25)
N920143	Breast abscess isolate	Breast abscess	-	+	Poor	+	+	+	(26)
SLci1	Clinical isolate #1	N.D.	+	+	Poor	+	+	+	This study
SLci2	Clinical isolate #2	N.D.	+	+	Poor	+	+	+	This study
SLci3	Clinical isolate #3	N.D.	++	+	Poor	+	+	+	This study
SLci4	Clinical isolate #4	N.D.	+	+	Poor	+	+	+	This study
SLci5	Clinical isolate #5	N.D.	++	+	Poor	+	+	+	This study
SLci6	Clinical isolate #6	N.D.	+	+	Poor	+	+	+	This study
SLci7	Clinical isolate #7	N.D.	++	+	Poor	+	+	+	This study
SLci8	Clinical isolate #8	N.D.	+	+	Poor	+	+	+	This study
SLci9	Clinical isolate #9	N.D.	+++	+	Poor	+	+	+	This study
SLci10	Clinical isolate #10	N.D.	-	+	Poor	+	+	+	This study
SLci50	Clinical isolate #50	N.D.	-	+	Poor	+	+	+	This study
SLci51	Clinical isolate #51	N.D.	+++	+	Poor	+	+	+	This study
SLci52	Clinical isolate #52	N.D.	++	+	Poor	+	+	+	This study
SLci53	Clinical isolate #53	N.D.	+	+	Poor	+	+	+	This study
SLci54	Clinical isolate #54	N.D.	+	+	Poor	+	+	+	This study
SLci55	Clinical isolate #55	N.D.	++	+	Poor	+	+	+	This study
SLci56	Clinical isolate #56	N.D.	-	+	Poor	+	+	+	This study
SLci57	Clinical isolate #57	N.D.	-	+	Poor	+	+	+	This study
SLci58	Clinical isolate #58	N.D.	+	+	Poor	+	+	+	This study
SLci59	Clinical isolate #59	N.D.	+	+	Poor	+	+	+	This study
SLci60	Clinical isolate #60	N.D.	+	+	Poor	+	+	+	This study
SLci61	Clinical isolate #61	Abscess	+	+	Poor	+	+	+	This study
SLci62	Clinical isolate #62	Abdomen aspirate	++	+	Poor	+	+	+	This study
SLci63	Clinical isolate #63	Abdomen wound	+++	+	Poor	+	+	+	This study
SLci64	Clinical isolate #64	Ankle bone and tissue	++	+	Poor	+	+	+	This study

[a] 1-2 colonies from an overnight TSB plate were patched onto 5% sheep blood agar. Radius of hemolysis was measured from the point of growth after 24 hours at 37°C. - not hemolytic; + mild hemolysis 0.5-1.5mm; ++ moderate hemolysis 2.0-2.5mm, +++ more hemolysis >2.5mm.

[b] Primers used are listed in Table 1.

[c] Several colonies of each isolate were pre-grown in 2mL C-TMS for at least 4 hours. Cultures were normalized to an OD_{600} of 1 and subcultured 1:400 into C-TMS:horse serum with or without 100μM $FeCl_3$ (excess iron data not shown) and incubated with aeration for 24 hours at 37°C 220 rpm. Poor growth OD_{600}<0.5; Good growth OD_{600}>1.0.

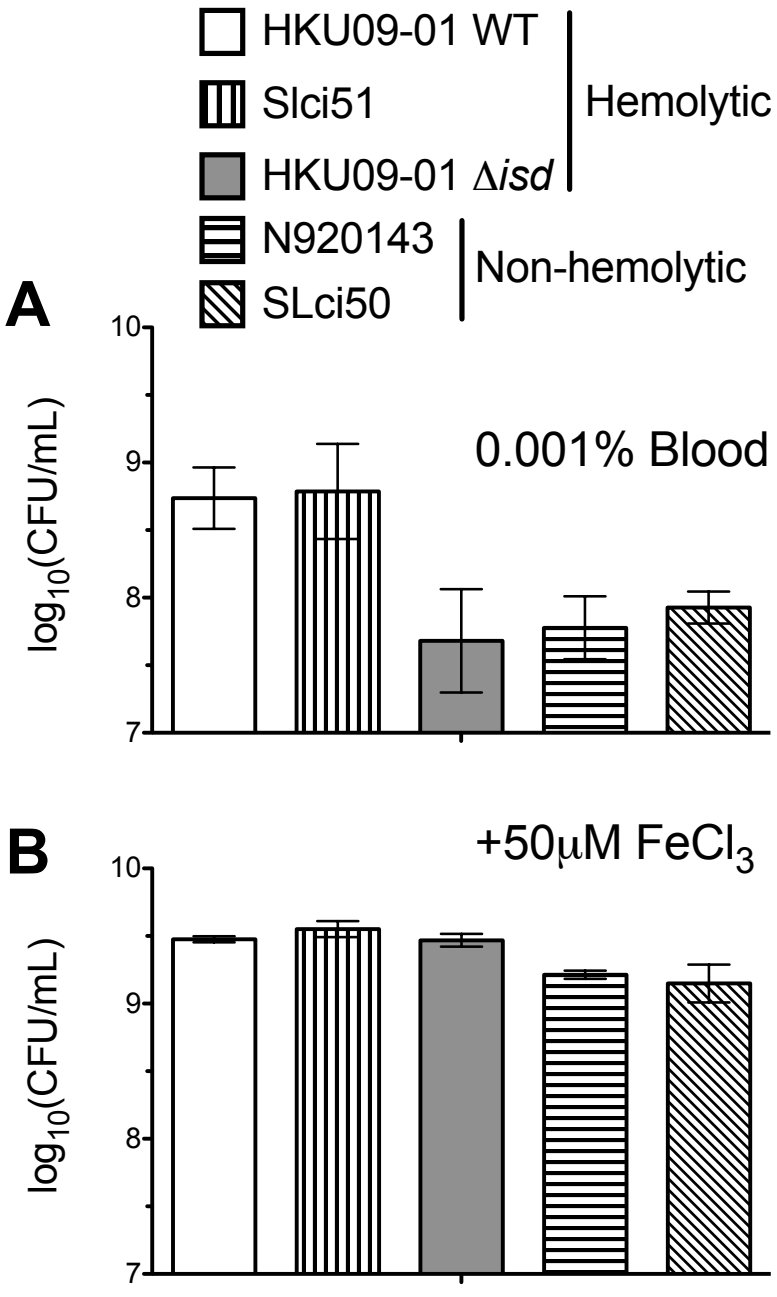
primers within both *sst* loci and others that would amplify across the midsection of the inexact, tandem duplicate *sst* loci, we found all isolates to contain both *sst* loci.

All clinical isolates grew poorly in C-TMS medium with 20% horse serum (serum), and none were able to achieve a biomass of OD_{600}>0.5. These isolates were able to grow well in serum with addition of excess iron (100μM $FeCl_3$), in which they attained a biomass of OD_{600}>1.0 (data not shown). Isolates had varying degrees of zones of hemolysis on blood agar. In comparison to HKU09-01, few had greater hemolysis (3/26), more had a similar level of hemolysis (7/26), most isolates were slightly less hemolytic (11/26), and a fraction were not hemolytic (5/27). The non-hemolytic clinical isolates did not exhibit hemolysis even during prolonged incubation (48 hours at 37°C). Previous studies reported that approximately 5% of *S. lugdunensis* isolates were non-hemolytic, whereas 19% of our isolates are non-hemolytic (55, 56).

3.3.3 Hemolytic clinical isolates are enhanced for growth in blood

The fact that we identified several non-hemolytic isolates in clinical manifestations led us to speculate whether hemolysis plays an important role for *S. lugdunensis* growth in a host environment. Several representative clinical isolates were assessed for growth in iron-restricted media supplemented with fresh whole human blood. Using a low concentration of blood, the hemolytic isolates, including HKU09-01, were able to achieve a much greater bacterial density than the non-hemolytic isolates. Whereas hemolytic isolates grew to a density of about 10^9 CFU/mL, non-hemolytic isolates, including N920143, only grew to 10^8 CFU/mL under the same conditions (Figure 3-2A). The growth difference is due to an inability of non-hemolytic isolates to obtain iron from blood, as addition of excess iron to the media enhanced growth of non-hemolytic isolates

Figure 3-2: Hemolytic *Staphylococcus lugdunensis* clinical isolates exhibit enhanced growth in blood compared to non-hemolytic isolates. (A) Bacterial cultures were grown in iron-restricted media supplemented with 0.001% whole human blood as a sole iron source. (B) Excess ferric chloride was added to blood media to ascertain non-hemolytic isolate impairment for iron acquisition from blood. Data are the average of three independent biological replicates, with error bars indicating standard error to the mean.

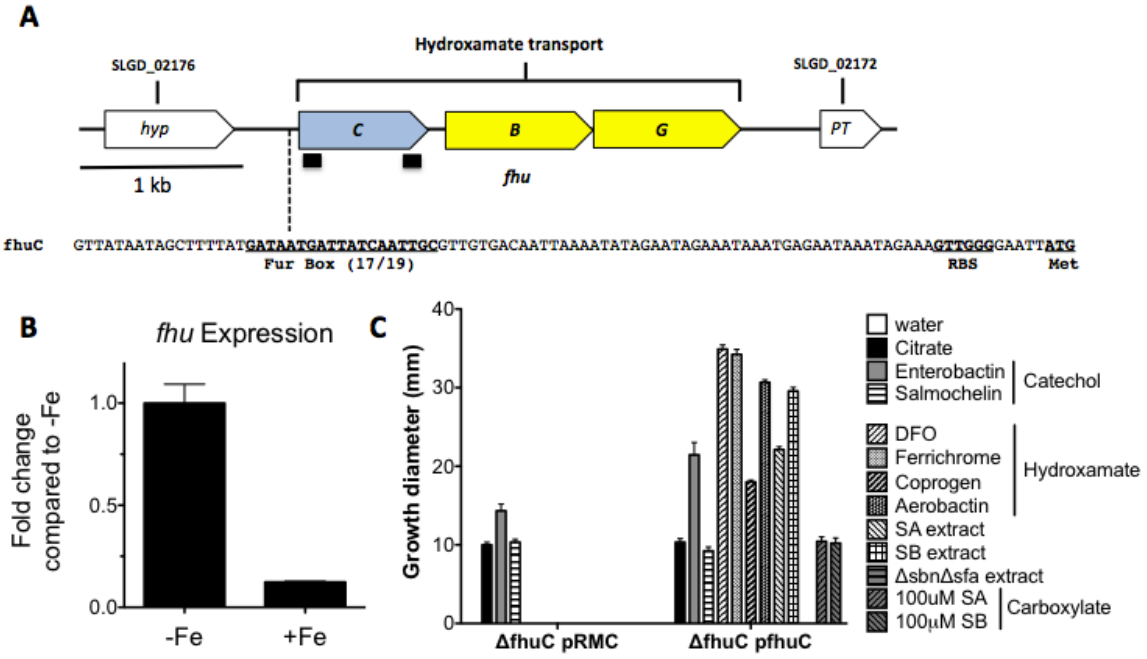

HKU09-01 WT
Slci51
HKU09-01 Δisd
Hemolytic
N920143
SLci50
Non-hemolytic
A
0.001% Blood
log10(CFU/mL)
10
9
8
7
B
+50μM FeCl3
log10(CFU/mL)
10
9
8
7

such to equal hemolytic isolate cell density (Figure 3-2B). An HKU09-01 *isd* mutant, although hemolytic, grew as poorly as the non-hemolytic strains (Figure 3-2A). The mutant was able to grow and exhibit hemolysis on blood agar plates (trypticase soy agar base medium)(data not shown), however its growth was hindered when blood was the sole source of iron, indicating that extracellular heme/hemoglobin are required for growth. We conclude that hemolysis and subsequent heme acquisition via Isd are required for optimal growth of *S. lugdunensis* in human blood.

3.3.4 *fhuC* is required for siderophore-mediated growth promotion of *S. lugdunensis*

Siderophores contribute to microbial growth in low-iron conditions by appropriating FeIII from host proteins such as transferrin and lactoferrin, and deliver it to microorganisms for growth. It is also common for microorganisms to acquire siderophores produced by heterologous organisms, a strategy that may be metabolically favorable. This would be particularly advantageous for *S. lugdunensis* because it does not produce a siderophore and, as such, this property would take on added importance to the biology of this microbe. In previous work, we showed that *S. lugdunensis* was able to acquire both *S. aureus*-produced SA and SB siderophores to promote its own growth in serum (28), however its ability to use other siderophores has not yet been investigated. Based on the presence of a canonical Fur box upstream of the *fhu* locus (Figure 3-3A), we tested and confirmed that the *fhu* genes are iron-regulated (Figure 3-3B). This suggests that in low-iron environments, *S. lugdunensis* may be able to use hydroxamate siderophores as a source of iron. To test this, we created an in-frame *fhuC* deletion in *S. lugdunensis* to determine whether this mutant would be debilitated for acquisition of

Figure 3-3: *Staphylococcus lugdunensis* FhuC is iron-regulated and required for uptake of hydroxamate and carboxylate siderophores. (A) Physical map of the ferric-hydroxamate uptake genes in *S. lugdunensis*. The sequence in between (and including) the black boxes underneath *fhuC* depict the chromosomal region deleted to produce a *S. lugdunensis* in-frame *fhuC* deletion mutant. The promoter sequence for the operon is shown, with the Fur box highlighted. (B) qPCR of *fhu* gene expression by wildtype *S. lugdunensis* grown overnight in C-TMS (-Fe) or C-TMS with 100 μM $FeCl_3$ (+Fe). Data was normalized relative to *rpoB* expression, and gene expression was set relative to that in C-TMS without added iron, set to 1 as the comparator. (C) Plate bioassays demonstrate that a *S. lugdunensis fhuC* mutant is able to use catecholamine siderophores, but unable to acquire hydroxamate-bound or staphyloferrin-bound iron. Water and ferric citrate were used as negative and positive controls, respectively. Hydroxycarboxylates staphyloferrin A (SA) and staphyloferrin B (SB) were administered as *Staphylococcus aureus* mutant-derived culture supernatants as well as *in-vitro* synthesized compounds, with the Δ*sbn*Δ*sfa* strain unable to produce either siderophore. DFO; desferrioxamine B. Data are the average of at least three independent biological replicates.

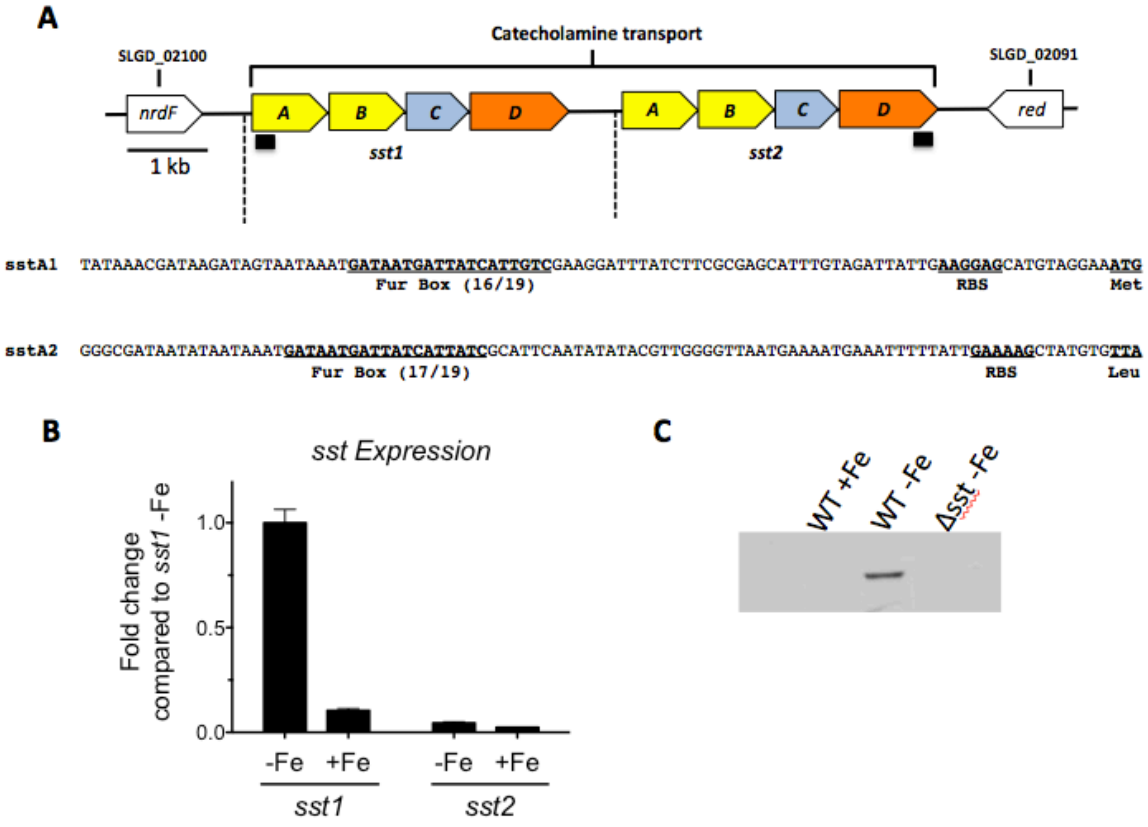

A
Hydroxamate transport
SLGD_02176
SLGD_02172
hyp
C
B
G
PT
1 kb
fhu
fhuC GTTATAATAGCTTTTATGATAATGATTATCAATTGCGTTGTGACAATTAAAATATAGAATAGAAATAAATGAGAATAAATAGAAAGTTGGGGAATTATG
Fur Box (17/19)
RBS
Met
B
fhu Expression
Fold change compared to -Fe
1.0
0.5
0.0
-Fe
+Fe
C
Growth diameter (mm)
40
30
20
10
0
ΔfhuC pRMC
ΔfhuC pfhuC
water
Citrate
Enterobactin
Salmochelin
Catechol
DFO
Ferrichrome
Coprogen
Aerobactin
Hydroxamate
SA extract
SB extract
ΔsbnΔsfa extract
100uM SA
100μM SB
Carboxylate

various siderophores. In agreement with its predicted role in ferric hydroxamate uptake, the *fhuC* mutant was unable to use a variety of hydroxamate siderophores as an iron source, yet retained the ability to use catecholamine siderophores and citrate for means of iron acquisition (Figure 3-3C). These *fhuC*-dependent phenotypes were complemented by supplying *fhuC in trans* into the mutant strain. Of note, we also found that the *fhuC* mutant was unable to utilize either SA or SB carboxylate siderophores (Figure 3-3C). These results also mirror those seen in *S. aureus*, where FhuC is also required for uptake of these endogenous siderophores (31, 37). Thus, FhuC plays an essential role in the acquisition of multiple types of siderophores in several pathogenic species of staphylococci.

3.3.5 *S. lugdunensis sst* genes are required for catecholamine acquisition and growth in serum

Catecholamine hormones enhance growth of pathogenic bacteria in serum by mediating iron release from transferrin (38, 40, 41, 57). Although catecholamines have little effect on *S. aureus* growth unless SA and SB production is impaired, they can stimulate *S. epidermidis* growth on transferrin (58, 59). Given the various growth-promoting effects of catecholamines on several staphylococci, and that *S. lugdunensis* does not produce a siderophore, we hypothesized that *sst* genes may contribute to iron acquisition by this species. Analysis of *sst* promoter regions identified Fur boxes in both, indicating that expression of *sst* loci would likely be iron-regulated (Figure 3-4A). qPCR revealed that *sst1* is highly upregulated in low-iron conditions compared to iron-replete conditions, whereas *sst2* gene expression was extremely low in either iron limited or iron-replete conditions (Figure 3-4B). Moreover, using antisera generated against *S. aureus* SstD, we

Figure 3-4: *Staphylococcus lugdunensis* Sst transporter expression. (A) Physical map of putative ferric-catecholamine genes in *S. lugdunensis*. The tandemly duplicated loci encode proteins where paralogs share ~70% identity (see Table 2). The sequence in between (and including) the black boxes underneath *sstA1-sstD2* depict the chromosomal region deleted to produce a *S. lugdunensis sst* deletion mutant. The promoter sequences for each gene set are shown, with Fur boxes highlighted. (B) qPCR of *sst1* and *sst2* gene expression by *S. lugdunensis* grown overnight in C-TMS (-Fe) or C-TMS with 100 μM $FeCl_3$ (+Fe). Data were normalized relative to *rpoB* expression, and expression was set relative to *sst1* in C-TMS without added iron, set to 1 as the comparator. (C) Western blot demonstrating iron-regulated expression of SstD1. Cultures were grown overnight in C-TMS (-Fe) or C-TMS with addition of 100 μM $FeCl_3$ (+Fe). Antisera raised against *S. aureus* SstD was used to assay for *S. lugdunensis* SstD.


A
Catecholamine transport
SLGD_02100
SLGD_02091
nrdF
A
B
C
D
A
B
C
D
red
1 kb
sst1
sst2
sstA1 TATAAACGATAAGATAGTAATAAATGATAATGATTATCATTGTCGAAGGATTTATCTTCGCGAGCATTTGTAGATTATTGAAGGAGCATGTAGGAAATG
Fur Box (16/19)
RBS
Met
sstA2 GGGCGATAATATAATAAATGATAATGATTATCATTATCGCATTCAATATATACGTTGGGGTTAATGAAAATGAAATTTTTATTGAAAAGCTATGTGTTA
Fur Box (17/19)
RBS
Leu
B
sst Expression
Fold change compared to sst1 -Fe
1.0
0.5
0.0
-Fe +Fe
-Fe +Fe
sst1
sst2
C
WT +Fe
WT -Fe
Δsst -Fe

demonstrated expression of one or both *S. lugdunensis* SstD proteins during iron-limited growth (Figure 3-4C) (41).

To test the functionality of the *sst* genes in catecholamine-iron acquisition, we constructed a mutant with both *sst* loci deleted, and assessed the growth of this mutant in catecholamine-supplemented media. The *sst* mutant was defective for growth compared to wildtype when grown in serum supplemented with the catecholamines dopamine, L-DOPA, epinephrine, and norepinephrine (Figure 3-5). The mutant grew comparably to wildtype in serum supplemented with DFO, owing to a functional Fhu transporter system (see above). Supplementation with either the catechol siderophore precursor 2,3-DHBA or the mammalian siderophore 2,5-DHBA did not promote *S. lugdunensis* growth. Thus, *sst* genes in *S. lugdunensis* are required for catecholamine-iron transport.

In an attempt to determine the relative importance of *sst1* and *sst2*, we sought to create individual *sst1* and *sst2* deletion mutants. However, for reasons we were not able to uncover, we were unable to create single *sst*-locus deletion mutants. We therefore chose to complement the *sst1*-*sst2* deletion mutant with vectors containing the individual gene sets. The *sst1* gene set complemented the catecholamine-dependent growth defect of the mutant (Figure 3-5), confirming its role in the process. Unfortunately, although we were able to clone the vector containing the *sst2* gene set into *E. coli* and *S. aureus*, we were unable to clone it into *S. lugdunensis* despite repeated attempts. *S. lugdunensis* transformants always contained deletions within the *sst2* region.

Since we were able to mobilize *sst2* into *S. aureus*, we were therefore able to test its role for catecholamine uptake in this organism. We cloned the vector, as well as that

Figure 3-5: *Staphylococcus lugdunensis sst1* is required for catecholamine-iron uptake. Growth of *S. lugdunensis* Δ*sst* was assessed compared to isogenic wildtype and Δsst complemented with *sst1* genes *in trans* (p*sst1*), in C-TMS with 20% serum supplemented with 50μM of each catechol compound indicated. Desferrioxamine B (DFO), a hydroxamate, was used as a positive control. Data are the average of at least three independent biological replicates.

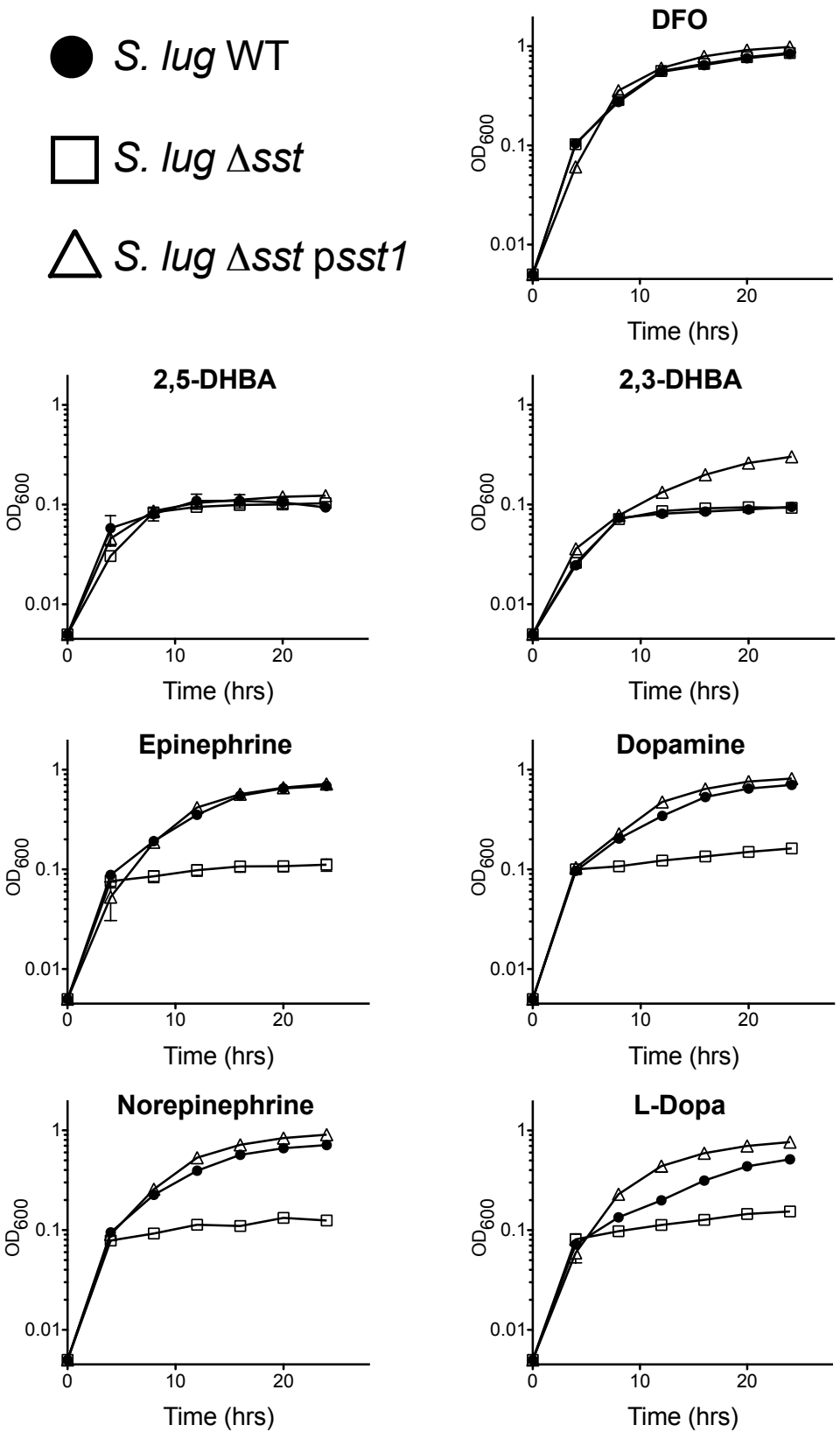
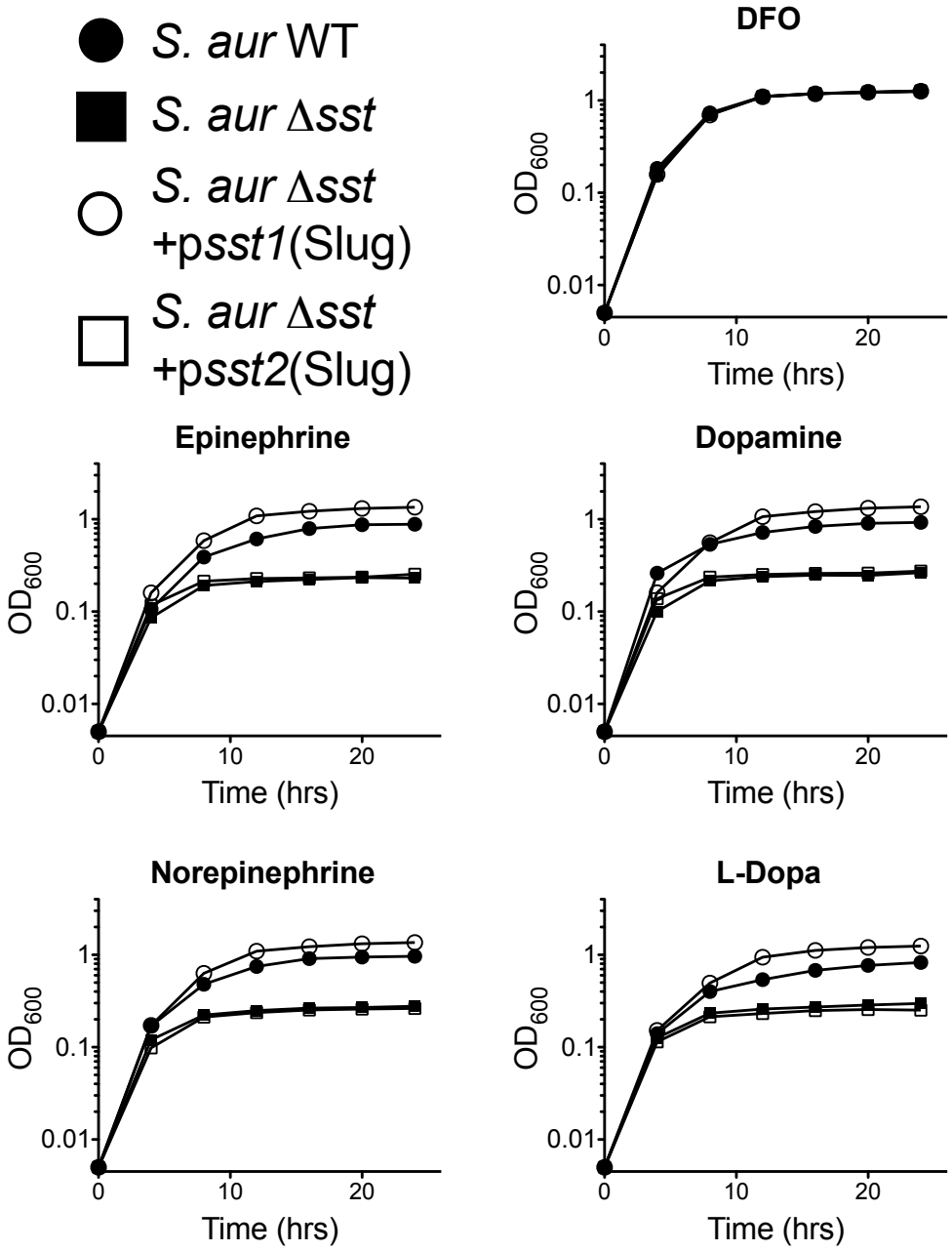

S. lug WT
S. lug Δsst
S. lug Δsst psst1
DFO
2,5-DHBA
2,3-DHBA
Epinephrine
Dopamine
Norepinephrine
L-Dopa
OD600
Time (hrs)
1
0.1
0.01
0
10
20

Figure 3-6: *Staphylococcus lugdunensis sstA1-D1* is required for catecholamine uptake by *S. aureus sfa sbn sst*. *sst1* and *sst2* loci from *S. lugdunensis* were introduced into *S. aureus sfa sbn sst* and analyzed for growth in C-TMS with 20% serum, supplemented with 50μM catecholamine hormones. *S. aureus sfa sbn* is depicted as the WT strain, and all depicted strains contain *sfa sbn* mutations. Desferrioxamine B (DFO) was used as a positive control. Data are the average of at least three independent biological replicates.

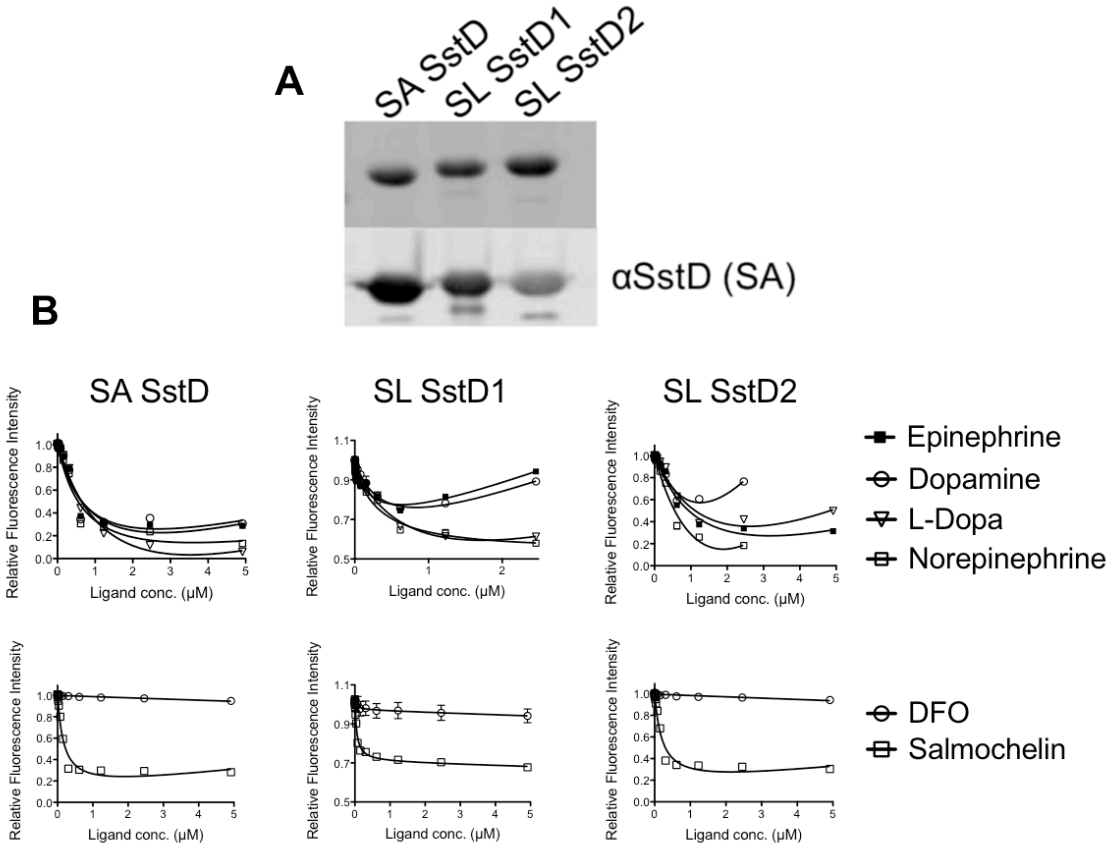

S. aur WT
S. aur Δsst
S. aur Δsst +psst1(Slug)
S. aur Δsst +psst2(Slug)
DFO
Epinephrine
Dopamine
Norepinephrine
L-Dopa
OD600
Time (hrs)
0.01
0.1
1
0
10
20

carrying *sst1*, into the *S. aureus sfa sbn sst* mutant (41). Vectors carrying *sst1* and *sst2* gene sets (p*sst1* and p*sst2*, respectively) from *S. lugdunensis* were mobilized into *S. aureus sfa sbn sst* and the strains were assessed for growth in serum supplemented with catecholamines and compared to *S. aureus sfa sbn* (annotated as wildtype for simplicity). *S. aureus sfa sbn sst* harboring p*sst1* grew much better than the vector control strain in serum supplemented with epinephrine, norepinephrine, dopamine or L-DOPA (Figure 3-6). The *S. aureus sfa sbn sst* mutant harboring p*sst2* was impaired for growth with all catecholamines, and grew similar to the vector control. All strains grew similarly when serum was supplemented with DFO, indicating an inability to attain iron from transferrin in the serum via catecholamines. Altogether these findings indicate that the Sst1 transporter is both necessary and sufficient for catecholamine-iron acquisition in *S. lugdunensis*.

3.3.6 SstD proteins vary in catecholamine binding affinities

To determine differences in substrate-binding affinity, *S. lugdunensis* SstD1 and SstD2 were overexpressed and purified (41). Antisera raised against *S. aureus* SstD were able to recognize both *S. lugdunensis* SstD1 and SstD2 purified proteins (Figure 3-7A). The three proteins were analyzed for substrate binding via intrinsic tryptophan fluorescence quenching. Bovine serum albumin fluorescence was not quenched with any tested ligands, serving as a protein negative control. The fluorescence of all three SstD proteins was quenched with added catecholamines, but not DFO (Figure 3-7B). *S. aureus* SstD was determined to bind each respective catecholamine with a similar dissociation constant as to a previous report (Figure 3-7C) (41). *S. lugdunensis* SstD1 was found to bind each of the catecholamine stress hormones analyzed, and the catechol(amine)

Figure 3-7: Substrate-binding dynamics of *Staphylococcus aureus* SstD and *Staphylococcus lugdunensis* SstD1 and SstD2. (A) Coomassie (top panel) and Western blot (bottom panel) of overexpressed and purified *S. aureus* (SA) and *S. lugdunensis* (SL) SstD homologs, cloned without lipobox motifs and overexpressed in *E. coli*. Proteins were assayed for using anti-*S. aureus* SstD antiserum. (B) Fluorescence quenching was used to determine binding affinity of SstD homologs for ferrated catecholamine hormones. (C) Table of K_D values for SstD-ferric catecholamine complexes. Data are the average of at least three independent biological replicates, with error bars representing the standard error to the mean.



C

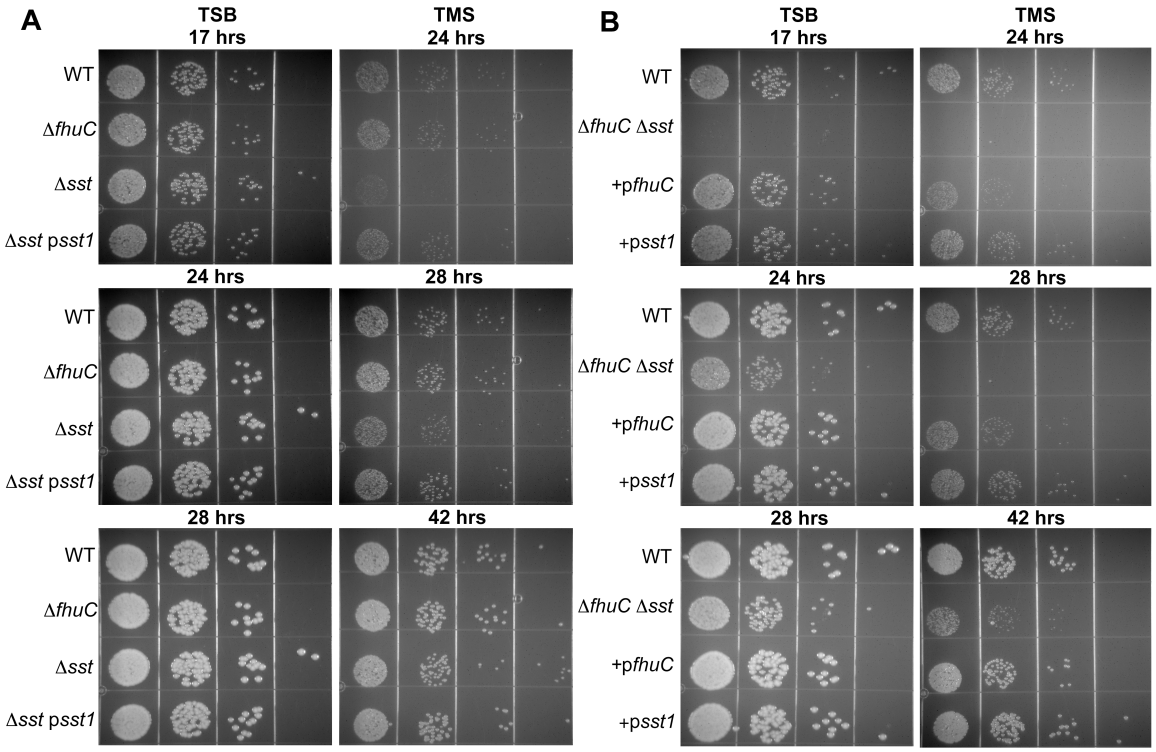
K_D +/- SD	Protein		
Ligand	**SA SstD**	**SL SstD1**	**SL SstD2**
Epinephrine	0.94 +/- 0.21	0.33 +/- 0.13	1.54 +/- 0.34
Norepinephrine	0.74 +/- 0.17	0.37 +/- 0.12	2.59 +/- 1.30
Dopamine	0.78 +/- 0.21	0.43 +/- 0.05	2.48 +/- 1.01
L-DOPA	1.56 +/- 0.28	1.02 +/- 0.41	2.14 +/- 0.58
Salmochelin	0.17 +/- 0.02	0.05 +/- 0.01	0.21 +/- 0.03

siderophore salmochelin, with greater affinity than the *S. aureus* homolog (Figure 3-7C). Dissociation constants for *S. aureus* SstD binding of ligands were 2-3x higher than that of *S. lugdunensis* SstD1. Both proteins had the least affinity for L-DOPA (other than DFO). *S. lugdunensis* SstD2 also bound these substrates, albeit more poorly than either of the other two proteins. Dissociation constants for SstD2 were over 4x greater than those of *S. lugdunensis* SstD1 for epinephrine, norepinephrine, dopamine, and salmochelin (Figure 3-7C). Consequently, *S. lugdunensis* SstD2 bound all catecholamine hormones and salmochelin with less affinity than either *S. lugdunensis* SstD1 or *S. aureus* SstD.

3.3.7 Role of *fhu* and *sst* in iron-restricted growth of *S. lugdunensis*

We next considered the possibility that the *fhu* and/or *sst* genes play a role in growth under iron starvation conditions, even in the absence of hydroxamates or catecholamines, respectively. Spot plating revealed that on TSB, there was no noticeable difference in growth between wildtype and *fhuC* and *sst* mutants (Figure 3-8A). When spotted on iron-restricted minimal salts medium (TMS), we noticed a reproducible growth delay for the *sst* mutant, a phenotype that was complementable with introduction of the *sst1* locus *in trans* (Figure 3-8A). We next coupled the *fhuC* and *sst* deletions together into one strain. This mutant, interestingly, was growth impaired not only on TMS minimal medium, but also TSB rich medium. This growth impairment was complemented by introduction of either *fhuC* or *sst1 in trans* (Figure 3-8B). On TMS medium, the double mutant was severely growth attenuated (Figure 3-8B). Together, these data demonstrate that both the *fhu* and the *sst1* loci play a role in vital nutrient acquisition for this species, both in the presence and absence of hydroxamates or catecholamines, respectively.

Figure 3-8: *Staphylococcus lugdunensis* Sst1 and FhuC contribute to growth in absence of catecholamines and hydroxamates. (A) Growth of Δ*fhuC* and Δ*sst* single gene set-deletion mutants was compared to wildtype and the Δ*sst* mutant complemented with *sst1* genes (p*sst1*), on rich media (TSB) and minimal media (TMS). (B) Growth of the Δ*fhuC* Δ*sst* double mutant was compared to wildtype and the Δ*fhuC* Δ*sst* double mutant complemented with either *fhuC* (p*fhuC*) or the *sst1* gene set (p*sst1*). One experiment that is representative of consistent results from several biological replicates is shown.

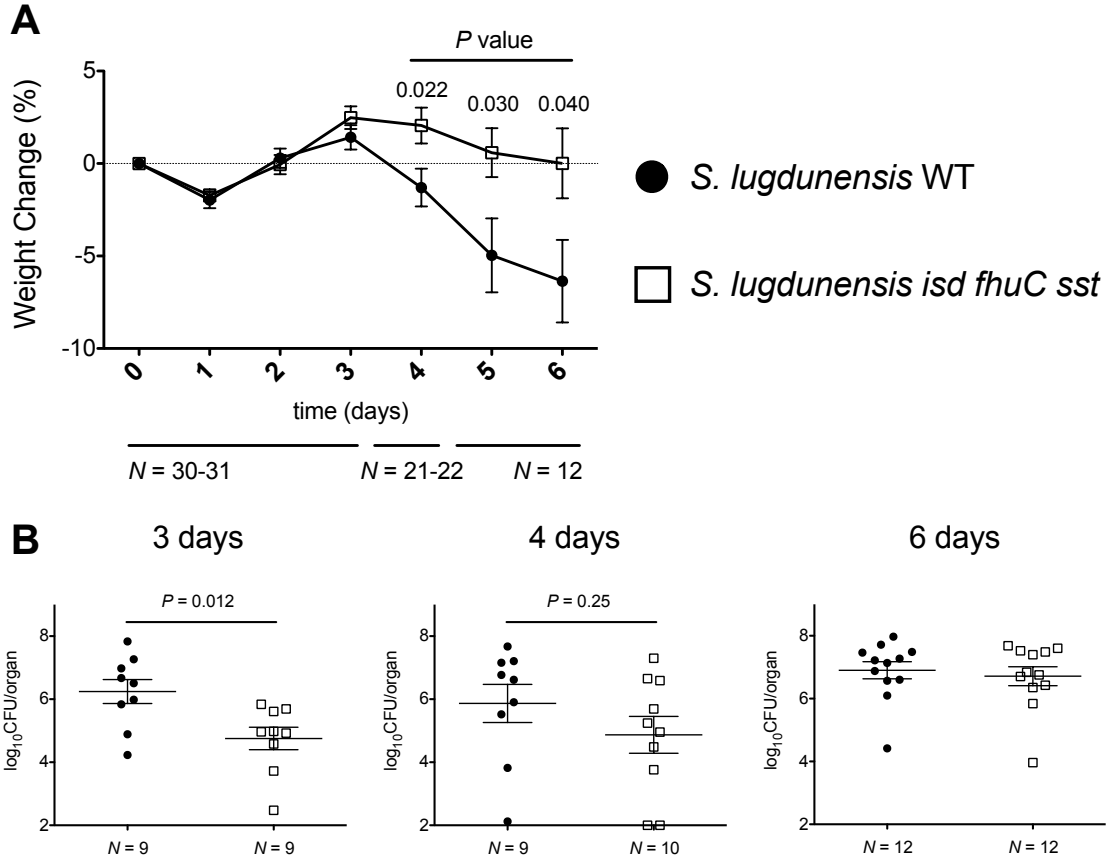

A
TSB
17 hrs
TMS
24 hrs
WT
ΔfhuC
Δsst
Δsst psst1
24 hrs
28 hrs
WT
ΔfhuC
Δsst
Δsst psst1
28 hrs
42 hrs
WT
ΔfhuC
Δsst
Δsst psst1
B
TSB
17 hrs
TMS
24 hrs
WT
ΔfhuC Δsst
+pfhuC
+psst1
24 hrs
28 hrs
WT
ΔfhuC Δsst
+pfhuC
+psst1
28 hrs
42 hrs
WT
ΔfhuC Δsst
+pfhuC
+psst1

3.3.8 Iron acquisition through Isd, FhuC and Sst contribute to host colonization and virulence

There is no model for *S. lugdunensis* systemic infection, with only two reports of animal infection available (to our knowledge). A catheter-induced rat endocarditis model showed that surface-anchored proteins, through Sortase A (SrtA), are required for *S. lugdunensis* pathogenesis (48). A recent article detailed a similar means to induce endocarditis in mice, in which induced inflammation was required to promote initial adherence of stationary-phase *S. lugdunensis* to aortic valve leaflets through von Willebrand factor (vWF) (60). In either infection, SrtA and vWF deficiency led to decreased bacteria in endocardial vegetations. Here, we modeled a *S. lugdunensis* systemic infection, without the use of a catheter. All mice survive the duration of the experiment. *S. lugdunensis* were administered to mice via tail-vein, after which infection was allowed to proceed over the course of several days. We noted that *S. lugdunensis*-infected mice do not become as sick as *S. aureus*-infected mice, even with greater CFU administration (61, 62). Nevertheless, the pathogenesis of *S. lugdunensis* murine infection was more similar to that of *S. aureus* infection rather than *S. epidermidis* infection, wherein we administer fewer bacteria which persist for a longer duration (63, 64).

Coinciding with previous studies of *S. lugdunensis* rodent infection (48, 60), our infected mice did not exhibit overt sickness relative to *S. aureus*-infected rodents (61, 62). To assess the role of the aforementioned iron acquisition pathways for colonization and virulence of *S. lugdunensis*, we combined *isd*, *fhuC* and *sst* mutations into one strain. Wildtype *S. lugdunensis*-infected mice lost significantly more weight than mice infected with a *isd fhuC sst* mutant during 4-6 days post-infection (Figure 3-9A). Mutant-infected

Figure 3-9: Iron acquisition through Isd, FhuC and Sst contributes to *Staphylococcus lugdunensis* pathogenesis. Female BALB/c mice were infected systemically with 2-3x10^7 CFU *S. lugdunensis* wildtype or an isogenic *isd fhuC sst* mutant. (A) Animal weight change was monitored over a period of 6 days, wherein animals were sacrificed for quantification of bacterial burden. (B) Bacterial load in the kidneys of infected mice was measured at various time points coinciding with weight change differences between the infection groups. The mean is depicted by a horizontal bar, with error bars corresponding to standard error to the mean for each group. Statistical analyses were performed using an unpaired Student's *t*-test. The limit of detection is the y-axis value at the origin.


A
P value
0.022
0.030
0.040
Weight Change (%)
5
0
-5
-10
0
1
2
3
4
5
6
time (days)
N = 30-31
N = 21-22
N = 12
S. lugdunensis WT
S. lugdunensis isd fhuC sst
B
3 days
4 days
6 days
P = 0.012
P = 0.25
log10CFU/organ
8
6
4
2
N = 9
N = 9
N = 9
N = 10
N = 12
N = 12

mice did not lose weight over the course of the experiment. The *isd fhuC sst* mutant was significantly impaired in its ability to colonize murine kidneys at 3 days post-infection compared to wildtype *S. lugdunensis.* However, after 4 days, the decreased bacterial burden was trending towards significance, and at 6 days, the bacterial burden was equivalent to wildtype. Therefore, it appears that the mutant is able to keep reproducing, presumably at a slower rate than wildtype, until the bacterial burden reaches wildtype level and plateaus 6 days post-infection (Figure 3-9B). Strikingly, there was a disconnect between weight loss and bacterial burden; at 3 days post-infection, there was no significant difference in weight loss, even though mice challenged with wildtype bacteria exhibited a significantly greater bacterial burden, while at 6 days post-infection, there was no difference in bacterial burden, in spite of the significantly greater weight loss displayed by mice challenged with wildtype *S. lugdunensis*. Given these findings, we conclude that these iron acquisition mechanisms impact the ability of *S. lugdunensis* to colonize host organs, and, based on weight loss, impact health.

3.4 Discussion

S. lugdunensis is capable to thrive in diverse infection niches within the human host and is able to cause unusually aggressive infections, which contrasts infections typical of CoNS. *S. lugdunensis* is unusual amongst the staphylococci in that it does not produce a siderophore. Accordingly, we showed that it is severely growth attenuated in animal serum unless it is supplemented with factors to compromise the iron-withholding capacity of transferrin (28). In this study, we have identified a single gene, *fhuC*, that is critical for iron acquisition from SA and SB (produced by other species of staphylococci),

as well as siderophores produced by fungi and Enterobacteriaceae (ie. ferrichrome, coprogen, aerobactin).

Catecholamine stress hormones are able to interact with and reduce iron within transferrin and other iron-withholding glycoproteins to render it available for intruding pathogens. In addition to siderophore acquisition, we found that *S. lugdunensis* is capable of catecholamine transport, which we have attributed to the *sst1* locus. *S. lugdunensis* is unique among staphylococci to carry duplicate *sst* regions, which was evident in all of our assessed clinical isolates (25, 26). This latter observation is in agreement with a recent study supporting *S. lugdunensis* as a clonal species (65).

Interestingly, *S. lugdunensis* SstD2, compared to the other SstD homologs, has inferior affinity for catecholamine substrates. Of interest, although we identified a Fur box in the promoter region of *sst2*, we were unable to identify a growth medium or conditions that resulted in expression of this locus. At present, it is unknown what purpose *sst2* serves for *S. lugdunensis*. Unlikely to function for inferior, redundant catecholamine transport, *sst2* may serve to transport a vitamin (ie. thiamine or folic acid), many of which contain similar structures to catechol. With the inability to produce a siderophore, *S. lugdunensis* appears able to adapt well in the presence of catecholamine xenosiderophores and stress hormones through a transporter with greater affinity than the *S. aureus* ortholog. It is noteworthy that for the *S. lugdunensis* Isd system, IsdB was found to bind hemoglobin with greater affinity than *S. aureus* IsdB (27, 66). Being endowed with seemingly fewer iron-acquisition strategies may have necessitated evolution of optimized iron-compound transport by *S. lugdunensis*, which may be advantageous to colonization. Large reservoirs of plasma catecholamines surround

mesenteric organs, kidneys and the liver in venous and arterial circuitry, and levels may spike in individuals undergoing therapeutic or recreational drug use (67). Conditions promoting elevated serum stress hormones are likely to enhance *S. lugdunensis* growth during infection. Furthermore, stress hormones have immunoregulatory roles and may alter immune cell function to further favor infection conditions (68, 69). The interplay between the effects of hormones on immune function and infection merits future investigation.

Another interesting finding from this study is that FhuC and Sst1 contribute to vital nutrient acquisition even in the absence of known substrates (Figure 3-8). This begs the question, in what form is iron coming into the cell? Is it complexed to an unknown chelating molecule? Are amino acids, short peptides, or vitamins able to coordinate iron to some degree? We are currently investigating these questions in more detail. It is interesting that these two transporters have evolved novel functions in addition to those hypothesized. *S. lugdunensis* IsdC, although necessary for heme acquisition, is also critical for biofilm formation (70). With seemingly fewer virulence determinants than *S. aureus*, *S. lugdunensis* has evolved for its expressed factors to not only bind substrates with greater affinity than homologous proteins in other organisms, but also to have those same transporters elicit additional functions.

Animal models for experimental *S. lugdunensis* catheter-induced endocarditis are published (48, 60). Both of these require surgical catheter insertion past the rodent aortic valve. However, many reports describe cases of *S. lugdunensis* native valve endocarditis following surgical procedures or skin trauma in dissociate regions of the body, indicating an ability to colonize internal organs in absence of surgical prosthesis insertion and

predisposing risk factors (9, 12–14). Therefore, organs other than the heart should not be disregarded, as these serve as not only sites of infection but also contribute as reservoirs for dissemination. In the systemic infection model used in this study, we do not typically recover *S. lugdunensis* CFU from murine hearts. Induced inflammation of the heart endothelial vasculature may be necessary to stimulate *S. lugdunensis* heart colonization (60). Nevertheless, we are able to consistently recover bacteria from the kidneys, and occasionally in smaller numbers from the liver and lungs. A mutant impaired for acquisition of heme, siderophores and catecholamine stress hormones is debilitated for growth in murine kidneys at early stages of infection, yet is still eventually capable of colonizing kidneys. Interestingly, despite an abundance of bacteria in the kidneys, mice are generally in good relative health. Albeit showing discomfort, sickness is in stark contrast to mice infected with *S. aureus*. Still, *S. lugdunensis*-infected mice lose weight, and the bacteria are able to persist longer than *S. epidermidis* in a similar model, giving credence to the notion that *S. lugdunensis* has elevated virulence compared to other CoNS (63, 64). Difference in weight loss between wildtype and mutant-infected mice does not coincide with differences in bacterial burden in the kidneys. These findings are unexpected, yet indicate that the surreptitious *S. lugdunensis* may reside quite amiably within host organs such as the kidneys yet not cause overt morbidity. Persistence may enhance dissemination to other organs via renal circulation. It may be that *S. lugdunensis* is also present in yet undetermined murine organs as well, or the mutant may elicit different pathology in kidneys. The genome of *S. lugdunensis* indicates an absence of many well-characterized *S. aureus* immune-evasion and toxin encoding genes. The less aggressive course of infection for *S. lugdunensis* as compared to *S. aureus*-infected mice

is undoubtedly attributed to these genetic differences, whereas the more aggressive course of infection for *S. lugdunensis* compared to other CoNS, such as *S. epidermidis*, largely remains to be elucidated. The less aggressive disease phenotype of systemic *S. lugdunensis* infection may allow the pathogen to remain undetected by the host immune system for a longer period of time, compared to *S. aureus*, providing greater capability for dissemination and persistence until (host) environmental factors become conducive to promote the severe pathogenesis *S. lugdunensis* is capable of causing.

It is striking that a proportion of clinical isolates are not hemolytic, yet nonetheless bear genes for procurement of heme as well as other iron sources. It may be that non-hemolytic isolates are preferentially found in co-infection, in specific host niches, or have different iron source preferences than hemolytic isolates. The relationship between hemolytic potential and iron source preference merits further investigation, as non-hemolytic isolates may thrive with non-heme iron sources compared to hemolytic isolates, which clearly thrive in comparison in the presence of human blood. Alternatively, hemolytic isolates may cause a more aggressive disease progression or be more fit for pathogenesis than non-hemolytic isolates during systemic infection.

In conclusion, we have identified the genetic means for *S. lugdunensis* to procure an assortment of diverse siderophores and catecholamine hormones through FhuC and Sst1, respectively, and shown them, along with Isd, to play a significant role in colonization and virulence. Other yet unidentified iron acquisition mechanisms must also be functional in the mammalian host and identifying these is a priority research area.

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4

Chapter 4

Discussion, conclusions and future directions

4.1 General overview

There exists limited molecular detail of *S. lugdunensis* genetic factors contributing to virulence. We, and others, continue to try to understand more about the lifestyle of this enigmatic pathogen. In this thesis, I have described several means by which *S. lugdunensis* acquires iron, including mechanisms for transport of staphyloferrin A (SA), staphyloferrin B (SB), hydroxamates, catecholamines and heme (1).

4.1.1 Defective staphyloferrin A biosynthesis

We have shown that *S. lugdunensis* grows poorly in animal serum, owing to its inability to produce a siderophore. Exogenously supplied siderophores or other iron sources promote growth in serum, indicating a parasitic predisposition to obtain transferrin-iron via environmentally scavenged metabolites. Despite this, the deletion in the SA biosynthetic locus has been retained across *S. lugdunensis* isolates, indicating it may be metabolically favourable for the species to divert precursor molecules that would have been used for SA biosynthesis for other uses, while simply feeding on xenosiderophores. Given the nature of cohabitation on skin with other staphylococci, which produce SA (SA is made by the majority of CoNS, including *S. epidermidis*) and SB, it is fitting to consider either of these compounds may be in abundance where *S. lugdunensis* is typically found (2–5). Recent insight into *S. aureus* metabolism has found that the Kreb's cycle-associated citrate synthase is required for synthesis of SA, but not SB. The iron-sparing response decreases Kreb's cycle activity under iron limitation, and millimolar glucose concentrations inhibit SA biosynthesis (6, 7). Nevertheless, SA production by *S. aureus* is required for causing pathogenesis during murine subcutaneous infection (8). These data, together with the predominance of SA production among skin colonizing

staphylococci, suggests a likelihood that SA is abundantly expressed and found on mammalian skin (9). It is therefore not surprising that some organisms, such as *S. lugdunensis*, may inhabit this niche while usurping this metabolite produced by other microbes.

4.1.2 Staphyloferrin transport

No remnants of SB biosynthesis genes are found in the *S. lugdunensis* genome, yet *sir* genes are present for transport of SB, in addition to *hts* genes for SA uptake. Both *hts* and *sir* loci have Fur boxes in their promoter regions and expression of both is iron-regulated. Genetic manipulation of *S. lugdunensis*, including generating mutations such to remove *hts* and *sir* genes, proved to be problematic. While means to genetically manipulate *S. aureus* are well established, there is a lack of detail for similar practices in other staphylococci (10, 11). Different cell wall architecture and genetic barriers hinder horizontal gene transfer, including intra-genus exchange of genetic material. Importantly, the work presented in this thesis and a limited number of other groups, has managed to overcome some of these limitations through development of tools and optimized processes to better study *S. lugdunensis*, which should be of use to other scientists as well, as the pathogen continues to gain notoriety in the clinic. After literature review and optimized procedures, we were able to obtain *S. lugdunensis* vector transformants and generate genetic deletion mutants. Being able to genetically manipulate *S. lugdunensis* allows us to determine the function and biological relevance of genes of interest. Analysis of *hts* and *sir* locus mutants showed that these are indeed required for acquisition of SA and SB, respectively, by *S. lugdunensis*. Polymicrobial infections involving *S. lugdunensis* also commonly include other staphylococci such as *S. epidermidis* and *S.*

aureus (12). When grown together in serum, *S. lugdunensis* is able to pirate SA and SB synthesized by *S. aureus* to promote its own growth. A *S. lugdunensis* Δ*hts* Δ*sir* mutant is impaired for growth in the same conditions, supporting our notions that cohabiting the same microenvironment with siderophore producers augments *S. lugdunensis* growth. SA biosynthesis is inhibited at blood-glucose concentrations, and SB is likely the more important of the two siderophores for internal infection of the host. *S. lugdunensis* is one of few staphylococcal species to encode the SB transporter, which may be relevant for persistence in host organs.

4.1.3 Heme acquisition

S. lugdunensis is unique among coagulase-negative staphylococci (CoNS) to encode the Isd pathway of heme-iron acquisition, albeit with several distinct proteins. IsdJ and IsdK have 2 and 1 NEAT domains, respectively, and are found in both membrane and supernatant fractions. The biological function of these potential hemophores, and interaction with other Isd proteins, has not been investigated, although both are able to bind heme but not hemoglobin (13). An Isd-associated autolysin (IsdP) alters processing and attachment of the heme-conduit protein IsdC to peptidoglycan (14). IsdC in *S. lugdunensis* is also distinct in its role for biofilm formation in this species (15). Surface-exposed IsdB is unable to bind mouse hemoglobin, but binds human hemoglobin with greater affinity than the *S. aureus* ortholog (13, 16). *S. lugdunensis* IsdJ is homologous to *S. aureus* IsdA, and both confer resistance to bactericidal lipids in addition to their respective roles in heme acquisition. The Isd system supports *S. lugdunensis* growth with low nanomolar concentrations of heme/hemoglobin, serving a high-affinity means to procure heme-iron in physiological conditions. Once infection has been established and

host cell lysis (including hemolysis) has taken place, conditions become non-physiological and heme concentration is increased. It is believed that other, non-Isd means of heme acquisition become more relevant in these conditions, as *isd* mutants are still able to use heme at greater concentrations. The mutant is able to use heme at lower concentrations in acidified media (same media used in heme/hemoglobin growth experiments in chapter 2, set to pH 6.4), and grows to more appreciable biomass sooner as well (data not shown). This growth is not seen with equimolar $FeSO_4$ in place of heme, indicating the metal chelator (EDDHA) and heme in the media have not degraded (data not shown). An acidic microenvironment may be caused by accumulation of metabolic end products or immune defense, which for staphylococci may be an indication of harsh conditions. These results may indicate that environmental pH (such as that commonly found in an abscess environment) has an effect on iron acquisition by staphylococci. A somewhat similar phenomenon affects siderophore acquisition by *Vibrio parahaemolyticus*, which upregulates enterobactin acquisition in response to alkaline pH (17). *S. lugdunensis*, like *S. aureus* and *V. parahaemolyticus*, is hemolytic and would likely need to adapt once substantial tissue damage has occurred. One may speculate that at high heme concentrations, high-affinity heme transport could cause heme internalization too quickly for proper mobilization/organization, such to cause toxicity (via free internal heme/iron, yet to be bound), and lower affinity heme uptake strategies may be necessary in addition to heme detoxification systems to mitigate heme toxicity.

4.1.4 Siderophore and catecholamine uptake

In addition to heme acquisition, *S. lugdunensis* uses the homodimeric FhuC ATPase to acquire siderophores. The *fhuCBG* locus is upregulated in response to iron limitation, and

although FhuBG comprise the permease component required for hydroxamate uptake, the FhuC ATPase is more promiscuous in its binding partners and also associates with HtsBC and SirBC. *S. lugdunensis*, much like *S. aureus*, relies on FhuC to acquire several different hydroxamate and carboxylate siderophores. In contrast to *S. aureus*, we identified two loci in *S. lugdunensis* that we presumed may be involved in catecholate acquisition. Paralogous proteins share approximately 70% sequence identity but little similarity in their regulation and substrate affinity. Sst1 is responsible for catecholamine acquisition as this locus complemented a *sst* mutant growth defect in presence of the catecholamine stress hormones epinephrine, norepinephrine, dopamine and L-DOPA. This transporter does not acquire all catecholates, as 2,3-DHBA and 2,5-DHBA could not be used as sources of iron. Interestingly, common catechol-type siderophores such as enterobactin, bacillibactin and salmochelin have catecholamine iron-coordinating moieties. The amine group in place of a carboxylic acid appears to be important for substrate recognition by SstD. In keeping with this, *S. aureus* SstD and *S. lugdunensis* SstD1, both of which bind and transport catecholamine-iron, have the least affinity of analyzed substrates for L-DOPA, which bears both amine and carboxylic acid moieties. The other analyzed hormones have only amine (dopamine), or amine and alcohol groups (epinephrine, norepinephrine) and are bound with greater affinity.

4.1.5 *S. lugdunensis* transporters are superior in function, and exhibit additional biological purposes compared to homologous systems in other organisms

At least two iron-acquisition mechanisms (Isd and Sst1) of *S. lugdunensis* have greater affinity for substrates than homologous systems in other organisms, namely *S. aureus*. The same may be true of other transporters, including Hts and Sir as well (although we

currently have no data to support this claim), giving the bacterium more of a competitive advantage. If *S. lugdunensis* has evolved more sophisticated/professional iron acquisition systems than other bacteria, it could use iron sources at lower concentrations than other organisms, including siderophore-producing microbes. Additionally, the lack of traditional virulence factors may be offset by multifunctional expressed factors that contribute to multiple different biological functions. Sst1 and FhuC impact *S. lugdunensis* growth in the absence of catecholamines and hydroxamates (or siderophores). On minimal media (TMS) the *sst* mutant is impaired for growth, and on rich media (TSB), a *fhuC sst* mutant is impaired for growth. The double mutant is even more-so attenuated on minimal media. Although the *fhuC* mutation has little impact on *S. lugdunensis* growth on these media, the combination of *fhuC* and *sst* mutations renders the strain much more attenuated for growth than the *sst* mutation alone. These findings are unexpected and signify that these transporters are more important to the inherent biology of this organism than previously thought. It may be that FhuC is involved in transport of other iron sources, including free iron, and Sst1 may transport catecholamine-like compounds that are able to chelate iron to some degree. The thought here being that TSB is iron-rich and should supply the bacterium such to limit expression of iron-regulated genes, unless iron cannot be taken up through FhuC, and iron-acquisition genes become expressed. In this case, Sst1 would be expressed and may overcome this deficiency. Both FhuC and Sst1 transporters are expressed in minimal media, however other nutrients are relatively rich and one or more of these may be additional substrates of Sst1. In addition to the multiple functions of certain *S. lugdunensis* Isd proteins, these two transporters also appear to exhibit novel functionality.

4.1.6 Live animal infection

During the course of this study, two *S. lugdunensis* animal infections were published, both being induced endocarditis models in rodents, and only sortase A was identified as a virulence determinant in the host (10, 18). We developed murine subcutaneous and systemic infection models in which we could recover viable bacteria from mouse organs. In a systemic murine infection model, the iron-acquisition systems we characterized *in vitro* did indeed contribute to the ability of *S. lugdunensis* to colonize host tissue and cause virulence *in vivo*. Although *S. lugdunensis* Isd is unable to bind mouse hemoglobin, other Isd proteins are involved in heme uptake, and may still be relevant in this infection model. FhuC and Sst are directed towards procurement of several prokaryote-produced and host-produced iron-binding compounds, respectively. A *isd fhuC sst* mutant is significantly debilitated for growth at early stages of colonization/infection, however is not eliminated from the host, and persists. The mutant is hindered for causing sickness, as mutant-infected mice do not lose weight as wildtype *S. lugdunensis*-infected mice do. Systemically infected mice do not typically exhibit *S. lugdunensis* heart valve colonization, although there is some evidence indicating that endothelial vasculature activation, through inflammation or damage, is required for adhesion, mediated by von Willebrand factor. Organs other than the heart contribute as not only infection sites but also reservoirs for dissemination, and should not be overlooked. A model of systemic infection with induced heart vessel inflammation may better mimic clinical courses of *S. lugdunensis* infective endocarditis.

4.1.7 Hemolysis

During the course of this research we were fortunate enough to obtain a collection of *S. lugdunensis* clinical isolates from London Health Sciences Centre - Victoria Hospital (London, Ontario, Canada). All assessed isolates grow poorly in serum, unless the serum is supplemented with exogenous iron sources, and all isolates contain genes for SB, heme and catecholamine uptake. Many of these clinical isolates are non-hemolytic, and these isolates are at a disadvantage for growth in human blood. Non-hemolytic isolates may be favoured in certain types of infection, polymicrobial infections, or they may have different nutrient preferences. In addition to the presence of a membrane-bound clumping factor, the varying hemolysis profiles add further doubt to clinical identification representing the true burden of *S. lugdunensis* infection.

4.2 Conclusions

Although *S. lugdunensis* does not produce a siderophore, it is able to acquire siderophores of all major types, in addition to heme-iron from human hemoproteins. Hydroxamates and/or hydroxycarboxylate xenosiderophores may be bound by receptors with greater affinity over that of competing organisms, such is true for heme/hemoglobin and catecholamines.

Non-hemolytic clinical isolates are impaired for iron acquisition from blood, compared to hemolytic isolates. Most host-iron is coordinated in hemoproteins within red blood cells. The frequency of non-hemolytic *S. lugdunensis* strains still capable of causing infection warrants research into possible reasons as to how these isolates are able to persist and cause disease.

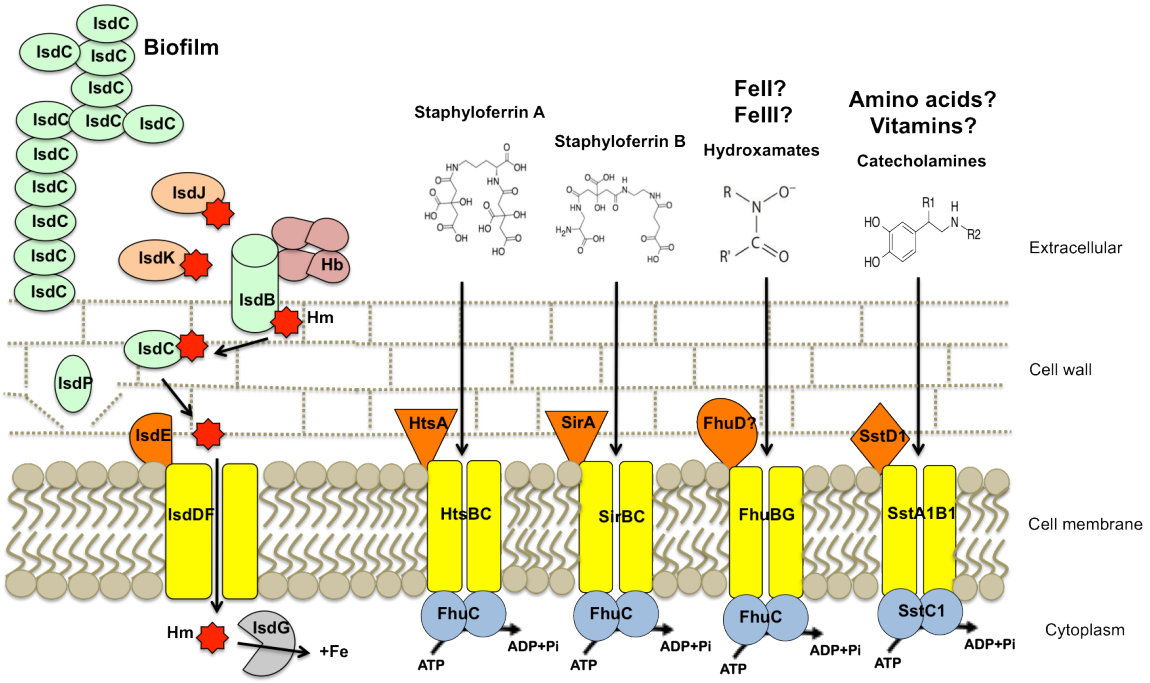
The transporters of *S. lugdunensis* are multifunctional, and although the bacterium may seemingly encode fewer traditional virulence factors than other nefarious pathogens, each of those expressed is able to accomplish additional biological functions for the pathogen, compared to homologous systems in other bacteria. This is true for Isd, FhuC and Sst1, with each of these transporters having functional pleiotropy important for the bacterium in various environmental conditions.

4.3 Significance of this study

Little is known about *S. lugdunensis* biology, and the molecular characterization of this species is in its infancy. This study builds on previous work by others describing several *S. lugdunensis* Isd proteins, adding to the comprehensiveness of this pathways description (13, 19). We are first to characterize multiple strategies of this organism to acquire a vital nutrient, and show that specific nutrient transporters contribute to host colonization and virulence. Several of these iron-acquisition mechanisms were shown to be important for novel functions in addition to those of homologous systems in other organisms.

In combination with discoveries by other groups, the findings of this study enable us to create a pictorial description of transport processes involving *S. lugdunensis* iron acquisition pathways (Figure 4-1). Although the majority of host-iron is kept within heme in hemoproteins, the vast majority of which reside in cells, *S. lugdunensis* has means to lyse these cells, extract heme from hemoglobin and funnel it into the bacterial cytoplasm for degradation. The absolute requirement of this precious nutrient necessitates successful pathogens elaborate many strategies to attain it from a variety of sources. Although a comparably smaller iron pool, transferrin-iron is labile, and along with other glycoproteins these iron sources comprise another important pool of nutrients.

Figure 4-1: Schematic of *Staphylococcus lugdunensis* transporters involved in nutrient acquisition. Isd proteins acquire heme (Hm)-iron(+Fe) from hemoglobin (Hb), are important for biofilm formation (IsdC) and protection from bactericidal lipids (IsdJ). Autolysin IsdP remodels peptidoglycan to facilitate IsdC movement. Staphyloferrin A and B carboxylate siderophores are transported through Hts and Sir, respectively. Hydroxamates are acquired through Fhu, which uses the FhuC ATPase for acquisition of these, as well as carboxylate siderophores. Catecholamines are transported via Sst1. FhuC and Sst1 are involved in acquisition of other nutrients as well, which is currently being examined in more detail.


IsdC
Biofilm
IsdJ
IsdK
Hb
IsdB
Hm
IsdC
IsdP
IsdE
IsdDF
Hm
IsdG
+Fe
Staphyloferrin A
Staphyloferrin B
FeII?
FeIII?
Hydroxamates
Amino acids?
Vitamins?
Catecholamines
HtsA
SirA
FhuD?
SstD1
HtsBC
SirBC
FhuBG
SstA1B1
FhuC
SstC1
ATP
ADP+Pi
Extracellular
Cell wall
Cell membrane
Cytoplasm

The many (seemingly) functionally redundant iron uptake strategies are expressed and required in different conditions. Obviously, they are likely to be expressed in low-iron conditions when the bacterium requires iron, however other mechanisms of regulation must also exist. Although the preferred iron source for *S. aureus* is heme, and siderophore biosynthesis is downregulated in the presence of heme, the iron source preference of *S. lugdunensis* is undetermined. Ever-changing microenvironments surrounding bacteria necessitate the ability of pathogens to adapt to new conditions, which may require other, more metabolically favourable means to acquire host nutrients. The host elicits functions to maintain homeostasis, while pathogens circumvent these to obtain host nutrients, producing different metabolite concentrations from/in various areas of tissue. It is likely that each iron-compound transporter is required for optimal exploitation of a specific niche. The iron acquisition strategies uncovered here are required for optimal exploitation of internal host organs (kidneys), and aid in the organisms ability to cause disease.

4.4 Areas of future investigation

4.4.1 Siderophore-iron removal

The intracellular fate of siderophores in staphylococci remain largely unknown. In *S. aureus*, oxidoreductase IruO and nitroreductase NtrA aid in iron removal from heme. IruO also facilitates iron release from ferric-desferrioxamine (hydroxamate siderophore), whereas NtrA from ferric-SA (20). *S. lugdunensis* encodes homologs to both of these in its genome. The IruO homolog (SLGD_00660) shares 68% identity and 84% similarity, whereas the NtrA homolog (SLGD_02019) shares 72% identity and 85% similarity. These may have similar or distinct functions compared to the *S. aureus* homologs. Ferric-

SB is also likely to undergo reduction to release iron, although there is no proposed reductase for this process as of yet. There is a greater likelihood of iron release from hydroxamate and carboxylate siderophores via reduction as opposed to degradation, which is more geared towards catecholamines which bind iron with greater affinity (21–23). Catecholamine-iron release also remains uninvestigated in staphylococci, and may be driven by a hydrolytic enzyme such as an esterase or amidase.

4.4.2 Biological differences between FhuC and Sst1

Characterization of additional substrates transported by FhuC and Sst1 is underway. This information will aid in uncovering the importance of the Sst system encoding its own ATPase. Several siderophore-acquisition transporters (Hts, Sir and Fhu; SA, SB and hydroxamate uptake), and possibly other iron-source transporters, use FhuC for energy to import iron-containing substrates. An ATPase associated with the Isd system remains to be elucidated, although heme is recognized by *S. aureus* (and possibly other staphylococci) in a different manner than other iron sources, so it is not surprising for the Isd transporter to use a different ATPase. Sst transports catecholamine siderophores and stress hormones. These siderophores may require degradative means to release iron, which contrasts from the other siderophores, however this is little motive to reason requirement of another ATPase specific for their transport. There must be other conditions facilitating differential expression of *fhu* and *sst*, and we find that Sst1 (and also FhuC) in *S. lugdunensis* transports more substrates than originally believed, and may be expressed to obtain these novel substrates. Additionally, the effects of catecholamine hormones on staphylococcal gene expression may be an exciting new research opportunity. It has been shown that catecholamines such as norepinephrine, epinephrine

and dopamine are sensed by adrenergic receptors (QseCE two-component system) on enteric microbes including enterohemorrhagic *E. coli* and *Citrobacter rodentium* to regulate virulence factor expression (24, 25). A similar phenomenon occurs in *Vibrio* species and may be widespread to include Gram-positive bacteria as well (26, 27). The fate of staphylococcal internalized catecholamine hormones remains unknown. It is obvious that there are biological differences between Sst and the other siderophore acquisition systems, which may be of importance on a global scale.

4.4.3 Characterize hemolysis

As elaborated in Chapters 1 and 3, factors affecting *S. lugdunensis* hemolysis are poorly understood. Although genes for hemolysins are present in all *S. lugdunensis* isolates, a fraction remain non-hemolytic. Is this due to a regulatory defect – possible repression of hemolysin genes, or overexpression of (metallo)protease to degrade secreted hemolysins? Furthermore, which hemolysin gene/s are responsible for lysing cells? The three suspected are β-hemolysin (SLGD_00006), hemolysin III (SLGD_00847) and the SLUSH peptides (SLGD_00440 – SLGD_00442). Although non-hemolytic strains are impaired for growth with blood as a sole source of iron *in vitro*, are they impaired for growth *in vivo*, compared to hemolytic isolates? It is possible that non-hemolytic isolates have a differential preference towards iron sources other than heme, or infection conditions favour losing hemolytic capability.

4.4.4 Improved animal models

Whereas *S. lugdunensis* disease severity mirrors or surpasses that of *S. aureus* in the clinic, it is inferior in causing disease in mice. We have developed subcutaneous and systemic *S. lugdunensis* murine infection models in which we recover great quantities of

viable bacteria from subcutaneous lesions, and kidneys. Presence of bacteria in liver and lungs varies over the course of our systemic infection (3-6 days), indicating that they may be cleared faster in these organs. It is also possible that the liver reservoir of *S. lugdunensis* are primarily sequestered by Kupffer cells, prior to dissemination to other organs (28). Heart endothelial activation, combined with systemic infection would be an interesting model to assess virulence factors that may be associated with *S. lugdunensis* heart colonization, persistence and virulence. It would be interesting to evaluate the herein discovered iron acquisition strategies for *S. lugdunensis* fitness in other host niches.

4.5 References

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Curriculum Vitae

Jeremy Brozyna

EDUCATION

05/2013 – 08/2016 **Graduate Studies - Doctor of Philosophy**
Department of Microbiology & Immunology,
University of Western Ontario, London, ON
Supervisor: Dr. David Heinrichs
Thesis title: Iron acquisition strategies employed by *Staphylococcus lugdunensis.*

09/2011 – 04/2013 **Graduate Studies - Master of Science (transfer)**
Department of Microbiology & Immunology,
University of Western Ontario, London, ON
Supervisor: Dr. David Heinrichs
Successfully transferred to PhD program (04/2013)

04/2011 Graduate **Undergraduate - BSc with Honours - Biochemistry Major**
Department of Molecular and Cellular Biology,
University of Guelph, Guelph, ON

04/2011 Graduate **Undergraduate - BSc with Honours - Microbiology Minor**
Department of Molecular and Cellular Biology,
University of Guelph, Guelph, ON

RESEARCH EXPERIENCE

05/2011 – 09/2011 **Research Assistant**
Department of Microbiology & Immunology,
University of Western Ontario, London, ON
Supervisor: Dr. David Heinrichs

RESEARCH EXPERIENCE (CONTINUED)

04/2010 – 09/2010 **Technical Specialist**
Assistive Dynamics Corporation, Brampton, ON
Supervisor: Maxine Williamson

01/2009 – 09/2009, **Laboratory Technician**
01/2008 – 05/2008 Research and Development,
Dawn Food Products Canada, Toronto, ON
Supervisor: Tina Kostantelou

TEACHING EXPERIENCE AND LECTURESHIP

2014, 2015 **Teaching Assistantship**
Microbiology Laboratory (3610F)
Department of Microbiology & Immunology,
University of Western Ontario, London, ON
Supervisor: Dr. Bryan Heit

2012, 2013 **Teaching Assistantship**
Biology of Prokaryotes (2100A)
Department of Microbiology & Immunology,
University of Western Ontario, London, ON
Supervisor: Dr. Susan Koval

Short lectures on theory and application to over 50 undergraduate students.

- **Pre-laboratory lecture**. 2015. Light Microscopy and Bacterial Stains.
- **Pre-laboratory lecture**. 2015. Environmental & Clinical Sampling of Microorganisms.
- **Lecture.** 2014. Environmental & Clinical Sampling of Microorganisms.
- **Lecture.** 2014. Antibiotic Resistance and Plasmids.
- **Pre-laboratory lecture.** 2013. Environmental & Clinical Sampling of Microorganisms.
- **Pre-laboratory lecture.** 2012. Environmental & Clinical Sampling of Microorganisms.

AFFILIATIONS

2016 – Present **Doctor of Philosophy**
Department of Microbiology & Immunology,
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2015 – Present **Canadian Society of Microbiologists (CSM)**

2014 – Present **American Society for Microbiology (ASM)**

2011 – Present **Alumnus**
Department of Molecular and Cellular Biology,
University of Guelph, Guelph, ON

PEER REVIEWED PUBLICATIONS

Brozyna JR, Sheldon JR, and Heinrichs DE. 2014. Growth promotion of the opportunistic human pathogen, *Staphylococcus lugdunensis*, by haem, haemoglobin, and co-culture with *Staphylococcus aureus*. *MicrobiologyOpen* 3(2):182-195. **[Peer-Reviewed]**

Heilbronner S, Monk IR, **Brozyna JR**, Heinrichs DE, Skaar EP, Peschel A, and Foster TJ. 2016. Competing for iron: Duplication and amplification of the *isd* operon in *Staphylococcus lugdunensis* HKU09-01 provides a competitive advantage to overcome nutritional immunity. **[Accepted – PLOS Genetics]**

Brozyna JR and Heinrichs DE. 2016. Impact of iron acquisition loci in *Staphylococcus lugdunensis* on growth *in vitro* and during infection. **[In Preparation – for submission to PLOS Pathogens]**

PRESENTATIONS

2016

Brozyna JR and Heinrichs DE. Mechanisms for host iron acquisition contribute to *Staphylococcus lugdunensis* infection and pathogenesis.

- American Society for Microbiology (ASM) Microbe 2016, Boston, MA, USA. **[Poster Presentation]**
- Canadian Society of Microbiologists (CSM) Annual Conference, Toronto, ON. **[Poster Presentation]**

PRESENTATIONS (CONTINUED)

2015

Brozyna JR and Heinrichs DE. Defining the genetic basis of growth promotion of *Staphylococcus lugdunensis* by hemoglobin, stress hormones, and siderophores.

- Infection and Immunity Research Forum, London, ON. **[Poster Presentation]**
- London Health Research Day, Infection and Immunity, London, ON. **[Poster Presentation]**

2014

Brozyna JR and Heinrichs DE. Iron acquisition mechanisms employed by *Staphylococcus lugdunensis*.

- Infection and Immunity Research Forum, London, ON. **[Poster Presentation]**
- BioMetals 2014, Duke University, Durham, NC, USA. **[Poster Presentation]**

2013 - 2014

Brozyna JR, Sheldon JR, and Heinrichs DE. Growth promotion of *Staphylococcus lugdunensis* by hemoglobin and co-culture with *Staphylococcus aureus*.

- London Health Research Day, Infection and Immunity, London, ON. **[Poster Presentation]**
- Infection and Immunity Research Forum, London, ON. **[Poster Presentation]**
- London Health Research Day, Infection and Immunity, London, ON. **[Poster Presentation]**

On behalf of **Assistive Dynamics Corporation**. **2010**. Newly developed assistive technology for upper cervical nerve injury.

- Abilities Expo: New York, NY, USA. **[Exhibition]**
- Abilities Expo: Chicagoland. IL, USA. **[Exhibition]**
- Durham Region Accessibility Expo. Oshawa, ON, Canada. **[Exhibition]**

SCHOLARSHIPS, AWARDS AND HONOURS

2015 – 2016 **Queen Elizabeth II Graduate Scholarship in Science and Technology**
Ontario Graduate Scholarship
Ontario Government Award ($15,000)

SCHOLARSHIPS, AWARDS AND HONOURS (CONTINUED)

2016 **Dr. FW Luney Graduate Travel Award in Microbiology & Immunology**
Travel award to present research at a scientific conference
Travel Award ($2,000)

2016 **ASM Student and Postdoctoral Travel Award**
Travel award to present research at ASM Microbe 2016
Travel Award ($500)

2014 **eBioscience Award for Best Graduate Poster Presentation**
9th Annual Infection and Immunity Research Forum
Best Graduate (>6 months) Poster Presentation ($100)

2011 – 2016 **Western Graduate Research Scholarship**
University of Western Ontario Internal Award
Tuition Scholarship ($37,600)

2011 **Dean's Honour List**
University of Guelph, Guelph, ON

2010 **Active Achievement Award**
Assistive Dynamics Corporation, Brampton, ON
Strong Performance Award ($1,000)

2009 **Co-operative Education Student of the Year Nominee**
University of Guelph, Guelph, ON

COMMITTEES, VOLUNTEER EXPERIENCE AND ACTIVITIES

2014 – 2016 **Elected Student Representative to the Research Committee**
Department of Microbiology & Immunology
University of Western Ontario, London, ON.
The Research Committee oversees the overall research goals, plans, and directions of the department. I have been elected to represent the interest of all Microbiology & Immunology graduate students and post-doctoral fellows to this faculty committee.

COMMITTEES, VOLUNTEER EXPERIENCE AND ACTIVITIES (CONTINUED)

2014 – 2016 **Representative to the American Society for Microbiology**

Microbiology & Immunology Department Representative
University of Western Ontario, London, ON.
American Society for Microbiology Student Member and liaison for the Microbiology & Immunology department.

07/2015 **American Society for Virology 34th Annual Meeting Volunteer**

University of Western Ontario, London, ON.

Cheerfully assisted in coordinating set-up, presentations, crowd movement and social events for 1200 conference attendees.

2014 – 2015 **Co-Chair**

Microbiology & Immunology Social Events & Contributions Committee

University of Western Ontario, London, ON.

Responsible for the initiation, planning and organization of departmental events for team-building, community service, and to foster internal collaboration. Includes coordinating and advising a team of graduate students to fully accomplish such functions, while managing a budget (over $5,000), chairing meetings and representing the committee at executive meetings.

2014 – 2015 **Student Representative to the Retreat Planning Committee**

Joint-Departmental Retreat Planning Committee

University of Western Ontario, London, ON.

The Joint-Departmental Retreat Planning Committee plans, organizes, and prepares the annual conference-style retreat for several departments at the University. This retreat fosters discussion and collaboration between scientific researchers at the University and infectious disease clinicians at three hospitals in London, ON.

2011 – 2014 **Ambassador**

Microbiology & Immunology Graduate Social Committee

University of Western Ontario, London, ON.

COMMITTEES, VOLUNTEER EXPERIENCE AND ACTIVITIES (CONTINUED)

2012 – 2014 **Team Captain (Rods & Cocci)**
Microbiology & Immunology Intramural Softball Team
University of Western Ontario, London, ON.

2012 – 2013 **Vice President of Public Relations and Promotions**
Infection and Immunity Research Forum
University of Western Ontario, London, ON.
Responsible for building, maintaining and overseeing all public and media promotional activities and relationships, as well as creation and distribution of media for conference marketing purposes for this graduate student-organized scientific conference.

2012, 2013 **Team Leader, World's Toughest Mudder**
24-hour military-style endurance race (four person team)

2012, 2013 **Team Leader, Tough Mudder**
12 mile military-style endurance race (four person team), qualifier for World's Toughest Mudder

2011 – 2012 **Organizing Committee Representative**
Infection and Immunity Research Forum
University of Western Ontario, London, ON.
Responsible for helping plan, organize and prepare a graduate student-organized scientific conference.

2011 – 2012 **Team Captain, Intramural Ball Hockey**
University of Western Ontario, London, ON.

2004 – 2006 **Programs Assistant**
Community Nursing Home Pickering, Pickering, ON.

2004 – 2006 **Secondary School Chemistry, Biology and Calculus Tutor**
St. Mary C. S. S. Pickering, ON.