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Molecular Investigation of Heme Acquisition in Staphylococcus aureus

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Abstract

Staphylococcus aureus is a notorious, Gram-positive, coccoid-shaped bacterium that is pathogenic to humans and animals. It is the culprit of many infectious diseases ranging from boils, impetigo, and food poisoning to more serious diseases such as endocarditis, osteomyelitis, and toxic shock syndrome. However, for *S. aureus* to survive in the host, nutrients and especially iron must be acquired. *S. aureus* expresses iron-regulated surface determinant (Isd) proteins that, together, function in the acquisition of heme iron. However, mutants devoid of IsdA or IsdE have only subtle defects in growth on heme as a sole iron source. Here I demonstrate that an *isd* locus deletion mutant (Δ*isd*) grows better than wildtype on heme-containing media, indicating the existence of non-*isd* mechanisms of heme acquisition. Transposon mutagenesis in the Δ*isd* background identified an insertion in *brnQ3*, encoding a putative branched chain amino acid (BCAA) transporter, which yielded a strain that grew extremely poorly, with an extended lag period, on media containing heme as a sole iron source. Expression of either *brnQ3* or *isdEF in trans*, or exogenously supplying a mixture of Ile/Leu/Val/Thr complemented the growth deficiency of the mutant. In comparison to wildtype *S. aureus*, the *isd brnQ3* mutant had impaired virulence in mice. Together, the data show not only that *isdEF* is active in heme uptake but also suggest that *brnQ3*, despite the presence of two additional paralogs, is important for uptake of BCAAs, and that intracellular BCAAs, which are an indicator of nutritional status, regulate expression of an alternate, non-Isd, heme uptake mechanism in *S. aureus*.

Key words: staphylococci, heme, branched-chain amino acid transporter, heme transport

Co-authorship

Portions of the work presented herein were contributed by Federico Beasley who kindly assisted with the murine sepsis model of infection as well as Alan Poole who contributed the real-time PCR data presented in the manuscript.

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List of Abbreviations

ABC	ATP-Binding Cassette
Amp	Ampicillin
bp	base pair
Cm	Chloramphenicol
DNA	Deoxyribonucleic acid
Dip	2,2-dipyridyl
EDDHA	Ethylene diamine-di(*o*-hydroxyphenol acetic acid)
Em	Erythromycin
kb	Kilobase
Km	Kanamycin
LB	Luria-Bertani broth
M	Molar
Mb	Megabase
mg	Milligram
ml	Millilitre
mM	Millimolar
Neo	Neomycin
nm	nanometer
OD	Optical Density
PCR	Polymerase Chain Reaction
Tet	Tetracycline
TMS	Tris-Minimal Succinate

TSB	Trypic Soy Broth
µg	Microgram
µl	Microlitre
UV	Ultra-Violet

Chapter 1 - Introduction

1.1. The Staphylococci

1.1.1. Description of the Genus

The linguistic root of the name *Staphylococcus* represents two Greek terms used to describe the appearance of these bacteria. The first term *staphylē* means "bunches of grapes" and the second term is *kókkos* which stands for "grain or berry". The resulting genus name *Staphylococcus* would therefore describe these bacteria as being irregular grape-like clusters of round, or cocoid, cells. *Staphylococcus* species are facultative anaerobic Gram-positive bacteria and are catalase-positive, unlike other Gram-positive cocci. Although *Micrococcus* is also catalase-positive, it is an obligate aerobe and can be distinguished from staphylococci using light microscopy. Species of staphylococci are fairly salt-tolerant (up to 10-15% NaCl) as well as being resistant to dry conditions. Staphylococci can also survive a broad pH range from 4.8 to 9.4 (36, 82). Staphylococci are separated into two categories based on the presence or absence of coagulase, a protein that enables the conversion of fibrinogen to fibrin. Coagulase causes fibrin to be deposited on *S. aureus* cells and protects the coated bacteria from attack by host cells (84). *S. aureus* colonies produce a golden yellow colour, due to the production of the pigment staphyloxanthin. The organism is the most pathogenic staphylococcal species and was one of the earliest bacterial pathogens identified. In contrast, the coagulase-negative staphylococci form white colonies and include lesser, yet clinically important, pathogenic species such as *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*, and *S. lugdunensis* (36). The genomes of staphylococci are within 2-3 Mb with a mol % G + C content of 30-39%. Prophages can also exist in the genomes but the presence of plasmids is rare. By using both DNA-DNA hybridization and 16s rRNA sequence

analysis, 41 species and 24 subspecies have been categorized within the genus *Staphylococcus* (36).

1.1.2. Association of *S. aureus* with Humans

S. aureus is considered to be a commensal bacteria, or part of the normal human flora. It can be persistently found in 20% of people; 60% are intermittent carriers and the remaining 20% are non-carriers (36, 82). Specifically, *S. aureus* colonizes the anterior nares and can often be dispersed onto healthy skin. It can also be a member of the throat, gut, underarm, groin and vaginal microflora (82). Colonization of the nasal mucosa is aided by the presence of a large number of surface adhesion proteins, some of which will be discussed in the next section. *S. aureus* poses the greatest threat of infection to immunocompromised patients and the elderly but young and healthy patients are no exception. Although most *S. aureus* infections are minor skin infections, the organism is capable of causing systemic infections including bacteraemia, endocarditis, pneumonia, muscle abscess, osteomylelitis, and bacterial arthritis if the epithelial barrier is ever breached. *S. aureus* also causes toxin-mediated illnesses such as toxic shock syndrome, acute food poisoning, staphylococcal enterocolitis, and infant scalded skin syndrome (36, 82).

1.1.3. Antibiotic Challenges with *S. aureus*

Traditional treatments for *S. aureus* infections were effective with administration of penicillin G, and this antibiotic was able to severely reduce the mortality rates of infected patients (47). However, since the first introduction of penicillin in the 1940s, antibiotic-resistant strains of *S. aureus* have emerged. By the 1950s, penicillin-resistant

S. aureus strains had reached communities and hospitals across the world (36). Despite development of next generation antibiotics to treat these infections, *S. aureus* resistant strains persist. Antibiotic resistant staphylococci can be either hospital-acquired or community-acquired. The difference is that hospital-acquired strains have higher frequencies of resistance than those community-acquired. The source of hospital-acquired infections can be due to transfer of bacteria between patients or between health care practitioners and patients. In addition, many infections tend to arise from surgical procedures (47). Since 2001, strains originating from hospitals have been causing an increasing number of community acquired *S. aureus* infections. Although most are mild skin lesions, cases of severe invasive pneumonia have also been reported (140). Moreover, the problem is that greater than 90% of *S. aureus* clinical isolates are resistant to penicillin derivatives or beta-lactam antibiotics, which include cephalosporins, carbapenems, cephamycins, and monobactams (47). The beta-lactam ring is the common constituent of these antibiotics, mimicking the D-Ala-D-Ala section of the N-acetylmuramic acid pentapeptide that is involved in crosslinking separate peptidoglycan units during cell wall synthesis. The crosslinking reaction is catalyzed by penicillin-binding proteins (PBPs) for which *S. aureus* contains four in total (36). Therefore, the beta-lactam ring serves as a PBP inhibitor. Beta-lactam resistant *S. aureus* produce beta-lactamase, which hydrolyzes the common beta-lactam ring of the antibiotics. With the beta-lactam ring destroyed, the antibiotic is rendered ineffective to act as a PBP inhibitor (36). The resistance to beta-lactam antibiotics prompted the development of methicillin, a semi-synthetic derivative that beta-lactamases have a difficult time hydrolyzing. Therefore, methicillin was free to bind PBPs. Unfortunately, with sufficient time and widespread use of methicillin, MRSA (Methicillin Resistant *Staphylococcus aureus*)

emerged. This resistance to methicillin is achieved through via the production of an alternative penicillin-binding protein (PBP2a), which permits continued cell wall synthesis while the native PBPs are bound up by methicillin (47). PBP2a is encoded by the *mecA* gene which is part of the staphylococcal chromosome cassette mec (SCCmec) and appears to have been introduced to *S. aureus* from coagulase-negative staphylococci such as *S. epidermidis*, *S. sciuri,* or *S. haemolyticus* (2, 59, 146). However, the exact origin and evolution of SCCmec remains unknown. The molecular features and genetic versatility of SCCmec allow potential for MRSA to obtain further resistance determinants through gene insertions, deletions, and rearrangements that can lead to multiple resistance phenotypes (61, 62). Not only are beta-lactams ineffective against MRSA, but these strains can have resistance to other classes of antibiotics including rifampin, fluoroquinolones, aminoglycosides, tetracyclines, and chloramphenicol (47). The situation with MRSA continues to worsen as 63% of all staphylococcal infections in 2004 were due to MRSA according to the Centers for Disease Control (36). Therefore, health care workers have turned to the last line of defense against clinical staphylococcal infections, which involves the use of glycopeptide antibiotics that include vancomycin and teicoplanin. These compounds bind to the D-Ala-D-Ala moiety of the N-acetylmuramic acid pentapeptide and block cell wall synthesis (47). Up until the late 1990s, vancomycin proved effective against *S. aureus*. However by 2002, the first cases of vancomycin-resistant *S. aureus* (VRSA) were reported by the Centers for Disease Control and Prevention. These VRSA strains have simply transitioned from being MRSA after gaining resistance to vancomycin (47). The appearance of such strains prompted the possibility of emerging *S. aureus* infections that would be untreatable by current antibiotics.

1.1.4. *S. aureus* Virulence Factors

S. aureus is known to be more pathogenic than its coagulase-negative brethren. Although categorized as being "coagulase-positive", *S. aureus* does not owe its pathogenic potential to only the coagulase enzyme since coagulase mutants show only a minor loss of virulence (5, 92). Therefore, to be an effective pathogen, *S. aureus* must possess a combination of other virulence determinants which some of the coagulase-negative species are lacking. For instanee, a comparison between the *S. aureus* and *S. epidermidis* genomes reveal that *S. aureus* hosts 18 genomic islands that encode exclusive virulence factors (50).

Table 1 summarizes a survey of some *S. aureus* virulence factors. As mentioned previously, *S. aureus* relies on various surface-related virulence factors for initial adhesion and eventual establishment of an infection. In addition, these surface virulence factors also help ward off host immune defences or can modulate the immune response. Surface exposed virulence factors are composed of cell wall anchored proteins or polysaccharides (36). The *S. aureus* cell wall contains the polysaccharide teichoic acid that contribute towards adherence onto nasal surfaces and endothelial cells (144, 145). Other components of the cell surface include lipoteichoic acid and peptidoglycan that are thought to work synergistically to recruit neutrophils and activate septic shock (67, 74). Many of the proteins on the cell surface of *S. aureus* are involved in protein-protein interactions with host serum or extracellular matrix proteins. Therefore, these adhesins are collectively called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and most of these proteins contain a signature cell wall anchoring sign

Table 1. Summary of *Staphylococcus aureus* virulence factors

Virulence factors of *S. aureus*	Gene	Function/Effect
Cell Wall components		
Teichoic acid		Nasal colonization
Lipoteichoic acid		Septic shock
Peptidoglycan		Septic shock
Surface adhesions		
Protein A	*spa*	Binds Fc portion of IgG molecules Inhibits phagocytosis
Clumping factors A and B	*clfA, clfB*	Adhesion to host tissues Resistance to phagocytic clearance
Fibronectin-binding proteins A and B	*fnbAB*	Adhesion to host tissues
Collagen adhesion	*cna*	Adhesion to collagen
Secreted adhesions		
Extracellular fibrinogen-binding protein	*efb*	
Extracellular matrix-binding protein	*emp*	Formation of staphylococcal communities that elicit abscess formation
Extracellular adhesion protein (EAP)	*eap*	Formation of pseudocapsule to allow bacterial resistance in host tissues
Exopolysacchardes		
Capsule	*cap*	Polysaccharide capsule production Inhibits opsonophagocytosis
Polysaccharide intercellular adhesion	*icaADBC*	Biofilm formation Resistance to phagocytosis and antimicrobial peptides
Exotoxins		
α-toxin	*hla*	Cell lysis
β-toxin	*hlb*	Cell lysis
δ-toxin	*hld*	Cell lysis
γ-toxin	*hlg*	Cell lysis and lysis white blood cells
Panton-Valentine leukotoxin	*lukFS-PV*	Damages leukocyte membrane
Exfoliative toxins	*etA, etB, etC, etD*	Staphylococcal scalded skin syndrome
Toxic shock syndrome (TSST)-1	*tstH*	Superantigenicity
Staphyloccal enterotoxins	*seA-E,seG,seI,seH, seJ, seV*	Superantigenicity
Enzymes		
Serine protease	*sspA*	Cleaves extracellular proteins
Cysteine protease	*sspB*	Degrades polypeptides
Iron Acquisition		
Iron-regulated surface determinants	*Isd* genes	Heme binding
Staphyloferrin A	*sfaA-D*	Siderophore
Staphyloferrin B	*sbnA-I*	Siderophore
Regulators		
Accessory gene regulator	*agr*	Quorum sensing Regulates expression of cell-surface and secreted proteins

Staphylococcal accessory regulator	*sar* genes	Regulation of Agr Regulation of extracellular and surface-associated virulence factors
Exoprotein expression	*saeRS*	Regulation of exotoxins

(usually with the motif LPXTG) that permits an enzyme called sortase A (and sometimes sortase B although with a different C-terminal anchoring motif; NPQTN) to covalently anchor the protein onto the peptidoglycan (87, 112). Examples of cell wall anchored proteins include Protein A (binds the Fc region of IgG antibodies and hence renders protection to the bacterium from antibody-mediated phagocytosis) (48), fibronectin-binding proteins FnbA and FnbB (30), and fibrinogen-binding proteins ClfA and ClfB (36). Aside from virulence factors that are tethered to cell surface, *S. aureus* is also armed with a plethora of secreted toxins that can independently cause disease despite the absence of the pathogen. Classic examples include exfoliative toxins ETA and ETB, which cause staphylococcal scalded skin syndrome (SSSS) with symptoms of sloughing off of the outer layer of skin (100). In addition, *S. aureus* exotoxins also include toxic shock syndrome toxin-1 (TSST-1), which is known as a superantigen, where T-cell activation causes massive cytokine storm to cause fevers, rashes, hypotension, multi-organ failure, and death (36, 133). Besides TSST-1, *S. aureus* can also produce other superantigens but these are also considered enterotoxins since they specifically cause food poisoning resulting in vomiting and diarrhea (133). However, a non-oral route of entry for such enterotoxins can still cause the classic superantigen symptoms (88). *S. aureus* is also capable of producing pore-forming toxins that create enlarged pores on the membranes of cells leading to osmotic imbalance and eventual cell lysis. Two popular toxins are the α- and β-toxins, which are protein complexes that are capable of destroying erythrocytes (36). The destruction of erythrocytes serves as an important nutritional strategy for heme-iron uptake which will be discussed in further sections. *S. aureus* also produces a δ-toxin which is a small peptide with the ability to lyse a large variety of cell types in addition to erythrocytes (41). Lastly, *S. aureus* can

produce γ-toxin and Panton-Valentine leukotoxins that are known to target and lyse leukocytes (102). The last category of *S. aureus* virulence factors are exoenzymes such as proteases, lipases, hyaluronidases, and nucleases. However, the contribution of these degradation enzymes to virulence remains unclear (36). Within the last decade, intense research regarding nutritional acquisition mechanisms by the staphylococci has revealed another set of determinants that contribute to virulence. Specifically, acquisition of the precious metal iro serves a critical role in the ability of *S. aureus* to establish an infection in iron-deprived environments such as the mammalian host.

1.2. Iron

1.2.1 Chemical and Physical Properties of Iron

Iron is an abundant element on Earth. Iron exists in many oxidation states with the most common being ferrous (Fe^{2+}) or ferric (Fe^{3+}) forms. In aerobic environments and at pH 7, iron is predominantly found in the ferric (Fe^{3+}) state with a soluble concentration of 1.4×10^{-9} M, rather than the more commonly reported value of 10^{-18} M (27, 104). The latter value incorrectly assumes that the major ionic species in solution is $Fe(OH)_3$ rather than the supposed $Fe(OH)^{2+}$ species. Regardless, the concentration of soluble iron is low enough that pathogens need to deploy iron acquisition mechanisms in order to survive, colonize a host, and to successfully establish an infection. However, once iron is acquired, it must be carefully controlled as the reducing conditions inside the cell convert Fe^{3+} to Fe^{2+}, thus allowing the possibility of Fe^{2+} iron to react with oxygen species to form hydroxide radicals (Fenton reaction), which damage cell membranes and DNA (142). Therefore, the challenge of iron acquisition for bacteria is

two-fold; iron scavenging must be efficient and iron stores inside the cell must be carefully regulated to avoid toxicity.

1.2.2. Iron in Biology

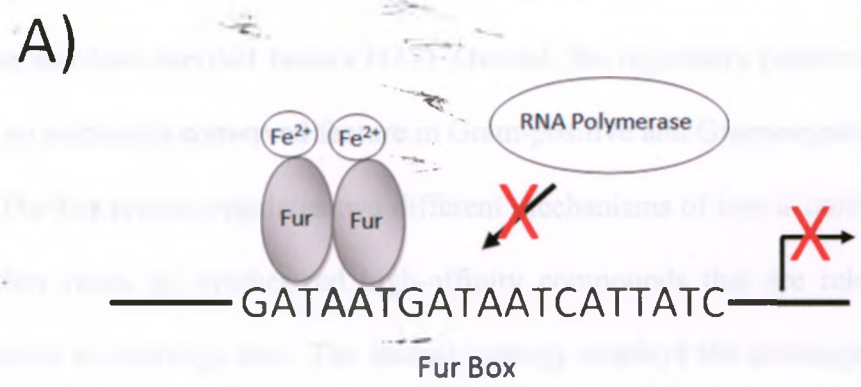
Iron is an essential requirement for the majority of all living organisms (142). Like many other metals, iron serves as a cofactor for many enzymatic reactions (amino acid, nucleotide, and DNA synthesis) and can also function as a structural component in proteins (121, 142). Furthermore, the catalytic potential of iron is harnessed through electron transport processes (121). Lastly, iron can participate in oxygen activation, peroxide reduction, and photosynthesis (142). Iron is needed to complete iron-sulfur clusters or catalytic centers of redox enzymes (142). Despite being a necessary nutrient, bacteria are constantly faced with the obstacle of accessing free iron in their host environment (142). Free iron is not readily accessible within host environments since mammals have developed many ways to sequester iron. First, iron is restricted in the host because it is bound intracellularly in the iron storage protein ferritin or to the hemoglobin of erythrocytes. Second, iron in extracellular fluids is also sequestered by host glycoproteins lactoferrin found in milk and mucosal secretions or transferrin found in blood and lymph (103, 142). Therefore, to limit the access of iron is to limit the growth of pathogens. As a result, the amount of free iron under physiological conditions is very low (see above) (27).

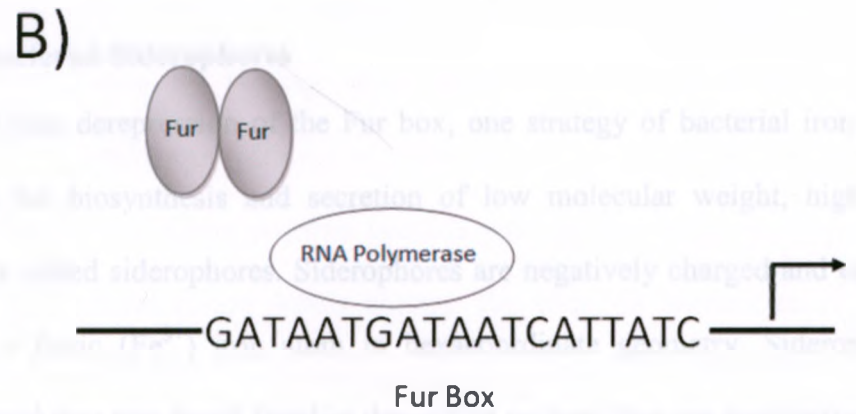
1.3. Bacterial Iron Uptake Systems

1.3.1. Regulation of Iron Uptake in Bacteria

As mentioned previously, the amount of readily available iron under physiological conditions is very low but 10^{-6} to 10^{-7} M iron is sufficient for pathogenic bacteria to grow (111). However, this does not suggest that a higher concentration of intracellular iron correlates with improved physiological status of the bacterium. The transport of iron by bacteria is very tightly regulated in order to maintain an appropriate intracellular concentration and to avoid accumulation of free iron in the cell which could be toxic to the organism (121). In order to maintain proper iron homeostasis, *S. aureus* and other bacteria rely on active transporters and efflux systems to maintain a non-toxic but sufficient concentration. A large number of genes can be expressed or repressed in response to intracellular iron levels via the action of regulatory proteins. Most genes encoding iron acquisition systems are regulated by the ferric uptake regulator (Fur) protein (104, 121, 142). A schematic is presented in Figure 1. The Fur protein was first identified 40 years ago as a repressor of iron-regulated genes in *Escherichia coli* and *Salmonella typhi* (142). The Fur protein is also found in *S. aureus* and regulates the expression of many operons, especially genes encoding iron acquisition systems (10, 37, 87, 147). The *fur* gene encodes an 18-kDa protein that is responsible for the iron-dependent repression of genes involved in iron acquisition. When iron is abundant, Fe^{2+} binds to a Fur homodimer (as a co-repressor) allowing the iron-proteincomplex to bind a 19-bp inverted repeat (5'-GATAATGATAATCATTATC-3') called the Fur box (121). The Fur box is a consensus sequence found in the promoter region of Fur-regulated genes. Under iron starved conditions, Fe^{2+} is not present to act as a co-repressor and therefore Fur cannot bind to the Fur box, resulting in derepresssion of Fur-regulated genes (allowing transcription of iron

Figure 1. Schematic Representation of Fur-Regulated Gene Expression. Fur is an 18-kDa protein and it forms a homodimer that binds to a 19-bp inverted repeat sequence known as the Fur box (GATAATGATAATCATTATC). A) Under iron-replete conditions, Fur binds to Fe^{2+} as a co-repressor which allows the complex to bind to the consensus DNA sequence (Fur box) causing a blockage for RNA polymerase to initiate transcription of the iron-regulated genes. B) Under iron-deplete conditions, Fe^{2+} is no longer present to act as a co-repressor and therefore Fur cannot bind the DNA. Without interference from Fur, RNA polymerase can now begin transcription of downstream genes. Note: the Fur protein binds onto the whole Fur box to regulate gene expression.


A)
Fe2+
Fe2+
Fur
Fur
RNA Polymerase
GATAATGATAATCATTATC
Fur Box


B)
Fur
Fur
RNA Polymerase
GATAATGATAATCATTATC
Fur Box

acquisition systems) (142). Moreover, in *S. aureus*, the Fur protein allows for detection o changes in the level of surrounding iron which would provide cells with the opportunity to respond by altering the abundance of many different virulence factors (137). In addition, *S. aureus* mutants lacking Fur are more sensitive to neutrophil killing and are less virulent in a murine pneumonia model, therefore indicating the important role Fur plays in regulating virulence and host survival factors (137). Overall, the regulatory properties of the Fur protein make it an extremely conserved feature in Gram-positive and Gram-negative organisms.

The Fur protein regulates two different mechanisms of iron acquisition. The first mechanism relies on synthesized high-affinity compounds that are released into the environment to scavenge iron. The second strategy employs the scavenging and uptake of heme-iron from various physiological sources (104, 142).

1.3.2. Bacterial Siderophores

Upon derepression of the Fur box, one strategy of bacterial iron acquisition is through the biosynthesis and secretion of low molecular weight, high affinity iron chelators called siderophores. Siderophores are negatively charged and each one wraps around a ferric (Fe^{3+}) iron atom in hexacoordinate geometry. Siderophores can be categorized into two broad families depending on how they are synthesized; specifically non-ribosomal peptide synthesis (NRPS) and NRPS-independent synthesis (NIS) (25). NRPS siderophores are synthesized by modular enzymatic platforms that condense individual units of nonproteinogenic amino acids through a series of chain elongation reactions (while remaining tethered to the enzymatic platform) and ending in a chain termination event that releases the siderophore (35). Well characterized NRPS siderophores include enterobactin, yersiniabactin, and vibriobactin (66, 90, 105). In

contrast, NIS siderophores are synthesized by single or multiple enzyme systems that condense individual units of dicarboxylic acids, diamines, and alcohol amines to form ester- or amide-based siderophores. Unlike the NRPS method, NIS synthesis does not utilize an assembly platform and allows intermediates of siderophore synthesis to remain freely dissociable from the biosynthetic enzymes (25). The model NIS siderophore is aerobactin from *Escherichia coli* (40). However, other NIS siderophore systems have also been studied including rhizobactin, achromobactin, vibrioferrin, and alcaligin (14, 49, 65, 81, 130). *S. aureus* produces two NIS siderophores, discussed in section 1.3.4.

1.3.3. Siderophore-Iron Transport

Once siderophores are synthesized inside the cell, they will be effluxed out to scavenge for iron. Siderophores in the extracellular milieu capture ferric iron (Fe3+), which is followed by binding onto dedicated Fe(III)-siderophore transporters for shuttling iron (and supposedly the siderophore molecule) back into the cell (9). Note that Gram-negative and Gram-positive bacteria use different systems for uptake of iron-loaded siderophores (more details below). However, all bacteria rely on at least one ATP-binding cassette (ABC) transporter to actively import the iron-siderophore complexes. An ABC transport system consists of a ligand-binding protein, a transmembrane permease, and an ATPase for ATP hydrolysis to provide the energy for shuttling of the substrate across the membrane. Note that all three components of an ABC transporter are intimately attached in order to couple ATP hydrolysis with the conformational changes necessary to pump the ligand into the cell (for review see (39)).

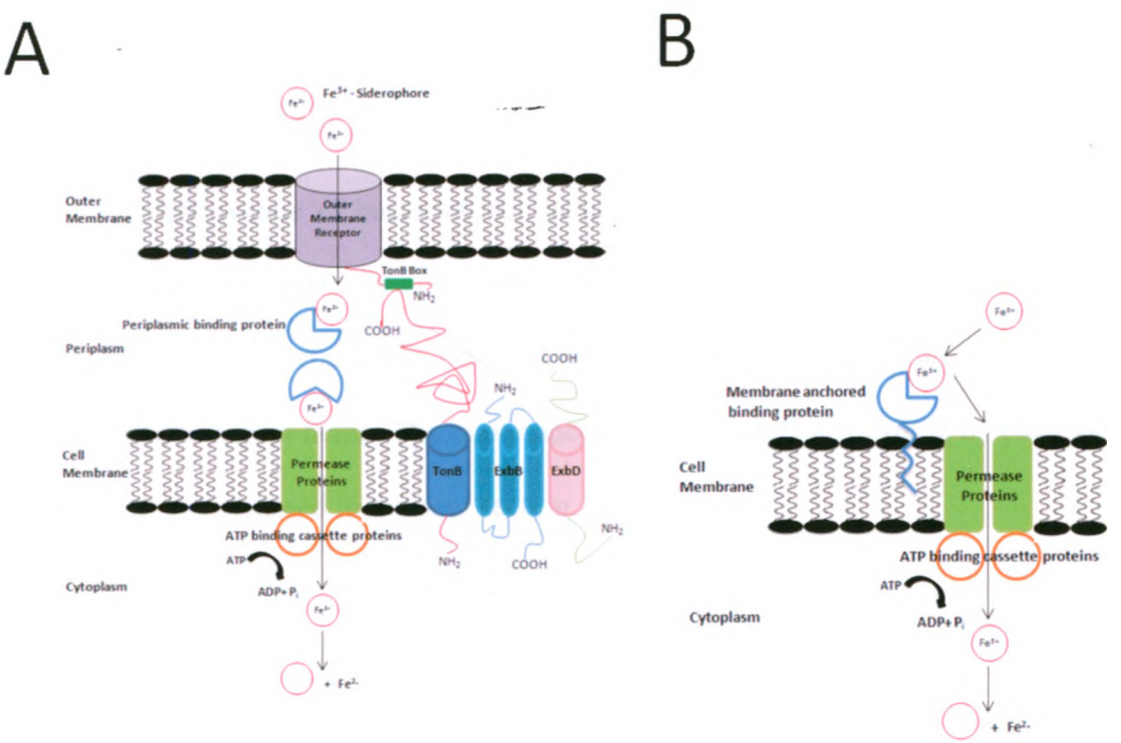
The siderophore transport system for Gram-negative bacteria includes an outer membrane receptor, a periplasmic binding protein, a TonB-ExbB-ExbD energy-transducing complex, and an inner membrane ABC transporter (71). In general, the iron-loaded siderophore initially binds the outer membrane receptor but in order for the siderophore to enter the periplasm, an energy transduction event must take place. This is achieved by the inner membrane TonB complex. The siderophore-iron complex cannot initially pass through the outer membrane receptor because the outer membrane receptor apparently is "corked" at its base by its own N-terminal domain and the C-terminal periplasmic extensions of the TonB protein, where the two domains exhibit protein-protein interactions (17, 75, 97). Therefore, without a conformational change to remove the "cork", the siderophore-iron complex will not enter the periplasm. The ExbB-ExbD complex utilizes the energy of the proton motive force to transduce enough energy to TonB to induce a conformational change between periplasmic extensions in TonB and the N-terminal domain of the outer membrane receptor. This effectively removes the N-terminal "cork" at the end of the outer membrane receptor to allow the siderophore-iron complex to freely pass into the periplasmic space (113, 135). The periplasmic binding protein then binds onto the siderophore and serve as the ligand-binding component of the inner membrane ABC transporter. This is followed by docking of the periplasmic binding protein onto the transmembrane permease and with the help of the ATPase (described above), the siderophore-iron complex is pumped across the inner membrane and into the cytoplasm (71). A schematic representation of siderophore-iron transport in Gram-negative bacteria is presented in Figure 2A.

In contrast, siderophore transport in Gram-positive bacteria employs only a lipid-anchored receptor protein (covalently linked to membrane lipids via acylation of a lipobox domain) and an ABC transporter. Due to lack of a periplasm in Gram-positive bacteria, the lipid-anchored receptor protein is analogous in function to the periplasmic binding proteins for Gram-negative bacteria. The siderophore-iron complex will bind onto the receptor lipoprotein, which serves as the ligand-binding protein for the ABC transport system. Once again, ATP hydrolysis permits entry of the siderophore-iron complex across the cell membrane and into the cell (9, 71). A schematic representation of siderophore-iron transport in Gram-positive bacteria is presented in Figure 2B.

1.3.4. Siderophore Systems of *S. aureus*

For *S. aureus*, there are two structurally and chemically characterized siderophores: staphyloferrin A (composed of two citrate molecules flanking a D-ornithine amino acid) and staphyloferrin B (a condensation between diaminopionate, citrate, diaminoethane, and α-ketoglutarate) (42, 58, 69, 89). Both belong to the NIS siderophore family. The staphyloferrin A biosynthetic and transport operons have been denoted as *sfa* and *hts*, respectively (10, 32). Similarly, the staphyloferrin B biosynthetic and transport operons have been named *sbn* and *sir*, respectively (37, 38). Furthermore, the biosynthetic pathways involving the enzymes and substrates to synthesize each siderophore have also been recently characterized (26, 32). Interestingly, deletion of one of the *sbn* genes leads to reduced virulence of *S. aureus* in a mouse model kidney infection (37), which further lends support to iron acquisition mechanisms as being virulence factors. Recently, high-resolution holo crystal structures of staphyloferrin A- and staphyloferrin B-bound HtsA and SirA high affinity siderophore receptor proteins

Figure 2. Schematic Representation of Iron-Siderophore Transport in Gram-Negative and Gram-Positive Bacteria. A) Schematic representation of siderophore-iron transport in Gram-negative bacteria. The siderophore initially binds with Fe^{3+} found in the environment and this siderophore-iron complex then binds to a dedicated outer membrane receptor. Transport of the siderophore-iron complex through the outer membrane receptor and into the periplasm requires the action of the TonB system (TonB, ExbB, ExbD), to supply the energy transduction to mobilize the siderophore-iron complex from the outer membrane receptor. Once in the periplasm, a periplasmic binding protein binds onto the siderophore-iron complex followed by docking of the protein onto the inner-membrane ABC transporter (composed of transmembrane permease and ATPase). The siderophore-iron complex is then actively pumped across the inner membrane and into the cytoplasm. Once inside the cell, iron is liberated from the siderophore. B) Schematic representation of siderophore-iron transport in Gram-positive bacteria, which unlike Gram-negative bacteria, lack an outer membrane. Therefore, the uptake of siderophore-iron only involves a membrane anchored substrate-binding protein and a membrane-associated ABC transporter. The figure is modified from (73).


A
B
Fe^{3+} - Siderophore
Outer Membrane
Outer Membrane Receptor
TonB Box
NH_2
COOH
Periplasmic binding protein
Periplasm
Cell Membrane
Permease Proteins
TonB
ExbB
ExbD
ATP binding cassette proteins
ATP
ADP+ P_i
Cytoplasm
+ Fe^{2-}
Membrane anchored binding protein

have been reported (51, 52). Although a third siderophore, tentatively named aureochelin, has been identified in culture supernatants, there has not yet been any further investigation into its chemical structure or biosynthetic genetic locus (34). It is interesting to note that staphyloferrin A is produced by all members of the staphylococci (coagulase-negative and –positive) and, despite earlier reports of staphyloferrin B production from coagulase-negative staphylococci (42, 89), only *S. aureus* is known to produce staphyloferrin B due to the absence of the *sbn* operon in many recently sequenced coagulase-negative staphylococcal genomes (9, 37).

1.3.5. Heme-Iron Transport

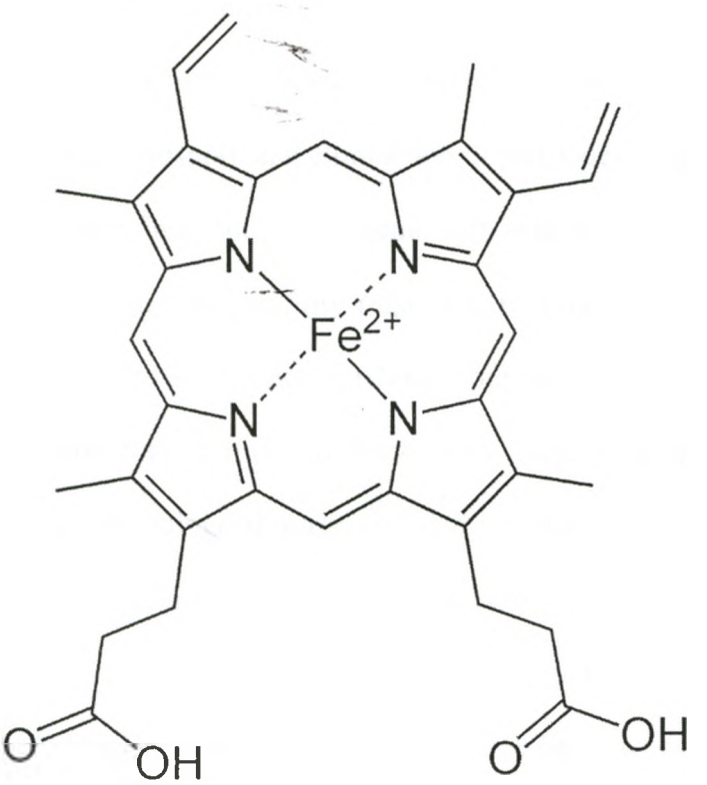
Another avenue of bacterial iron acquisition during infection of a mammalian host is through the uptake of heme-iron. Other than through the use of siderophores, another prerequisite to successful bacterial pathogenesis appears to include the ability to scavenge and transport heme. The importance of heme and its acquisition by pathogenic bacteria will be discussed in the next section.

1.4. Heme

1.4.1. General Properties of Heme

As mentioned previously, free iron in the human body is sequestered by both intracellular and extracellular proteins. Heme is the most abundant source of intracellular iron accounting for roughly 80% of the total available iron pool (53, 111, 127). The structure of heme is shown in Figure 3. Technically, heme refers to the ferrous form of iron (Fe^{2+}) bound to the tetrapyrrole ring (a macrocyclic porphrin), whereas

Figure 3. Chemical Structure of Iron-Protoporphyrin XI (heme). The molecule contains a central iron atom fixed into a heterocyclic organic ring called a protoporphyrin. Heme refers to the ferrous form of iron (Fe^{2+}) bound to the protophorphyrin, whereas hemin refers to the same molecule with the exception of bound ferric iron (Fe^{3+}). Heme is a lipophilic molecule with a low molecular weight (~600 Da) that can potentially allow for easy insertion within cell membranes that may impair lipid bilayers and organelles. The iron atom in heme forms complexes with oxygen molecules, which are carried throughout the body by the hemoglobin in the red blood cells. Figure obtained from (108).


N
N
Fe2+
N
N
O
OH
O
OH

hemin contains the oxidized ferric form of iron (Fe^{3+}) (135). Regardless, both forms are commonly referred to as "heme", so this terminology will be used throughout the remainder of this thesis. Heme is a lipophilic molecule with a low molecular weight which allows for easy insertion between membranes, potentially leading to impairment of lipid bilayers and organelles (135). When iron is encased by the tetrapyrrole ring to form heme, the catalytic potential of iron increases by 5- to 10- orders of magnitude (1). Due to the overall catalytic activity of heme, it is involved in many vital processes such as oxygen storage, oxygen transport, and elimination of peroxides as well as oxygen free radicals (1). Just like free iron, too much heme can lead to toxicity and, therefore, intracellular heme levels must be carefully regulated. Hemoglobin is one of the most significant biological examples of a hemoprotein. Four heme molecules are found in each molecule of hemoglobin in order to function as oxygen transporters within red blood cells (142). Other physiological sources of heme include haptoglobin-hemoglobin, hemopexin, albumin, and other hemoproteins. Haptoglobin-hemoglobin is a tetrameric glycoprotein containing haptoglobin bound tightly to hemoglobin after it is released via hemolysis in serum (142). Hemopexin also sequesters free heme when it is released into plasma. In addition, albumin is the most prominant plasma protein (about 60% of total protein found in serum) and also functions as a serum heme carrier, albeit with a much lower affinity (135).

Due to the insolubility of iron and in the context of bacteriology, bacteria often absorb heme-iron more easily than inorganic iron (128). This uptake of heme-iron can be achieved through several sources. *Yersinia enterocolitica* for example, has a HemR receptor that is capable of recognizing both heme and hemoglobin while HmbR in

Neisseria meningitidis is more specific for hemoglobin usage (142). However, a distinction between heme and hemoglobin utilization is unclear because hemoglobin often rapidly oxidizes to methemoglobin as soon as red blood cells are lysed (142). Moreover, methemoglobin has a lower affinity for heme due to its rapid dissociation into αβ dimers, therefore, the release of free heme becomes more likely (142). Furthermore, some bacteria do not possess the biochemical machinery to synthesize heme and therefore require large amounts of heme for growth and such bacteria can include species of *Haemophilus*, *Porphyromonas*, *Prevotella*, *Bacterioides*, some mycobacteria, *Legionella*, and *Bartonella* (128).

1.4.2. Heme Transport in Gram-Negative Bacteria

Heme-iron acquisition in Gram-negative bacteria is achieved via a diverse and intricate set of mechanisms. The first mechanism of heme transport is achieved by direct heme transfer using outer membrane receptor proteins to capture free heme or heme bound to hemoproteins (hemoglobin, hemopexin, or hemoglobin-haptoglobin complex). Briefly, bacteria secrete hemolysins that lyse red blood cells causing the release of hemoglobin from which heme will then be released (via an unknown mechanism) from hemoglobin. The free heme will be captured by outer membrane receptors and the heme will be shuttled into the periplasm to be loaded onto a periplasmic heme binding protein (135). The process of heme transfer from the outer membrane receptor to the periplasmic binding protein is an energy-dependent process that requires the TonB-ExbB-ExbD inner membrane complex. The process of heme transport into the periplasm is analogous to how siderophore-iron complexes are transported (see section 1.3.3 Siderophore-iron transport) but the siderophore-iron ligand is replaced with heme in this

scenario (71). Once heme is bound to a periplasmic binding protein, the protein will dock onto an inner membrane ABC transporter so that the heme can be actively transported into the cytoplasm (135). Such direct heme transfer mechanisms have been well characterized in *Yersinia enterocolitica* (via the *hemR-hemSTUV* system) (126), *Yersinia pestis* (via the *hmuRSTUV* system) (63), *Shigella dysenteriae* (via the *shuASTUV* system) (18, 45), and *Pseudomonas aeruginosa* (via the *phuRSTUVW* system) (72, 136). Interestingly, only recently was the first heme transport system characterized in *E. coli* K12 by Létoffé *et al.* (2006). Since *E. coli* does not encode its own outer membrane heme receptor, this transport system utilizes a dipeptide transporter (periplasmic and inner membrane components) that is coupled to a foreign outer membrane heme/hemophore receptor HasR, which originates from *Serratia marcesens* (more details below). Specifically, this transport system in *E. coli* is set up with a dipeptide inner membrane ATP-binding cassette transporter (DppBCDF) along with two interchangeable periplasmic peptide/heme binding proteins which are either DppA (L-alanyl-γ-D-glutamyl-meso-diaminopimelate binding protein) or MppA (dipeptide binding protein) that can bind to heme for active transport into the cytoplasm (76). Therefore, the dual role of heme and peptide transport for this unique system implies that peptide and heme ligands can serve as antagonists for each other in the periplasm.

Another heme transport mechanism in Gram-negative bacteria involves the use of hemophores, secreted proteins that scavenge free heme or extract heme from hemoproteins in the extracellular milieu and return to the cell via recognition of a specific outer membrane. Heme is then transferred from the outer membrane receptor to the inner membrane ABC transporter in a TonB-dependent process as described earlier

(135). The best characterized hemophore system is the *hasADEB* system from *S. marcescens* although this system is also found in *P. aeruginosa*, *P. fluorescens*, *Y. pestis*, and *Y. enterocolitica* (23, 135). In this system, HasA is the hemophore that captures extracellular heme and donate it to the outer membrane receptor HasR (19). Another yet less understood hemophore system is the *hxuCBA* system from *Haemophilus influenzae.* In contrast to HasA, the hemophore HxuA is capable of scavenging heme from heme-hemopexin complexes only rather than free heme or that bound to other hemoproteins (93). HxuB serves to release the hemophore HxuA from the cell and HxuC is predicted to be the outer membrane receptor for HxuA or to serve as a low affinity binder to free heme or other hemoproteins (93).

A third characterized method of heme uptake in Gram-negative bacteria involves the use of bipartite receptors such as the HpuAB system of *Neisseria meningitidis* (80). This system involves HpuA and HpuB as outer membrane receptors to bind heme, hemoglobin, or hemoglobin-haptoglobin complexes and heme is shuttled into the cell via energy transduction using the TonB system (78, 79).

1.4.3. Heme Transport in Gram-Positive Bacteria

Heme transport is just as important to Gram-positive bacteria as it is to Gram-negative bacteria. A few example of Gram-positive heme transport systems to be described here employ the use of multiple cell wall anchored proteins that relay heme across the cell wall towards a dedicated ABC transporter. *B. anthracis* encodes an iron-regulated surface determinant (*isd*) operon that consists of genes *isdC*, *isdX1*, *isdX2*, *isdE2*, *isdE1*, *isdF*, *srtB*, *isdG*. This operon contains three transcriptional units, where

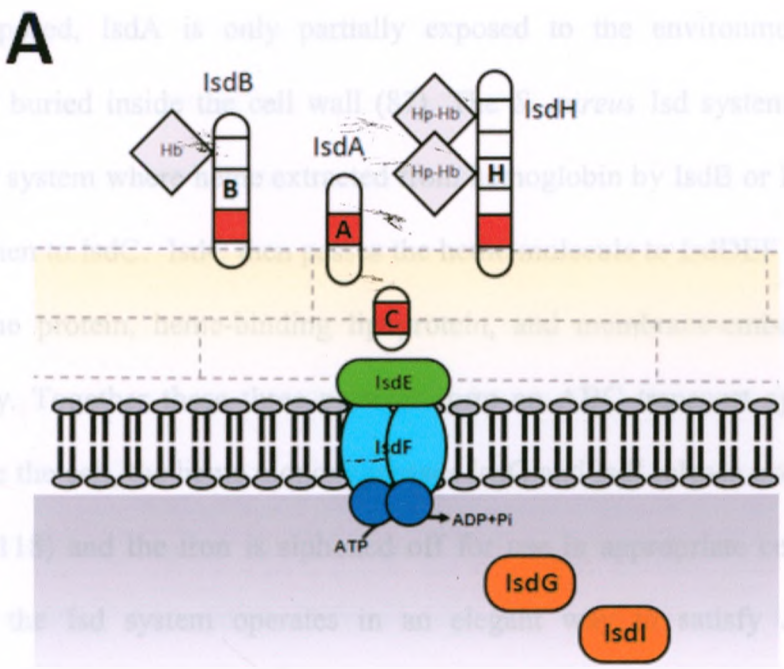
each unit is flanked by a Fur-box (86, 117). Specifically, the gene *isdC* encodes a heme-binding protein, *isdX1* and *isdX2* each encode two hemophores, and the remaining genes *isdE*, *isdE2*, and *isdF* encode an ABC-type iron transporter. An additional part of the operon is the *srtB* gene that encodes a sortase that anchors IsdC to the cell wall via an NPKTG motif. Lastly, *isdG* encodes a monooxygenase protein that is predicted to degrade the heme molecule once it is inside the cell (86). Under iron-limiting conditions, the *isd* genes are expressed and *B. anthracis* secretes two NEAT (NEAr-iron Transporter)-domain containing hemophores (IsdX1 and IsdX2) into the environment to scavenge heme from hemoglobin (46, 85). Proteins containing the NEAT domain are involved in heme acquisition (86, 87). Unlike *S. aureus,* the heme-binding proteins are all tethered to the cell wall (more details to follow), the secreted proteins IsdX1 and IsdX2 of *B. anthracis* return to the cell after binding heme and interact with cell wall anchored IsdC (via the NEAT domains of each protein). Here they unload the heme and initiate heme import into the cell (46, 85). Once the heme molecule is inside the cell (after being relayed from IsdC to IsdEF), IsdG will liberate the iron from the heme for *B. anthracis* to use (46). Genes have been found in *Listeria monocytogenes* genomes that share homology with proteins of the Isd system (16). The Isd system for *S. aureus* will be covered more extensively in the following section.

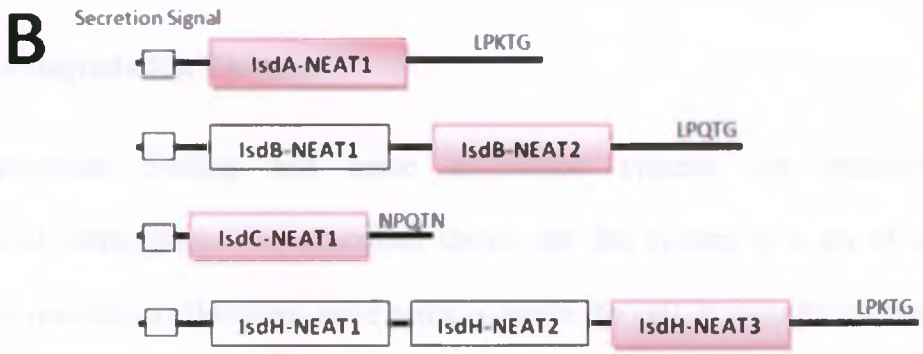
Streptococcus pyogenes has a heme acquisition system similar to the Isd system. It consist of two streptococcal surface heme-binding proteins, Shr and Shp as well as a heme-specific ATP-binding cassette transporter HtsABC (not to be confused with staphyloferrin A transporter of *S. aureus*) (148). In brief, Shr binds heme and passes it to Shp, which then relays the heme molecule to heme-binding receptor lipoprotein HtsA for active transport across the cell envelope (135, 148).

1.4.4. Heme Transport in *S. aureus* - The Isd System

The majority of iron found in the host is bound by heme proteins and heme has been shown to be the preferred source of iron (119). One well charcterized heme uptake system in *S. aureus* and other microorganisms (*B. anthracis, L. monocytogenes*) is the Isd system. A schematic representation of the Isd system is presented in Figure 4A. It was first identified by Taylor and Heinrichs (2002) and Mazmanian *et al.* (2003), where factors responsible for binding hemoglobin and transport of heme-iron into the cytoplasm were found (87, 132). The *isd* operon of *S. aureus* contains nine genes transcribed by five transcriptional units (*isdA, isdB, isdCDEFsrtBisdG, isdH,* and *isdI*) (53, 87, 132). All five transcription units are regulated by Fur in response to iron limitation (36, 87). Note that all of the *S. aureus* Isd proteins (IsdA, IsdB, IsdC, and IsdH) contain one to three copies of a NEAT domain (53). A schematic representation of the NEAT domains is presented in Figure 4B. In addition to the NEAT domain for heme binding, each Isd protein also contains a secretion signal and a sortase anchoring signal (53, 54). IsdA, IsdB, and IsdH are receptor proteins for heme that are covalently anchored to the cell wall by sortase A through recognition of a conserved LPXTG motif at the C-terminal end. In contrast, IsdC is covalently anchored to the cell wall by sortase B through recognition of a conserved NPQTN motif at the C-terminus (86, 87). IsdA is known to bind hemoglobin, transferrin as well as the extracelluar matrix proteins fibronectin and fibrinogen (132). On the other hand, IsdH is able to bind hemoglobin, haptoglobin, and a hemoglobin-haptoglobin complex (43). The binding of IsdH to hemoglobin-haptoglobin complexes is significant because upon erythrocyte lysis, large

Figure 4. Schematic Representation of the Isd (iron-regulated surface determinant) System and the NEAT Domains. A) The Isd system consists of nine cell surface (cell wall anchored and membrane-embedded) proteins involved in heme uptake. IsdA, IsdB, IsdC, IsdH are cell-wall anchored surface receptors. These four proteins are covalently anchored to the peptidoglycan cross-bridges through the recognition of a C-terminal Sortase anchoring signal. IsdC is anchored by sortase B and the remaining three proteins are anchored by sortase A. IsdB also binds onto hemoglobin through one of the NEAT domains such that heme can be extracted for passage to IsdC. IsdH also possesses NEAT domains that bind to haptoglobin and haptoglobin-hemoglobin complexes for heme extraction. IsdH then transfers the extracted heme to IsdC. IsdEF are the binding protein and permease components of an ABC transporter, respectively. Note that the ATPase component of the Isd system is not yet clearly defined. Also, IsdG and IsdI are cytoplasmic heme-degrading enzymes. Fig. 4A courtesy of Dr. Heinrichs. B) Schematic representation of the NEAT domains found in IsdA, IsdB, IsdC, and IsdH. The shaded NEAT domains are responsible for heme-binding. IsdA and IsdC contain one NEAT domain, IsdB contains two NEAT domains, and IsdH contains three NEAT domains. The heme binding NEAT domains are shown in red. Fig 4B is modified from (53).


A
IsdB
Hb
B
IsdA
A
Hp-Hb
Hp-Hb
IsdH
H
C
IsdE
IsdF
ADP+Pi
ATP
IsdG
IsdI


B
Secretion Signal
IsdA-NEAT1
LPKTG
IsdB-NEAT1
IsdB-NEAT2
LPQTG
IsdC-NEAT1
NPQTN
IsdH-NEAT1
IsdH-NEAT2
IsdH-NEAT3
LPKTG

amounts of hemoglobin become sequestered by serum haptoglobin and therefore the ability to capture this protein complex broadens the availability of iron in serum (36). IsdB is an additional hemoglobin binding protein (53, 138). In contrast to IsdB, which is surface exposed, IsdA is only partially exposed to the environment and IsdC is completely buried inside the cell wall (87). The *S. aureus* Isd system is essentially a heme relay system where heme extracted from hemoglobin by IsdB or IsdH is passed to IsdA and then to IsdC. IsdC then passes the heme molecule to IsdDEF which constitute a membrane protein, heme-binding lipoprotein, and membrane-embedded permease, respectively. Together these three proteins form an ABC transport system (53, 134). Once inside the cell, the heme monoxygenases IsdG and IsdI release iron from the heme molecule (118) and the iron is siphoned off for use in appropriate cellular processes. Therefore, the Isd system operates in an elegant way to satisfy *S. aureus*'s iron requirements.

1.4.5. Heme Degradation Enzymes

Hemogoblin binding and heme acquisition systems are required for staphylococcal pathogenesis. As described above, the Isd system is a set of unique heme-uptake machinery. However, once heme is inside the cell, it must be processed in order to free the iron such that the metal can be used for numerous cellular purposes. Imported heme is degraded by two *S. aureus* cytoplasmic heme oxygenases (IsdG and IsdI), yielding a novel end product staphylobilin, carbon dioxide and free iron (108). In contrast, heme oxygenases from other bacteria such as *C. diphtheriae* degrade heme into biliverdin. Staphylobilin is a yellow chromophore, that differs from the blue-green

biliverdin chromophore produced by other bacteria. Moreover, the *S. aureus* heme oxygenases are structurally distinct from other characterized heme oxygenases (108). Although IsdG and IsdI are proteins with a high degree of similarity (78% amino acid similarity) perhaps redundant sharing functions, and despite both being transcriptionally regulated by Fur, they are differentially regulated (106, 116). The difference lies in that IsdG is robustly expressed in an iron-deplete environment even if heme is still present (via posttranscriptional regulation) whereas IsdI is expressed in an iron-deplete environment that is also devoid of heme (106). Perhaps, *S. aureus* produces two heme-degrading enzymes, as a way of maximizing heme-iron acquisition depending on the ecological niche and environmental conditions. Also, it is interesting that Reniere *et al.* (2007) showed that *S. aureus ΔisdGI* mutants still exhibit growth on heme as a sole iron source, suggesting that additional heme-degrading enzymes must be present. It is therefore tempting to speculate that possession of multiple heme-degrading enzymes permits *S. aureus* to avoid heme toxicity upon encounter with high levels of exogenous heme, such as during the establishment of blood-borne infections (107). It is also interesting to note that there is considerable conservation of the IsdG/IsdI enzyme family in *B. anthracis* and *L. monocytogenes* (106).

Recently, Létoffé *et al.* (2009) discovered two genes, *yfeX* and *efeB,* in *E. coli* that encode enzymes that are capable of extracting iron from heme without interrupting the structure of the tetrapyrrole ring. These enzymes belong to the family of dye-decolorizing peroxidases (Dyp-peroxidases) (77). YfeX is a cytoplasmic protein, whereas EfeB has a twin arginine signal peptide that allows it to be exported to the periplasm by the twin arginine translocation (TAT) system, known to transport folded

proteins across the cytoplasmic membrane (77, 129). Also, the gene *efeB* belongs to an iron-regulated *efeUOB* operon that is involved in the uptake of Fe^{2+} under acidic conditions (21). Apparently, YfeX and EfeB are highly conserved and widespread in Gram-positive and Gram-negative bacteria. In fact, an operon found in *S. aureus* strain Newman (*NWMN_0336, NWMN_0337, NWMN_0338*) contains genes that are homologous to a receptor lipoprotein, dye-decolorizing peroxidase, and iron permease, respectively. Further studies will be required to confirm if the products of this operon in *S. aureus* contribute to heme-iron acquisition.

1.4.6. The Role of Heme Acquisition in Staphylococcal Infections

The Isd system has been shown to be important for *S. aureus* growth using heme as the sole iron source. The Isd system is also known to contribute towards virulence. Various genetic inactivations such as that of genes *isdA*, *isdG* or *isdI* lead to a phenotype of impaired growth in iron-restricted media even when heme was supplied as the sole iron source (54, 106). Furthermore, mutations of *isdA*, *isdB*, *isdC*, *isdG* or *isdI* in *S. aureus* Newman resulted in reduced bacterial load in the heart, kidney, liver, and spleen abscess models (106, 138). Specifically, inactivation of IsdA lead to a decreased bacterial load in the heart and liver of mice, whereas IsdB inactivation only impaired growth in the heart alone (99, 106). Also, IsdG and IsdI each contribute to virulence in heart tissues, but IsdG is important in kidney infections (106). With the vast number of virulence experiments involving *isd* mutants of *S. aureus*, it is certain that the Isd system plays a pivotal role in pathogenic infections.

Besides heme scavenging, certain Isd proteins can perform alternative functions that can aid in infection establishment. Research has demonstrated that IsdA can interact with many host components such as proteins found in serum and the extracellular matrix. Therefore, IsdA is a likely candidate that can promote nasal colonization (31). In addition, increased expression of IsdA changes the hydrophobicity of the *S. aureus* cell surface, which leads to increased resistance of *S. aureus* to host innate mechanisms (28, 29). Therefore, IsdA appears to be a versatile protein that suits *S. aureus* at different stages of colonization and infection.

1.4.7. Heme Regulation in *S. aureus* - the HssRS and HrtAB Systems

As much as heme is the preferred iron source, heme also becomes toxic at high concentrations (139). Torres *et al.* (2007) discovered a system called the heme sensor system (HssRS) that responds to heme exposure and activates the expression of a heme-regulated transporter or effluxer (HrtAB) (139). The HrtAB transport system is composed of the ATP-binding protein HrtA and the permease HrtB (139). HrtAB allows *S. aureus* to avoid heme toxicity by effluxing excess heme in the cytoplasm to the extracellular environment before toxic levels of heme accumulate within the cell. Interestingly, *S. aureus* exposure to exogenous heme under iron-rich conditions results in 45-fold up-regulation of *hrtAB* (139). As mentioned, the expression of HrtAB is regulated by HssRS, which is a two component system comprised of HssR (response regulator) and HssS (histidine kinase) (139). Heme in the environment activates the membrane-localized histidine kinase HssS leading to autophosphorylation of a specific histidine residue. HssS then phosphorylates the cytoplasmic response regulator HssR at

an aspartate residue. The phorphorylated HssR will bind to the direct repeats within the *hrtAB* promoter to induce expression of the HrtAB effluxer system (124, 125). Note that hemoglobin and blood exposure alone can activate *hrtAB* expression but iron cannot. Although HssRS-dependent HrtAB activation is achieved through recognition of metal-bound porphyrins, even metal-induced cellular stress does not activate HrtAB (139). It has been shown that *S. aureus ΔhrtA* or *ΔhssR* mutants have a phenotype of increased liver virulence as well as increased secretion of virulence factors (139). The enhanced virulence is due to overexpression of HrtB and in the absence of HrtA, excessive permease insertions into the cell membrane occur. This leads to increased pore formation and an increase in secretion of virulence factors (4). Fortunately for an *S. aureus ΔhrtA* mutant, the damaged and permease-laden membrane also triggers *S. aureus* to increase the expression and secretion of immunomodulatory factors (4). Similarly, the pathogens *B. anthracis* and *C. diphtheriae* also resist heme toxicity by using the HssRS and HrtAB systems (15, 124). Moreover, HssRS and HrtAB are conserved across Gram-positive pathogens such as *S. epidermidis, L. monocytogenes, and Enterococcus faecalis* (139). However, the *hss* and *hrt* genes cannot be found in the genomes of non-pathogenic commensals such as *B. subtilis* (123). Overall, the coordinated activity of HssRS and HrtAB allow *S. aureus* to sense when heme levels approach toxicity in a certain environment and to initiate a response that would prevent itself from succumbing to the very nutrient that it requires.

1.5. Nutritional Regulation

1.5.1. CodY-Global Pleiotropic Regulator

CodY is a global transcriptional regulator that was first identified in *B. subtilis* as a nutritional repressor of the dipeptide permease operon (dpp) (120). CodY is highly conserved in low G+C Gram-positive organisms that regulate expression of genes involved in metabolism, sporulation, and virulence in response to nutrient limitation (12, 22, 56, 60, 83, 84, 101, 115, 122). CodY also controls more than a hundred genes that are typically repressed during rapid growth phases and are induced when cells are in poor growth conditions (91, 122). This includes genes that encode for extracellular degradative enzymes, transport systems, catabolic pathways, branched-chain amino acid synthesis, antibiotic synthesis, peptide uptake systems, extracellular proteases, the Agr system, stress response, and iron uptake (8, 60, 91, 115, 122). In *B. subtilis*, CodY is a GTP-binding protein where GTP activates the repressor function of CodY through interaction with GTP-binding motifs (122). Although CodY regulates many genes, many of these genes may not necessarily be direct targets of CodY, for CodY binds only in proximity to the regulatory regions of about 70 operons (122). However, the genes for the biosynthesis of branched-chain amino acid (BCAAs, e.g. isoleucine, leucine, valine, and threonine) are among the direct targets of CodY control and BCAAs serve as effector molecules as they activate the repressor function of CodY (122). The reason why CodY and BCAAs are linked may be due to the fact that BCAAs are used preferentially over other amino acids or that there is a greater availability of BCAAs to control gene expression for cells in stationary phase (91). Therefore, during rapid growth in a rich medium, cells will use presynthesized amino acids and use CodY to repress

relevant biosynthetic pathways. However, when the supplies of amino acids decrease, CodY will undergo derepression of BCAA biosynthesis genes such that the cell can produce more BCAAs. Additionally, as in *S. aureus* and other Gram-positive organisms, CodY is known to regulate the expression of many transport systems including those for amino acids and oligopeptides, among others (83). One interesting note is that dipeptides containing BCAAs are potently more effective at activating CodY as in the case of *L. lactis* (56). Notably, Létoffé *et al.* (2006) found that a dipeptide permease was involved in heme uptake in *E. coli* thus prompting the possibility of similar permeases in *S. aureus* as well as a potential link between BCAAs, dipeptides, and heme.

1.6. Research Objectives

The goal of this research was to identify other heme uptake systems in *S. aureus* aside from the already well-characterized Isd system. The *first objective* was to identify a potential non-Isd heme acquisition system through screening of a random transposon mutagenesis library using a Tn*917* transposon in the *S. aureus* strain RN6390 *Δisd* background. The screening was achieved after identifying mutants that failed to grow using heme as the sole iron source. The rationale for the possibility of an alternative heme uptake system in *S. aureus* arises from the observation that a mutant strain containing deletions of *isdB*, *isdA*, *isdC*, *isdD*, *isdE*, *isdF* and *isdH* (such a mutant was dubbed Δ*isd*) was still capable of acquiring and utilizing heme as a sole iron source for growth. The *second objective* of this research was to identify and characterize the potential transposon mutants that are debilitated in using heme as the sole iron source through growth analysis, nutrient plate bioassays, and various other experiments that assessed the physiology of such *S. aureus* transposon mutants. From screening the transposon mutants, several grew poorer than *S. aureus* RN6390 Δ*isd* in the presence of heme as a sole iron source. In fact, one mutant in particular (Tn #13) showed the most drastic phenotype, failure to grow on a heme-containing medium even after 24 hours of incubation. Therefore, the *third objective* was to further characterize transposon Tn#13 in greater detail.

Chapter 2 - Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Bacterial strains used in this study are described in Table 2. Bacteria were cultured at 37°C unless otherwise indicated. Antibiotics were used at the following concentrations: ampicillin (100 μg/ml) and erythromycin (300 μg/ml) for *E. coli* selection; chloramphenicol (5 μg/ml), tetracycline (4 μg/ml), kanamycin (50 μg/ml), neomycin (50 μg/ml) and erythromycin (3 μg/ml) for *S. aureus* selection. For genetic manipulations, bacteria were grown in Luria-Bertani broth (Difco) for *E. coli* or tryptic soy broth (Difco) for *S. aureus*. Iron restricted media (TMS) was prepared according to (114) and contained 150μM 2,2-dipyridyl (Dip) (Sigma). Briefly, 1 litre of TMS was made with 40 ml 25x Tris salts (125g NaCl, 92.5 g KCl, 27.5 gNH_4Cl, 3.55 g $NaSO_4$, 6.80 g KH_2PO_4), 12.1 g Tris base, 16.6 g Sodium Succinate, 10 g Casamino acids (Difco). The TMS was then pH to 7.4 before bringing it to 1 litre with ddH_20. Postautoclaving, the following vitamins and animo acids (filter sterilized) were added back to the TMS (2 ml of 10 mg/ml Tryptophan, 2 ml of 11 mg/ml Cystein, 1 ml of 16.9 mg/ml Thiamin, 1 ml of 1.23 mg/ml Nicotinic acid, 1 ml of 0.5 mg/ml Panthotenic acid, 1ml of 0.01 mg/ml Biotin, 1 ml of 11.1mg/ml $CaCl_2 \cdot 2H_20$, 1 ml of 95.3mg/ml $MgCl_2 \cdot 6H_20$). All solutions and media were made with water purified through a Milli-Q water purification system (Millipore). Solid media were obtained by addition of 1.5% w/v Bactoagar (Difco).

2.2. DNA Methodology

2.2.1. Plasmid Isolation from *E. coli*

All plasmids used in this study are listed in Table 3. Plasmid DNA were prepared from *E. coli* using E.Z.N.A Miniprep Kit (Omega Bio-tek) according to the

TABLE 2. Bacterial strains used in this study

Bacterial strains	Description[a]	Source or reference
E. coli		
DH5α	F- ϕ80 Δ(*lacZ*)M15 *recA1 endA1 gyrA96 thi-1 hsdR17*(r_k^- m_k^+) *supE44 relA1 fhuA2* Δ(*argF-lacZ*)U169 *phoA glnV44*	Promega
S. aureus		
RN4220	r_k^- m_k^+; accepts foreign DNA	(70)
RN6390	Prophage-cured laboratory strain	(98)
Newman	Wildtype clinical isolate	(44)
H1262	Newman Δ*htsABC*::Tet; Tet^R	(10)
H1400	RN6390 Δ*isd*::Km; Km^R	This study
H1532	Newman Δ*isd*::Km Δ*isdH*::Spec; Km^R $Spec^R$	This study
H2245	RN6390 *brnQ3*::Tn*917*; Em^R	This study
H2235	RN6390 Δ*isd*::Km *brnQ3*::Tn*917*; Km^R Em^R	This study
H2240	RN6390 Δ*isd*::Km *brnQ3*::Tn*917*; Km^R Em^R	This study
H2248	Newman *brnQ3*::Tn*917*; Em^R	This study
H2249	Newman Δ*isd*::Km Δ*isdH*::Spec *brnQ3*::Tn*917*; Km^R $Spec^R$ Em^R	This study
H2274	Newman Δ*isd*::Km Δ*isdH*::Spec *brnQ3*::Tn*917* with pCC10; Km^R $Spec^R$ Em^R Cm^R	This study
H2276	Newman Δ*isd*::Km Δ*isdH*::Spec *brnQ3*::Tn*917* with pLI50; Km^R $Spec^R$ Em^R Cm^R	This study
H2277	Newman *brnQ3*::Tn*917* with pLI50; Em^R Cm^R	This study
H2278	Newman Δ*isd*::Km Δ*isdH*::Spec with pLI50, Km^R $Spec^R$ Cm^R	This study
H2309	Newman WT with pLI50; Cm^R	This study
H2323	RN6390 Δ*brnQ3*::Tet; Tet^R	This study
H2324	Newman Δ*brnQ3*::Tet;Tet^R	This study
H2325	Newman Δ*isd*::Km Δ*isdH*::Spec Δ*brnQ3*::Tet; Tet^R	This study
H2353	Newman Δ*isd*::Km Δ*isdH*::Spec, Tn*917* mutant with pALC2073	This study
H2354	Newman Δ*isd*::Km Δ*isdH*::Spec, Tn*917* mutant with pCC12	This study
H1262	Newman *ΔhtsABC::*Tet; Tet^R	(10)
H2350	Newman Δ*isd*::Km Δ*isdH*::Spec *brnQ3*::Tn*917ΔhtsABC::*Tet; Km^R $Spec^R$ Em^R Tet^R	This study
H2351	Newman Δ*isd*::Km Δ*isdH*::Spec *ΔhtsABC::*Tet; Km^R $Spec^R$ Tet^R	This study

[a]Abbreviations: Ap^R, Cm^R, Em^R, Km^R, Lc^R, $Spec^R$, and Tet^R, resistance to ampicillin, chloramphenicol, erythromycin, kanamycin, lincomycin, spectinomycin, and tetracycline, respectively.

Table 3. Plasmids used in this study

Plasmid	Description[a]	Source or reference
pLTV1	Temperature-sensitive vector containing Tn*917*-LTV1; Em^r Ap^r Cm^r	(20)
pLI50	*E.coli/ S. aureus* shuttle vector; Ap^R Cm^R	(73)
pALC2073	*E. coli/S. aureus* shuttle vector; Ap^R Cm^R	(7)
pCC10	pLI50 derivative expressing *S. aureus brnQ3*; Cm^R	This study
pCC12	pALC2073 derivative expressing *S. aureus isdEF*; Cm^R	This study
pDG1513	pMTL22 derivative that carries a tetracycline resistance cassette; Ap^R Tet^R	(57)
pDG780	pBluescriptKS+ derivative that carries a kanamycin resistance cassette; Ap^R Km^R	(57)
pMAD	Temperature-sensitive Gram-positive bacteria suicide vector; Em^R Ap^R	(3)
pSHU15	pMAD derivative containing Δ*brnQ3*::Tet; Em^R Tet^R	This study

[a]Abbreviations: Ap^R, Cm^R, Em^R, Km^R, Lc^R, $Spec^R$, and Tet^R, resistance to ampicillin, chloramphenicol, erythromycin, kanamycin, lincomycin, spectinomycin, and tetracycline, respectively.

manufacturer. To summarize the procedure, approximately 2.5 ml of stationary phase culture of *E. coli* was harvested by centrifugation and resuspended in 250 µl of solution I/RNase A solution (50 mM Tris, pH 8.0, 20 mM EDTA, 100 µg/ml RNase A). Cell lysis was accomplished by adding 250 µl of solution II (200 mM NaOH, 1% [w/v] SDS) and gently inverting the tube. To neutralize the solution, 250 µl of solution III (guanidine hydrochloride with acetic acid) was added to the lysate, mixed by inversion, and centrifuged for 10 minutes at 15,000 x g to pellet insoluble material. The resulting supernatant was applied to the E.Z.N.A spin column and centrifuged at 13, 000 x g for 1 minute. Five hundred microlitres of Buffer HB was used to wash the DNA miniprep column. Finally, the column was subsequently washed with 700 µl of DNA wash buffer before centrifuging at 13, 000 x g for 1 minute to remove excess ethanol. Plasmid DNA was then eluted from the column into a fresh microfuge tube by addition of 50 µl of ddH20 followed by centrifugation (13, 000 x g for 1 minute).

2.2.2. Plasmid Isolation from *S. aureus*

The same procedure followed as described for *E. coli* was used for *S. aureus* plasmid DNA extraction but with the following modifications. Cells were resuspended in 250 µl of solution I/RNase A solution with 50 µg/ml of lysostaphin (Sigma) added to it. The cell suspension was then incubated at 37°C for 20 minutes prior to addition of solution II.

2.2.3. Isolation of Chromosomal DNA from *S. aureus*

Chromosomal DNA was obtained from *S. aureus* by pelleting 500 µl of overnight stationary phase cells grown in TSB culture. 200 µl of STE (75 mM NaCl, 25 mM EDTA, 20 mM Tris pH 7.5) was added to the cells along with 50 µg/ml of

lysostaphin dissolved in 20 µl of STE. The cell resuspension was incubated at 37°C for 1 hour. After, 20 µl of 10% sodium dodecyl sulfate and 20 µl of proteinase K (New England Biolab) were added to the cell suspension and incubated at 55 °C overnight. The next day, 80 µl of 5M NaCl was added and mixed by inversion. 320 µl of 25:24:1 phenol/ chloroform/ isoamyl alcohol (Invitrogen) was added and was allowed to sit on benchtop for 30 minutes. The aqueous layer was removed after the mixture was spun at 12,000 rpm for 10 minutes. An addition of 300 µl of 24:1 of chloroform/ isoamyl alcohol was added and then spun at 12,000 rpm for 10 minutes. The second aqueous layer, was removed and 600 µl of isopropanol was added to the aqueous layer, which was then stored for 1 hour at -20 °C. After the 1 hour, the mixture was spun at 12,000 rpm for 5 minutes. The resulting pellet was washed with 700 µl of 70% ethanol and centrifuged again. The ethanol was carefully pipetted out and allowed to dry in the speed-vacuum (Eppendorf) for 10 minutes or until the ethanol was fully evaporated. Lastly, the pellet was resuspended in 100 µl of ddH_20.

2.2.4. Restriction Enzyme Digestion

Restriction enzymes were purchased from New England Biolabs or Roche Diagnostics. Reactions were typically carried out in 30-40 µl over a 2 hour incubation at the appropriate temperature (generally 37°C). Digested DNA was subsequently cleaned using QIAquick PCR purification kit (QIAgen) as described by the manufacturer.

2.2.5. DNA Ligation

DNA fragments were ligated in a 20 µl reaction volume using (110) protocol. Reactions were carried out using the T4 DNA ligase (Roche Diagnostics) in accordance with the manufacturer's recommendations.

2.2.6. Recombinant DNA Methodology

Routine manipulation of recombinant DNA was performed as described (110). Plasmid DNA was isolated from bacteria using QIAprep mini-spin kits (Qiagen) as directed. For plasmid isolation from *S. aureus*, cells were incubated for 30 minutes at 37°C in P1 buffer (Qiagen) containing 50 mg ml^{-1} lysostaphin (Sigma Aldrich) prior to addition of lysis buffer P2 (Qiagen). Restriction endonucleases were purchased from New England Biolabs. T4 DNA ligase, and *Pwo* polymerase were purchased from Roche Diagnostics, and oligonucleotides were purchased from Integrated DNA Technologies. Antibiotics were purchased from Bioshop Canada Inc.

2.2.7. Agarose Gel Electrophoresis

Agarose gel electrophoresis was used for the separation and analysis of DNA fragments. Agarose gels (0.8% w/v) were prepared using 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) to which 1:10000 stock dilution of SYBR Green (Invitrogen) was added. DNA samples to be separated in the gel were mixed with loading dye (5% glycerol, 0.04% bromophenol blue, 0.04% xylene cyanol, 10 mM EDTA, pH 7.5) prior to being loaded in the gel. Electrophoresis was typically carried out at 110 V for 20 minutes. The 1kb-Plus ladder (Invitrogen) was used as a standard reference marker for estimation of the size of the DNA fragments. Following electrophoresis, DNA fragments were visualized using a ChemiDoc XRS imaging unit and Quantity One software (Bio-rad).

2.2.8. Isolation of DNA Fragments from Agarose Gels

Desired DNA fragments were visualized under longwave UV light (365 nm) and excised from agarose gels following electrophoresis. DNA was isolated using QIAquick PCR purification kit (Qiagen) as described by the manufacturer.

2.2.9. Polymerase Chain Reaction (PCR)

PCR reactions were carried out in 50 µl reactions containing: DNA template, 1x PCR buffer, 200 µM dNTP mix (Roche Diagnostics), 12 pMols of forward and reverse primers, and 0.5 units of either PwoI DNA polymerase (Roche Diagnostics) or Taq DNA polymerase (GenScript). PCRs were performed using the GeneAmp PCR system (Perkin-Elmer), DNA engine Gradient Cycler (Bio-rad) or the MJ Mini Personal Thermal Cycler (Bio-rad). Oligonucleotides primers were obtained from Integrated DNA technologies.

2.2.10. DNA Sequencing

DNA sequencing was performed at the DNA Sequencing Facility at the Robarts Research Institute (London, Ontario, Canada) or the York Sequencing Facility (Toronto, Ontario, Canada), with sequencing reactions prepared according to their guidelines.

2.2.11. Computer Analysis

DNA sequence analysis, sequence alignments, and oligonucleotide primer design were carried out using the Vector NTI Suite 7 software package (Informax, Inc.,

Bethesda, Maryland). BLAST searches were performed using tools available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/).

2.3. Transformation and Transduction Methodologies

2.3.1. Preparation of Transformation Competent *E. coli*

E. coli DH5α $CaCl_2$ competent cells were prepared as follows. An overnight, stationary phase culture of *E. coli* DH5α was diluted 1:100 into 400 ml of fresh LB and grown to an OD_{600} of approximately 0.5 and placed on ice for 20 minutes. The cells were then harvested by centrifugation and resuspended in 100 ml of ice-cold 100 mM $CaCl_2$ plus 15% glycerol incubated on ice for 30 minutes. Cells were again collected by centrifugation and resuspended in 4 ml of 100 mM $CaCl_2$ with 15% glycerol, and stored as 100 µl aliquots at -80 °C.

2.3.2. Transformation of $CaCl_2$ Competent *E. coli*

To transform $CaCl_2$ competent cells *E. coli* DH5α, purified plasmid DNA or ligation mixtures were added to a 100 µl aliquot of competent cells and kept on ice for 30 minutes after which cells were subjected to a heat shock treatment at 42°C for 2 minutes immediately followed by a 1 minute incubation on ice. An 900 µl aliquot of LB broth was added to the tube, mixed, and the cells were allowed to recover for 1 hour at 37°C before being plated on selective media and incubated overnight.

2.3.3. Preparation of transformation Competent *S. aureus*

Strains of *S. aureus* were made competent for transformation via electroporation as follows. An overnight culture of *S. aureus* was diluted 1:100 into 250 ml of fresh TSB and grown to an OD_{600} of approximately 0.3 at which point cells were put in ice for 10 minutes. Cells were then harvested by 7000 rpm centrifugation and resuspended in 25 ml of ice cold 0.5 M sucrose. The cells were centrifuged a second time but resuspended in 3.13 ml of 0.5 M sucrose. The cells were then allowed to sit on ice for 20 minutes before centrifuging and were resuspended in 2.5 ml of 0.5 M sucrose. After the final wash, the cells were stored in 60 µl aliquotes at -80 °C.

2.3.4. Transformation of Electrocompetent *S. aureus*

Electrocompetent *S. aureus* were transformed using purified plasmid DNA (typically 5 µl from a E.Z.N.Z miniprep). DNA was added to a tube of competent cells and allowed to incubate on ice for 30 minutes before being transferred to an ice cold electroporation cuvette (2mm, Bio-rad) for electroporation. Electroporation was performed using a Bio-Rad Gene Pulser II with settings at 2.5 KV, 200 mA, and 25 Ω. 900 µl of TSB was immediately added to pulsed cells, which were then allowed to recover for a minimum of 1 hour at 37 °C or 30 °C (depending on plasmid) before being plated onto appropriate selective media.

2.3.5. *S. aureus* Phage Lysate Preparation

UV induction was used to obtain bacteriophage 80α lysates from RN2564. The bacteriophage lysate were filter sterilize through a 0.45-µm-pore size filters (Millipore).

For routine preparation of infective lysates, 500 µl of an overnight culture of *S. aureus* was diluted 1:1 in fresh TSB supplemented with 2.5 mM $CaCl_2$ and incubated at 37 °C for 10 minutes at which point 300 µl of phage 80α were added. The mixture was allowed to incubate for 15 minutes at 37 °C before being added to 20 ml of TSB plus 2.5 mM $CaCl_2$ and incubated with slow shaking (90 rpm) at 37 °C until lysis of the culture was observed. Following lysis, cell debris were removed by centrifugation and phage lysate was sterilized by passage through a 0.2 µm filter (Millipore). All phage lysates were maintained at 4 °C.

2.3.6. Phage Transduction of *S. aureus*

Tranductions were carried out as previously described by Novick (95). Briefly, a 5 ml culture of the recipient strain was grown in TSB plus 2.5 mM $CaCl_2$ to an OD_{600} of 0.9, at which time 1 ml of culture was harvested by centrifugation and resuspended in 500 µl of fresh TSB plus 2.5 mM $CaCl_2$ and divided into 100 µl aliquots. Various dilutions of phage 80α lysates were added to each aliquot and incubated for 20 minutes at 37 °C, following which 10 µl of 1M sodium citrate was added to halt the phage infection. Cells were then plated on appropriate selective media for incubation.

2.4. Transposon Mutagenesis

2.4.1. Generation of a Tn*917*-LTV1 Random Transposon Library

A Tn*917*-LTV1 random transposon insertion library was constructed in *S. aureus* RN6390 *Δisd* by using the temperature-sensitive vector pLTV1 and the procedure by Watson, Antonio, and Foster (143).

2.4.2. Mutant Selection

A Tn*917*-pLTV1 random transposon library of *S. aureus* RN6390 *Δisd* (constructed as described above) was screened by patching colonies onto erythromycin- and lincomycin-containing iron-restricted TMS agar with no supplementation or supplemented with Ethylene ethylenediamine-di-o-hydroxyphenylacetic acid (EDDHA) (LGC Promochem) at 15 μM. TMS agar plates supplemented with EDDHA were additionally supplemented with or without 2 μM heme (Sigma). After overnight incubation at 37 °C, corresponding cell patches that were unable to grow on selection plates containing heme were restreaked onto the same TMS media containing with or without the addition of heme to confirm observed growth phenotypes.

2.5. Transposon Mutant Analysis

2.5.1. Bacterial Growth Curves

Bacteria were initially grown for approximately 10 hours in TMS liquid media then further grown for approximately 14 hours in the presence of 150 μM 2,2-dipyridyl (Sigma-Aldrich). Cells were washed 3x in saline and 10^7 CFU of each strain were inoculated into TMS liquid media containing 15 μM EDDHA with or without 2 μM Heme. Cultures were grown with continuous shaking at 37°C in a Bioscreen C machine (Growth Curves, USA) and bacterial growth was measured at 600 nm every 30 minutes for 48 hours.

2.5.2. Plate Bioassays

Bacteria are pre-grown as described above. After washing the cells 3x with saline, 1 x 10^7 CFU ml^{-1} of cells were seeded into molten TMS agar containing 7.5 μM

EDDHA for Newman strains and 10 μM EDDHA for RN6390 strains. Concentrations of haemin as indicated in the text were spotted onto sterile paper discs and were then placed onto the agar. Plates were incubated for 48 hours at 37 °C. Growth promotion was quantified by measuring the radius of growth around the disc after 24 hours and 48 hours.

2.6. Locus Deletion and Construction of Complementation Vectors

2.6.1. *NWMN_1040-1045 (isd)* Locus Deletion

The *NWMN_1040-1045* knockout allele consisted of a kanamycin resistance cassette, which was excised from pDG780 (57) with *Bam*HI and *Sal*I restriction enzymes, followed by flanking DNA sequences that were homologous to regions upstream of *NWMN_1040* and immediately downstream of *NWMN_1045*. This knockout allele was initially subcloned into pBC SK (+). Using *Kpn*I and *Xba*I, the *NWMN_1040-1045*::*Kan* knockout allele was excised and cloned into the temperature-sensitive *E. coli/S. aureus* shuttle vector pAUL-A (24), and then passaged through *S. aureus* RN4220 prior to being transduced into *S. aureus* RN6390. Recombinant RN6390 was cultured at 30 °C to mid-log phase before the incubation temperature was shifted to 42 °C. The bacteria were further incubated for 16 hours before being plated onto tryptic soy agar (TSA) containing kanamycin. Colonies were screened for sensitivity to erythromycin, which indicates a loss of the pAUL-A backbone DNA after integration of the knockout allele into *S. aureus* chromosome via double homologous recombination. The *NWMN_1040-1045* deletion was mobilized to other *S. aureus* backgrounds by transduction using 80α phage (95) as described above. Oligonucleotide primers used in this study are listed in Table 4.

TABLE 4. Oligonucleotides used in this study

Oligonucleotides	Description[a]
To sequence Tn*917* insertion site	CCATACGCAAGACCAATCACTCTCGGAC
Cloning of *brnQ3* from *S. aureus* into pLI50 (F'1320-pLI50)	ATTAGAGAATTCGAACTGTAGAAATGTCACGTA (forward, *Eco*RI)
Cloning of *brnQ3* from *S. aureus* into pLI50 (R'1320-pLI50)	TTGAGATCTAGATAGTATTAATGGCGAGACTCCT (reverse, *Xba*I)
Cloning of *brnQ3* from *S. aureus* (F'NWMN_1320 upstream arm-*Sal*I)	TTGTCGACGATTGAACGAGAACAACGAGATGC (forward, *Sal*I)
Cloning of *brnQ3* from *S. aureus* (R'NWMN_1320 upstream arm-*Pst*I)	TTCTGCAGGCGTAAACCCAATGACCCATG (reverse, *Pst*I)
Cloning of *brnQ3* from *S. aureus* (F'NWMN_1320 downstream arm-*Pst*I)	TTCTGCAGCAACGATATTAGGCTATCTAGTCGGC (forward, *Pst*I)
Cloning of *brnQ3* from *S. aureus* (R'NWMN_1320 downstream arm-*Sma*I)	TTCCCGGGAGCTCGTTGAGCTGCTATTTTCC (reverse, *Sma*I)
Cloning of *isdH* from *S. aureus* (F'isdH-SacI)	GAGCTCTTCAGGTGGTAGCACAA (forward, *Sac*I)
Cloning of *isdH* from *S. aureus* (R-isdH-*Sal*I)	GTCGACTAGCGTTGACAGGTGC (reverse, *Sal*I)
Cloning of *isdB* from *S. aureus* (F'isdB-*Kpn*I)	TTGAGAGGTACCTTGCTAAACAGCAAGAACAAG A (forward, *Kpn*I)
Cloning of *isdB* from *S. aureus* (R'isdB-*Sal*I)	TTGAGAGTCGACCCAACAACTGCTTCATCAAAAA (reverse, *Sal*I)
Cloning of *isdF* from *S. aureus* (F'isdF/srtB-*Xba*I)	TTGAGATCTAGAATTGTGCCACATGTGGTTAGAA (forward, *Xba*I)
Cloning of *isdF* from *S. aureus* (R'isdF/srtB-*Bam*HI)	TTGAGAGGATCCTGTGTTTTTCATAAAACGATTG (reverse, *Bam*HI)
Cloning of *isdE* and *isdF* from *S.aureus* into pALC2073 (F'isdE-*Kpn*I)	TTGAGAGGTACCACAGCAGAAAGAGAGGTAACC AA (forward, *Kpn*I)
Cloning of *isdE* and *isdF* from *S. aureus* into pALC2073 (R'isdF-*Sac*I)	TTGAGAGAGCTCCAGTTGTTGTACCGTTTTAGATT (reverse, *Sac*I)
Real-Time PCR	
NWMN_1320-512-For	CTGGAAAGGGCAATGAAGCAC
NWMN_1320-706-Rev	CTGCAATTAAACCAGCAGTCAAAG

[a] Restriction endonuclease recognition sequences are underlined.

2.6.2. *isdH* Gene Deletion

The *isdH* gene sequence was first amplified from the *S. aureus* RN6390 chromosome, using forward and reverse primers containing homologous isdH gene sequence which were flanked at the 5'ends with *Sac*I and *Sal*I restriction sites, respectively. The PCR product was digested with *Sac*I and *Sal*I and ligated into *SalI*/*Sac*I-digested pBCSK(+). Spectinomycin cassette from plasmid pDG1726 (57) was obtained through gel extraction (Qiagen) after being digested with *Pst*I and *Hind*III restriction enzymes. The spectinomycin cassette was then cloned into pBCSK-isdH digested with *Hind*III and *Pst*I. The disrupted *isdH* gene knockout allele was introduced into the temperature-sensitive *E. coli/S. aureus* shuttle vector pAUL-A (24), and then passaged through *S. aureus* RN4220 prior to being transduced into *S. aureus* RN6390. Recombinant RN6390 was cultured at 30 °C to mid-log phase before incubation temperature was shifted to 42 °C. The bacteria were further incubated for 16 hours before being plated onto tryptic soy agar (TSA) containing spectinomycin. Colonies were screened for sensitivity to erythromycin, which indicates a loss of the pAUL-A backbone DNA after integration of the knockout allele into *S. aureus* chromosome via double homologous recombination. The *isdH*::spec deletion was mobilized to other *S. aureus* backgrounds by transduction using 80α phage (95) as described above.

2.6.3. *NWMN_1320* Gene Deletion

The *NWMN_1320*::Tet knockout allele consisted of a tetracycline resistance cassette, excised from plasmid pDG1513 (57) with restriction enzyme *Pst*I, flanked by DNA sequences homologous to regions upstream and downstream of *NWMN_1320*. The knockout allele was cloned to the temperature-sensitive *E. coli/ S. aureus* shuttle vector

pMAD (3), and then passaged through *S. aureus* RN4220 prior to being transduced into *S. aureus* RN6390. Recombinant RN6390 was cultured at 30 °C to mid-log phase before incubation temperature was shifted to 42 °C. The bacteria were further incubated for 16 hours before being plated onto tryptic soy agar (TSA) containing tetracycline. Colonies were screened for sensitivity to erythromycin, which indicates a loss of the pMAD backbone DNA after integration of the knockout allele into *S. aureus* chromosome via double homologous recombination. The *Δ1320*::tet deletion was mobilized to other *S. aureus* backgrounds by transduction using 80α phage (95) as described above.

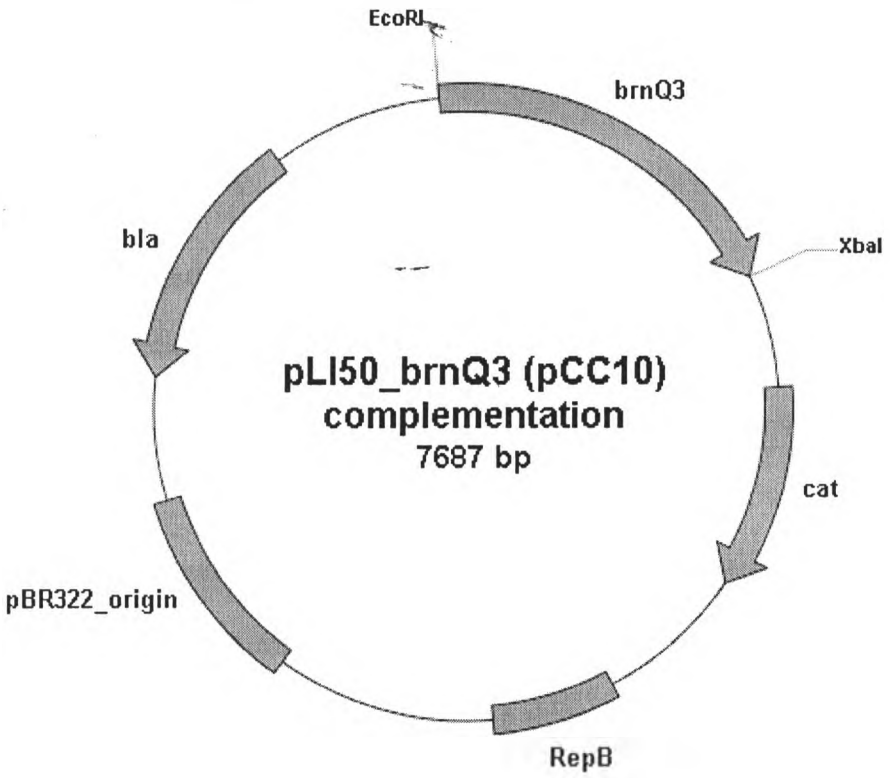
2.6.4. Construction of *NWMN_1320* Complementation Vector (pCC10)

To create the *NWMN_1320* complementation vector, a 1982 bp PCR product was amplified from the Newman chromosome using primers F'1320-pLI50 and R' 1320-pLI50. The PCR product and pLI50 vector were digested with *Eco*RI and *Xba*I. The digested PCR product and vector were ligated and transformed into DH5α. Ampicillin resistant DH5α colonies were screened for a plasmid with the appropriate size (~ 7.4-kb). The plasmid with the correct size was then sent to a sequencing facility to confirm whether it contained the correct sequence. Once confirmed, the plasmid was introduced into *S. aureus* RN4220 by electroporation and chloramphenicol resistant colonies were selected for. Physical map of pCC10 is shown in Figure 5.

2.6.5. Construction of IsdEF Complementation Vector (pCC12)

To create the IsdEF complementation vector, a 1922 bp PCR product was amplified from the Newman chromosome using primers F'isdE-*Kpn*I and R'isdF-*Sac*I.

Figure 5. Physical Map of Wild-Type *brnQ3* Complementation Plasmid pCC10. A fragment containing *brnQ3* upstream regulatory sequence and the *brnQ3* coding region was cloned into the *Eco*RI and *Xba*l site of *E.coli/ S. aureus* shuttle vector pLI50. The plasmid carries an ampicillin resistance marker (*bla*) for selection in *E. coli* and a chloramphenicol resistance marker (*cat*) for selection in *S. aureus*. The pBR322 origin of replication allows for stable plasmid maintenance in Gram-negative species, and RepB allows for stable replication in Gram-positive bacteria.


EcoRI
brnQ3
XbaI
cat
RepB
pBR322_origin
bla
pLI50_brnQ3 (pCC10)
complementation
7687 bp

The PCR product and pALC2073 vector were digested with *Kpn*I and *Sac*I. The digested PCR product and vector were ligated and transformed into DH5α. Ampicillin resistant DH5α colonies were screened to see if they contained a plasmid with the appropriate size (~ 7.4-kb). The plasmid with the correct size was then sent to sequencing facility to confirm whether it contained the correct complementation sequence. Once confirmed, the plasmid was introduced into *S. aureus* RN4220 by electroporation and chloramphenicol resistant colonies were selected for. Physical map of pCC12 is shown in Figure 6.

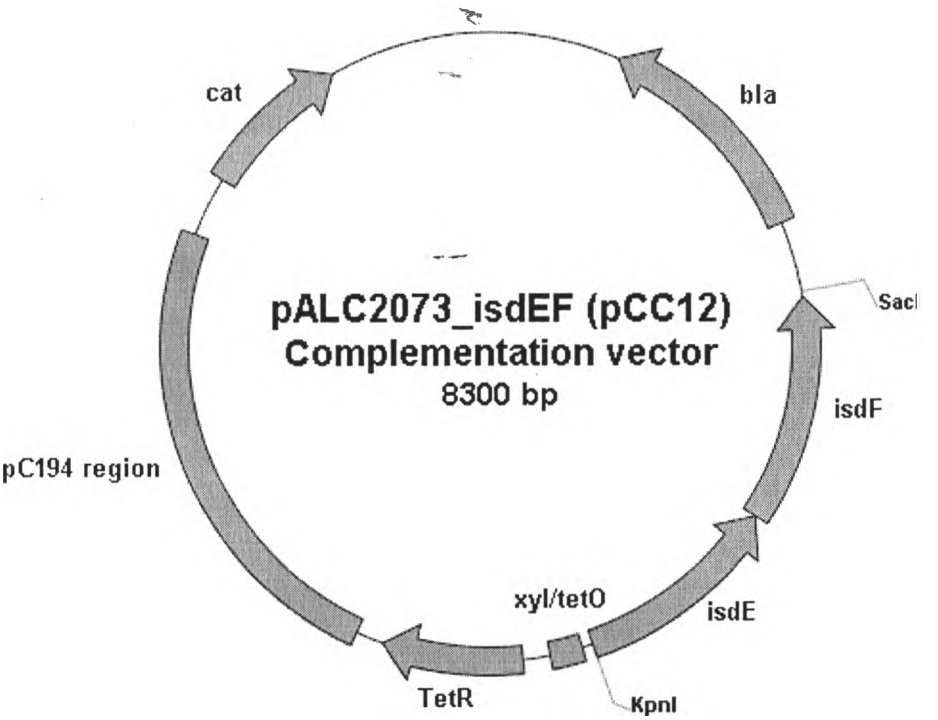
2.7. Real-Time Quantitative Polymerase Chain Reaction

Oligonucleotides used for real-time PCR are listed in Table 4. For real-time PCR analysis, each culture was grown in duplicate, and for each sample, RNA extraction was performed in triplicate. To prepare cDNA, 500 ng of total cellular RNA was reverse-transcribed using Superscript™ II reverse transcriptase (Invitrogen) according to the manufacturer's recommendations. One µg of cDNA was amplified in a Rotor-Gene 6000 (Corbett Life Science) using the iScript One-Step RT-PCR Kit with SYBR Green (BioRad). Each sample was quantified in relation to *rpoB*, and quantification was performed using the standard curve method.

2.8. Murine Systemic Model of Infection

For animal infections, all protocols were reviewed and approved by the University of Western Ontario's Animal Use Subcommittee, a subcommittee of the

Figure 6. Physical Map of *IsdE* and *IsdF* Complementation Plasmid pCC12. PCR amplified isdE and isdF coding regions were cloned into *Kpn*I and *Sac*I site of the *E.coli/ S. aureus* shuttle vector pALC2073. The plasmid carries an ampicillin resistance marker (*bla*) for selection in *E. coli* and a chloramphenicol resistance marker (*cat*) for selection in *S. aureus*. The *tetR* gene encode for TetR repressor and *xyl/tetO* promoter is a tetracycline-inducible promoter.


cat
bla
pALC2073_isdEF (pCC12)
Complementation vector
8300 bp
SacI
isdF
pC194 region
xyl/tetO
isdE
TetR
KpnI

University Council on Animal Care. Seven week old female immunocompetent BALB/c mice were purchased from Charles River Laboratories (Wilmington, Massachusetts) and housed in microisolator cages. Bacteria were grown to an OD_{600} of 3.0 in TSB, pelleted by centrifugation, and washed twice in saline. Bacterial saline suspensions were administered via 100 µL tail vein injections (5×10^6 CFU/injection). At 96 hour following challenge, mice were euthanized via intraperitoneal injection of pentobarbital. Kidneys, livers, and hearts were excised and placed in phosphate-buffered solution containing 0.1 % v/v Triton X-100. Organs were homogenized for 10 seconds and bacterial loads were calculated following serial dilution of the suspension and drop plating on TSB agar plates. Data are presented as $\log_{10}$ CFU recovered per organ.

Chapter 3 - Results[1]

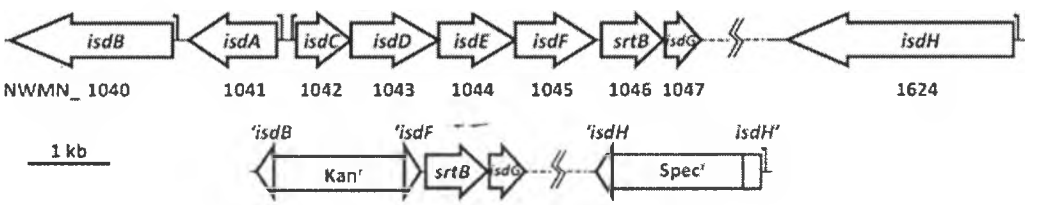
[1] The work presented in this chapter has been submitted for publication in Molecular Microbiology (MMI-2010-10641)

3.1 Deletion of the Isd locus Results in Increased Growth of *S. aureus* on Heme as a Sole Iron Source

The Isd series of proteins have been the subject of intense investigation since their discovery by Tauch, *et al.* (133). Together, these studies have established the role of Isd proteins in heme binding and transfer. Surprisingly, however, and despite their demonstrated role in heme binding and trafficking, single *isd* gene knockouts in *S. aureus* show minimal phenotypic changes versus wild type when assessing growth on heme as a sole iron source (54, 55). This leads to two possible hypotheses. The first is that other heme binding Isd proteins expressed on the surface of *S. aureus* compensate for the loss of any single Isd protein. In another scenario, there exists an alternative, non-Isd route of heme entry into the cell, which masks any observable effect from the loss of individual Isd proteins. To examine which scenario was more likely, we constructed a strain, Newman Δ*isd* (strain designation H1532, see Table 2), negative for all *isd* genes encoding proteins that would be cell wall anchored or membrane-embedded (see Fig. 7A and B). The annotation *Δisd* will be used throughout the remainder of this thesis to suggest a deletion of IsdA through IsdF including IsdH (*ΔisdΔisdH).* The *isdI* gene was left intact so as to allow utilization of heme as an iron source. The *srtB* and *isdG* genes were also left intact but due to the removal of their promoter (upstream of *isdC*) in the mutant, they would not likely be expressed. In comparison to its parental wildtype strain, the mutant displayed no obvious growth deficiency in TSB or TMS media lacking heme (Fig. 8). We then tested this strain for its ability to grow in the presence of heme as a sole iron source. Surprisingly, Newman Δ*isd* consistantly grew better than wildtype in

Figure 7. Generation of the Isogenic Δ*isd* Knockout Mutant in *S. aureus* Strain Newman. A) Physical map of the *isd* locus and the *isdH* gene in *S. aureus* illustrating the construction of the Δ*isd* mutant. Coding regions are denoted by arrows, promoters are denoted with small black arrows, and the annotated gene numbers below the coding regions are from the *S. aureus* Newman genome. The Isd locus consist of eight genes (*isdB, isdA, isdC, isdD, isdE, isdF, srtB, isdG*) and *isdH* is encoded elsewhere on the chromosome. isdB, isdA, and isdCDEFG are three independently transcribed units that are regulated by its own ferric uptake repressor (Fur) promoter. Primers used for the construction of the mutant are described in the Materials and Methods section. B) Polymerase chain reaction analysis confirms the presence of *isd* genes in wildtype and their absence in the mutant. Newman chromosome was used as template for amplicons loaded in lanes 1, 3, 5 and 7, and Δ*isd* chromosome was used as template for PCR reactions loaded in lanes 2, 4, 6 and 8. PCR reactions were performed with oligonucleotides that should amplify regions across *sbnH* 1228kb (lanes 1 and 2, as control), *isdB-isdA* 1406bp (lanes 3 and 4), *isdA-isdC* 1212bp (lanes 5 and 6), and *isdE-isdF* 1184bp (lanes 7 and 8). As independent confirmation of the Δ*isd* and *isdH* mutations, amplification from the mutant chromosome across deleted regions were performed and sequencing confirmed the junctions points to antibiotic resistance.

A


isdB
isdA
isdC
isdD
isdE
isdF
srtB
sdG
isdH
NWMN_ 1040
1041
1042
1043
1044
1045
1046
1047
1624
1 kb
'isdB
'isdF
'isdH
isdH'
Kan'
srtB
sdG
Spec'

B

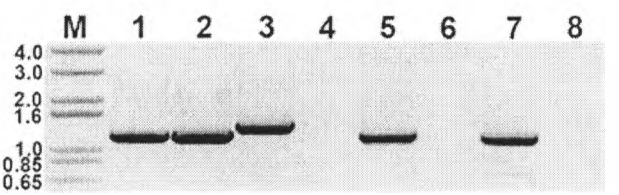
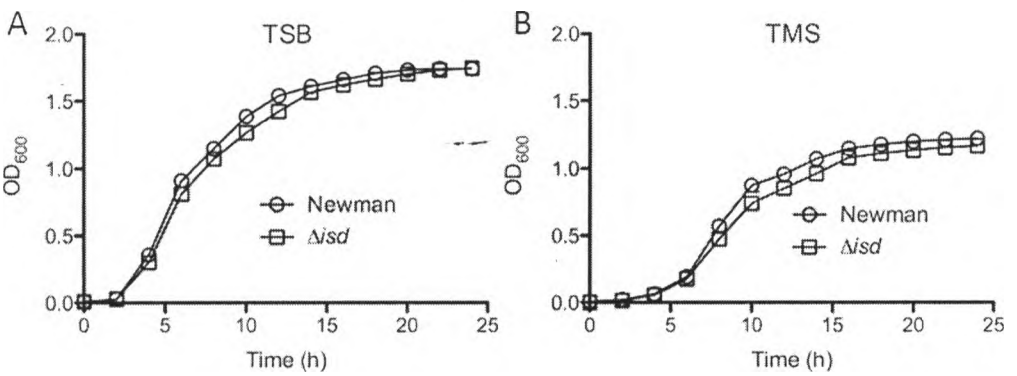

M
1
2
3
4
5
6
7
8
4.0
3.0
2.0
1.6
1.0
0.85
0.65

Figure 8. *In-vitro* Growth curve Analysis of WT Newman Versus Newman *Δisd* in Tris-minimal succinate and Tryptic soy broth A) Growth curve analysis of Newman WT (parental) versus Newman *ΔisdΔisdH* grown in Tryptic soy broth. B) Growth curve of Newman WT and Newman *ΔisdΔisdH* grown in Tris-minimal succinate (TMS) media. Error bars represent standard deviation; $n = 3$. Data are representative of three independent experiments.


A
TSB
B
TMS
OD600
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
Time (h)
Newman
Δisd

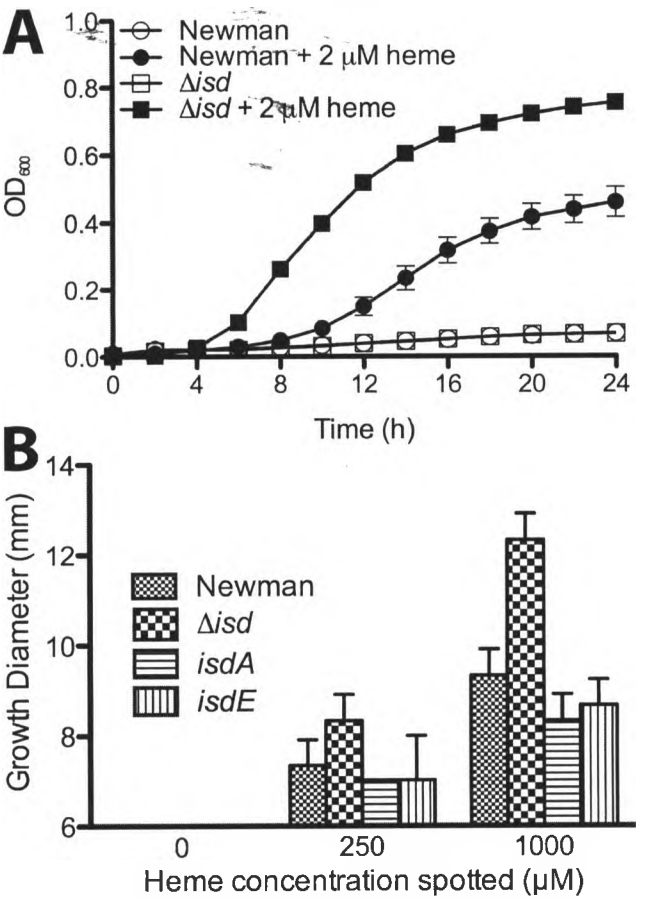
two different conditions tested; liquid growth and a plate bioassay (Fig. 9), leading us to conclude that there is a non-Isd route of heme entry into *S. aureus*.

3.2. Screening of a Tn*917* Insertion Library for Mutants Growing Poorly on Heme as a Sole Iron Source

To identify genes involved in non-*isd*-mediated heme trafficking, a Tn*917* insertion library was generated in a Δ*isd* genetic background. For this, we constructed the Δ*isd* deletion in strain RN6390 since this strain lacks prophage and in order to avoid non-random transposition events as prophage were known hotspots for transposon insertion; Newman contains four prophage (6). Moreover, the RN6390 Δ*isd* strain, as in the Newman genetic background, grew better on heme as a sole iron source than did the RN6390 isogenic parent strain (data not shown). The base growth medium used for Tn*917* mutant screening was chemically-defined Tris-minimal succinate (TMS) containing EDDHA, a non-metabolizable iron chelator, at a concentration of 15 μM, which severely restricted growth of the bacteria, despite their capability to synthesize the iron-chelating siderophores staphyloferrin A and staphyloferrin B. Importantly, the incorporation of 2 μM heme into this medium promoted good growth (data not shown). This concentration of heme, from a range that was tested, was empirically determined to promote optimal growth in the media used for our studies.

Screening of several thousand Tn*917* insertion mutants resulted in the identification of several that grew poorer than did RN6390 Δ*isd* in the presence of heme, and one (Tn#13) that did not grow on the heme-containing plate after 24 h incubation (data not shown). Given the drastic phenotype associated with mutant Tn#13, we chose

Figure 9. Heme-Dependent Growth Response of Newman and Newman Δ*isd* in Iron Restricted Chemically-Defined Media (TMS). A) Liquid culture growth curve of Newman WT and Newman *ΔisdΔisdH* grown in TMS media supplemented with or without 2 μM heme. B) Plate bioassay experiment of Newman WT, Newman *ΔisdΔisdH,* Newman *ΔisdA*, Newman *ΔisdE* measuring the growth on heme (0, 250, 1000 μM) as the sole iron source. High concentrations of heme are spotted on the disks in order to see evidence of bacterial growth on the plate because the majority of heme remains adhered to the paper disks. The growth around the disk was measured after 48 hours. Error bars represent standard deviation; $n = 3$. Data are representative of three independent experiments.


A
Newman
Newman + 2 μM heme
Δisd
Δisd + 2 μM heme
OD600
1.0
0.8
0.6
0.4
0.2
0.0
0
4
8
12
16
20
24
Time (h)
B
Growth Diameter (mm)
14
12
10
8
6
Newman
Δisd
isdA
isdE
0
250
1000
Heme concentration spotted (μM)

to characterize this mutant in further detail for this study. To confirm the phenotype of Tn#13, the transposon insertion was transduced from the mutant back into RN6390 Δ*isd*, and also into Newman Δ*isd*, and a similar lack of growth on the EDDHA and heme-containing plates was observed in both cases, linking the growth phenotype to the Tn*917* insertion. *S. aureus* strain Newman and derivatives were used for the remainder of these studies.

3.3. Identification of the Tn*917* Insertion Site

Sequencing from Tn#13 genomic DNA using a primer that hybridized near the end of the transposon (Table 4) and primed outward into the *S. aureus* chromosome revealed that Tn*917* had inserted into the NWMN_1320 coding region. BLAST searches of the databases showed that the NWMN_1320 gene product exhibited 31% identity and 54% total similarity to the BrnQ proteins of *Salmonella enterica* sv. Typhimurium and *E. coli*, 32% identity and 52% total similarity to the BrnQ protein of *Corynebacterium glutamicum*, and 35% identity and 55% total similarity to the BraB protein in *Pseudomonas aeruginosa, respectively*. BrnQ and BraB proteins are involved in the Na(+)-coupled import of branched chain amino acids (BCAAs) (64, 131). Hydropathy profiles indicate that the proteins are likely to contain 12 membrane-spanning domains. The gene has been annotated in the *S. aureus* COL genome as *brnQ3* since there are two additional *brnQ* paralogs in *S. aureus*. Pairwise alignments demonstrated that the three BrnQ paralogs shared approximately 30-35% identity and 50-55% total similarity across the total length of the proteins. The *S. aureus brnQ3* gene was previously identified in screens for salt-sensitive mutants (141), but its function as a branched-chain amino acid carrier protein has not been formally tested.

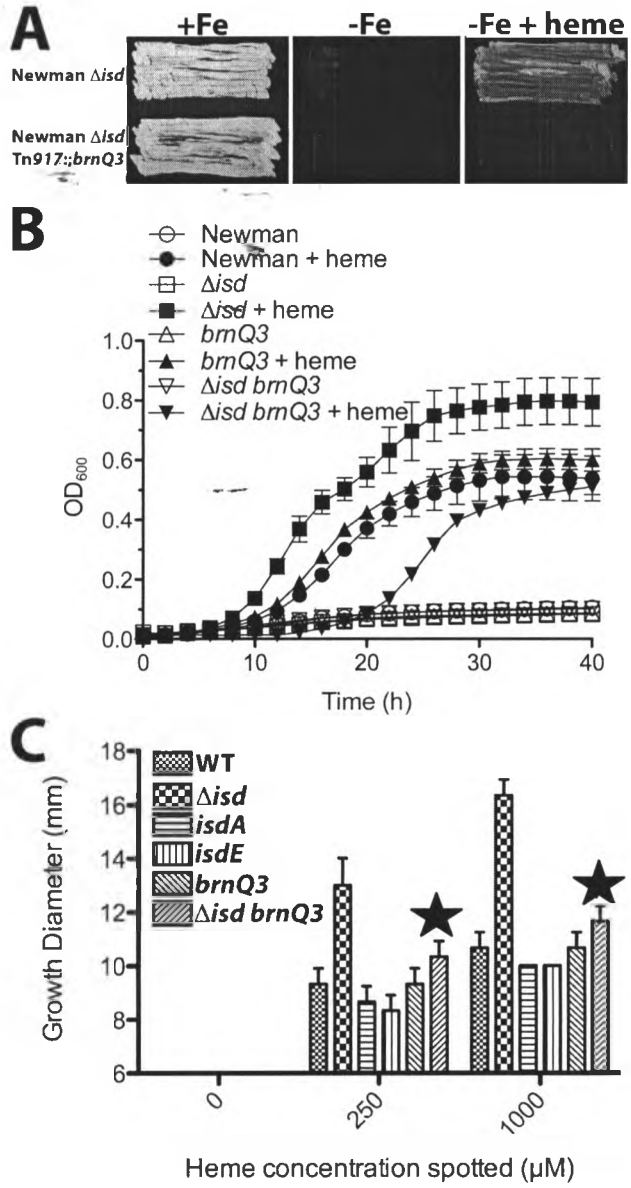
3.4. Characterization of the *S. aureus* brnQ3::Tn*917* Insertion Mutation

The *brnQ3* gene (*NWMN_1320*) encodes a predicted product of 447 amino acids in length with a predicted mass of 48.8 kDa and an estimated pI of 10.0. Like other BrnQ homologs, hydrophobicity analyses suggested that the protein is likely to contain 12 membrane-spanning domains. The last 5 amino acids in the native protein sequence are …^{443}KYQQE^{447}. Sequence analysis from the genome of the mutant showed that the Tn*917* insertion yielded wildtype BrnQ3 sequence right up to the final amino acid and generates a translational fusion such that the fusion protein encoded by the mutant ends …^{443}KYQQGVPSAYEEFVSIRNRFKNFAVILYI^{471}.

3.5. *S. aureus* BrnQ3 and Isd Each Contribute to Growth in the Presence of Heme as a Sole Iron Source

In Figure 10A, growth of Newman Δ*isd* is compared to that of Newman Δ*isd* Tn*917*::*brnQ3* on the three types of solid media that were used in the transposon screening. Newman Δ*isd* Tn*917*::*brnQ3* has no detectable growth deficiency on unchelated TMS (i.e. no EDDHA added) (Fig. 10A, column labeled +Fe) or rich media such as tryptic soy broth agar (data not shown). Neither strain grew on the chelated media (-Fe); however, and only Newman Δ*isd* grew in the presence of heme as a sole iron source over a 24-hour incubation period (Fig. 10A). We also performed similar growth assays in liquid culture media. Notably, we observed a pronounce lag phase for Newman Δ*isd* Tn*917*::*brnQ3* (approaching 24 hours) but when incubation of bacterial cultures was allowed to proceed beyond 24 hours, we consistently observed that the Newman Δ*isd* Tn*917*::*brnQ3* began to grow in iron-restricted media containing 2 μM

Figure 10. Selection for a Mutant with Impaired Growth in Media Containing Heme as a Sole Iron Source. A) Newman *ΔisdΔisdH* and Newman *ΔisdΔisdH Tn917::brnQ3* were streaked on TMS plates without EDDHA chelation (+Fe), TMS plates containing 10 μM EDDHA (-Fe), or TMS plates containing 10 μM EDDHA plus 2 μM heme. Plates were incubated for 24 hours at 37 °C. B) Heme-dependent liquid growth of Newman WT, Newman *ΔisdΔisdH*, Newman *Tn917::brnQ3*, Newman *ΔisdΔisdH Tn917::brnQ3* over a 40 hour incubation at 37 °C, highlighting that Newman Δ*isd brnQ3*::Tn*917* begins to grow after approximately 24 hours incubation at 37 °C. (C) Disk plate bioassays of Newman WT, Newman *ΔisdΔisdH*, Newman *Tn917::brnQ3*, Newman *ΔisdΔisdH Tn917::brnQ3* incubated at 37°C. The growth around the heme (0, 250, 1000 μM) spotted disks were measured after 48 hour incubation. The stars indicate that although we report diameters of growth of 10-12 mm, the growth was extremely poor and sparse compared to the growth of the other strains tested, which had heavy growth within the diameters indicated. Error bars represent standard deviation; $n = 3$. Data are representative of three independent experiments.

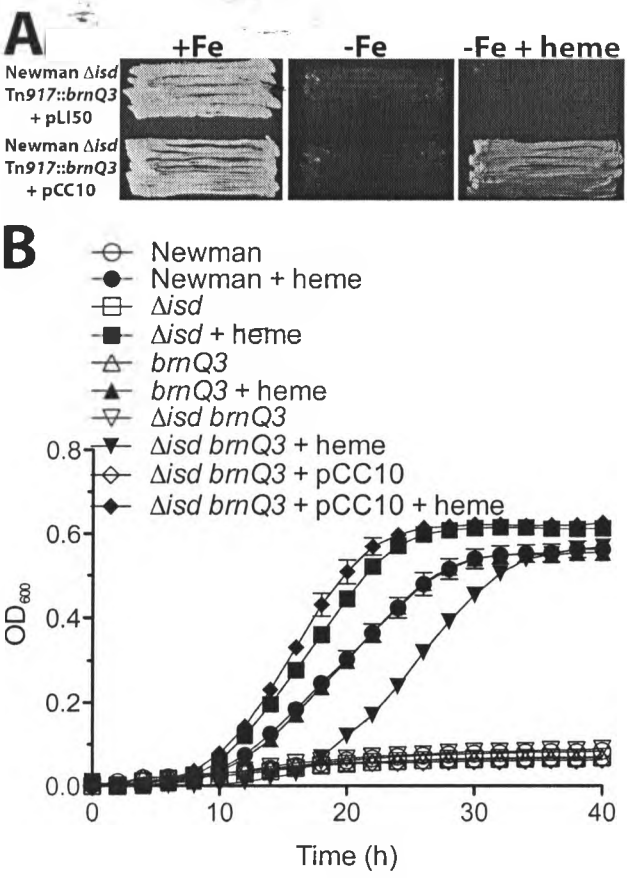

A
+Fe
-Fe
-Fe + heme
Newman Δisd
Newman Δisd Tn917::brnQ3
B
Newman
Newman + heme
Δisd
Δisd + heme
brnQ3
brnQ3 + heme
Δisd brnQ3
Δisd brnQ3 + heme
OD600
Time (h)
C
WT
Δisd
isdA
isdE
brnQ3
Δisd brnQ3
Growth Diameter (mm)
Heme concentration spotted (μM)

heme, eventually attaining close to the same final biomass as that of Newman (Fig. 10B). To examine the role of *brnQ3* in heme trafficking in wildtype *S. aureus* (i.e. a strain capable of expressing Isd proteins), we transduced the mutation into strain Newman and found that there was no obvious growth defect in comparison to wildtype Newman growth in the presence of heme as a sole iron source (Fig. 10B), suggesting that, as predicted, Isd proteins are involved in heme shuttling and function to compensate for the loss of *brnQ3*.

We then used a third heme growth promotion assay, supplying heme on paper disks and assessing growth halos around the disks as a measure of heme utilization as an iron source (see Fig. 10C). In this assay, as in the liquid cultures, there was no difference between growth of Newman and Newman Tn*917*::*brnQ3* and a larger growth halo for Newman Δ*isd* after 48 hours incubation at 37 °C. Single gene knockouts in *isdA* and *isdE* indicate slight decreases in growth on heme, as reported previously (54, 55). Moreover, while no growth of Newman Δ*isd Tn917*::*brnQ3* was observed around the heme spotted discs at 24 hours (data not shown), there was noticeable but very sparse growth around the disc (observed across a 10-12 mm diameter) after 48 hours incubation for the Newman Δ*isd*Tn*917*::*brnQ3* strain (Fig. 10C, denoted with an asterisk).

In order to verify that this phenotype was due to the loss of *brnQ3* function, we cloned the *brnQ3* gene, along with 375 bp of upstream DNA to ensure the inclusion of the *brnQ3* promoter, into the *E. coli-S. aureus* shuttle vector pLI50 thus creating plasmid pCC10. Introduction of pCC10 into Newman Δ*isd* Tn*917*::*brnQ3* complemented the observed growth deficiency on heme on solid and in liquid media (Fig. 11A and 11B) and, as seen in the liquid assay, resulted in a growth phenotype in the presence of 2 μM

Figure 11. The *brnQ3* Gene *in trans* Complements the Heme-Dependent Growth Defect of Newman Δ*isd brnQ3*::Tn*917*. The heme-growth defect of the Newman Δ*isd brnQ3*::Tn*917* in both A) solid and B) liquid media is complemented by *brnQ3* provided *in trans* on plasmid pCC10. All strains contained either vehicle control (pLI50) (not indicated in figure) or pCC10 (pLI50 containing *brnQ3*) (indicated in figure), such that growth of all strains was performed in the presence of chloramphenicol. Error bars represent standard deviation; $n = 3$. Data are representative of three independent experiments.


A
+Fe
-Fe
-Fe + heme
Newman Δisd Tn917::brnQ3 + pLI50
Newman Δisd Tn917::brnQ3 + pCC10
B
Newman
Newman + heme
Δisd
Δisd + heme
brnQ3
brnQ3 + heme
Δisd brnQ3
Δisd brnQ3 + heme
Δisd brnQ3 + pCC10
Δisd brnQ3 + pCC10 + heme
OD600
0.0
0.2
0.4
0.6
0.8
0
10
20
30
40
Time (h)

heme that was identical to the phenotype observed for a Δ*isd* deletion mutant (i.e. grew better on heme than wildtype Newman).

Since the Tn*917* insertion occurred right at the 3' end of *brnQ3* (recall from above that the insertion site was in front of the last codon), it was possible that the mutant had a partially functional BrnQ3 protein that, over time, would eventually allow growth on heme. To assess this, we deleted the *brnQ3* gene and replaced it with a tetracycline resistance cassette, creating the Newman *brnQ3*::Tet strain called H2324. This mutation was then transferred into the Δ*isd* background. The phenotype, as illustrated in Figs. 10 and 11, for strains containing the *brnQ3* deletion was identical to that of strains containing the *brnQ3*::Tn*917* insertion mutation (data not shown), suggesting that the transposon insertion resulted in non-functional BrnQ3 protein. As was the case for strains containing Δ*isd brnQ3*::Tn*917*, plasmid pCC10 also complemented the heme growth defect of Δ*isd brnQ3*::Tet (data not shown). To assess whether strains carrying deletions in *isd* and *brnQ3* built up genetically stable suppressor mutations that, during growth beyond 24 hours, resulted in the ability to grow in the presence of heme, we took Newman Δ*isd* Tn*917*::*brnQ3* that had grown 48 hours in TMS + EDDHA in the presence of 2 μM heme (i.e. cells that grew after a long lag phase), and subcultured these cells back into the same media. We observed an identical long lag phase where greater than 24 hours incubation was required to observe appreciable growth (data not shown).

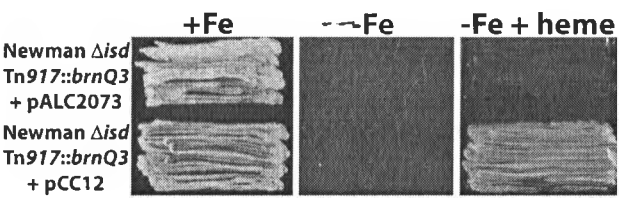
The *brnQ3* mutation produces the heme growth deficiency phenotype in the *isd* knockout background but not in wildtype *S. aureus* backgrounds. To assess whether this

phenotype depended on a complete absence of Isd proteins, or only a subset of Isd components, we chose to first assess whether or not the IsdEF membrane transporter would be sufficient to allow heme utilization in the absence of *brnQ3*. The IsdE protein is a high-affinity receptor for heme (55) and IsdF is the permease component of an ATP-binding cassette (ABC)-family transporter. As shown in Fig. 12, expression of *isdEF* (from plasmid pCC12) did complement the heme growth deficiency of Newman Δ*isd* Tn*917*::*brnQ3*, suggesting that heme readily passes through the cell wall to the IsdEF transporter. Given the subtle phenotypes associated with *isdE* mutations in *S. aureus* (55), this experiment is the first solid demonstration that IsdEF functions in heme import.

3.6. Deletion of HtsABC Does Not Affect Growth on Heme as a Sole Iron Source

The iron-regulated *htsABC* operon, adjacent on the *S. aureus* chromosome to the staphyloferrin A biosynthetic locus, encodes an iron-regulated ABC transporter that is involved in the capture and uptake of iron bound to the siderophore staphyloferrin A (10). The HtsA lipoprotein belongs to the class III ligand binding protein family, and binds Fe(III)-staphyloferrin A with high affinity into a positively-charged binding pocket that provides several specific contacts with the Fe(III)-staphyloferrin A complex (53). The *htsABC* locus, however, was initially proposed to be involved in heme uptake in *S. aureus*, based on the observation that strains bearing mariner transposon insertions in *htsB* or *htsC* preferentially accumulated iron bound to transferrin over heme, suggesting a deficiency in heme-iron accumulation (119). To assess this further, we took advantage of the growth assays developed in this study to examine if HtsABC played a role in growth of *S. aureus* on heme as an iron source. In our previous studies, we

Figure 12. Expression of IsdEF *in trans* (pCC12) Complements the Heme-Dependent Growth Defect of Newman Δ*isd brnQ3*::Tn*917*. All strains were grown on TMS media containing chloramphenicol, and either without addition of EDDHA (left column labeled +Fe), with addition of 10 μM EDDHA (center column labeled -Fe), or with addition of 10 μM EDDHA plus 2 μM heme (right column). Plates were incubated for 24 hours at 37 °C.


+Fe
-Fe
-Fe + heme
Newman Δisd Tn917::brnQ3 + pALC2073
Newman Δisd Tn917::brnQ3 + pCC12

generated an *htsABC* deletion (10). We mobilized this deletion, via transduction, into the Δ*isd*, *brnQ3*, and Δ*isd brnQ3* genetic backgrounds and repeated the three growth assays described in Figure 10. The addition of the *htsABC* deletion did not change the growth kinetics of any of the strains in comparison to their HtsABC-competent isogenic parents (data not shown).

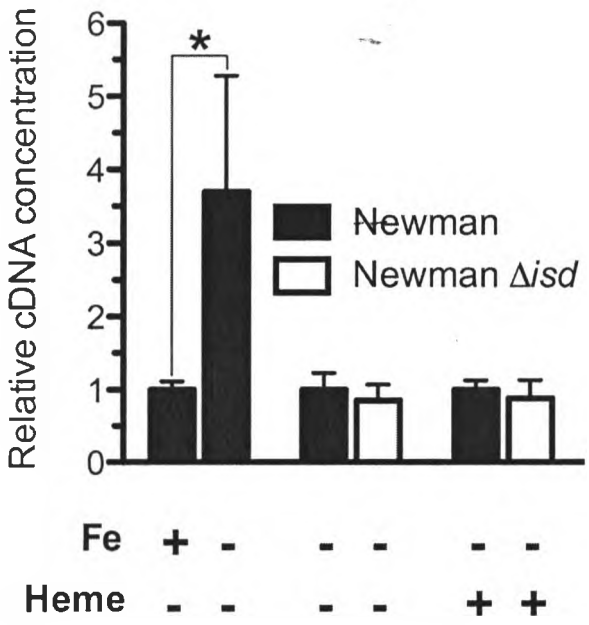
3.7. *brnQ3* Transcription is Enhanced in Response to Iron Limitation

It was of interest to determine whether the *brnQ3*-dependent growth response was due to changes occurring in the transcriptional levels of *brnQ3*. To characterize this, quantitative real-time RT-PCR (qPCR) was performed. The results, shown in Figure 13, demonstrated that transcription of *brnQ3* in Newman was iron-regulated, although not nearly to the level observed for other known iron-regulated transcripts such as *isdA* or *fhuC* (>20-fold; data not shown). The results also showed that there was no change in the levels of *brnQ3* transcript in response to deleting *isd*, either in iron-restricted cells or iron-restricted cells exposed to heme for 1 hour.

3.8. A *brnQ3* Mutation is Not Associated with Increased Sensitivity to NaCl

S. aureus is a NaCl tolerant bacterium, capable of growing in the presence of 2 M NaCl. In previous work, it was shown that a transposon disruption 337 nucleotides downstream of the *brnQ3* initiation codon (the full gene contains 1341 nucleotides) yielded a strain (parent strain RN450) that was sensitive to salt (141). We therefore tested for this phenotype with both the *brnQ3* transposon mutant and the deletion mutant. In contrast to the previous study, we found that Newman *brnQ3* mutants (both

Figure 13. Quantitative Real-Time RT-PCR Results for *brnQ3* Transcript Levels. RNA was isolated from Newman and Newman Δ*isd* grown in iron-restricted TMS media to an OD of 0.8, before splitting the cultures into three aliquots and exposing the cells for one hour further incubation (prior to RNA extraction) to i) no further additions, ii) 2 μM heme, or iii) 10 μM $FeSO_4$. Transcript levels were normalized using transcript levels for *rpoB* and calibrators (first bar in each of the three comparison groups shown) were arbitrarily set to 1. Data presented are the means of two independent experiments each done in triplicate and error bars indicate standard deviation. The difference in *brnQ3* transcript levels between samples indicated with an asterisk are statistically significant with *P* value less than 0.05 as determined by Student's unpaired *t* test.


Relative cDNA concentration
6
5
4
3
2
1
0
*
Newman
Newman Δisd
Fe + - - - - -
Heme - - - - + +

the transposon mutant and the mutant containing the complete gene deletion, in both the Newman and Newman Δ*isd* backgrounds), in comparison to wildtype, showed no evidence of increased salt sensitivity on solid media containing up to 2 M NaCl (data not shown). The results were the same in three different growth media; tryptic soy broth, TMS (i.e. succinate as the primary carbon source) or the defined media used in the study describing the *brnQ3* phenotype (i.e. glycerol as the primary carbon source) (141).

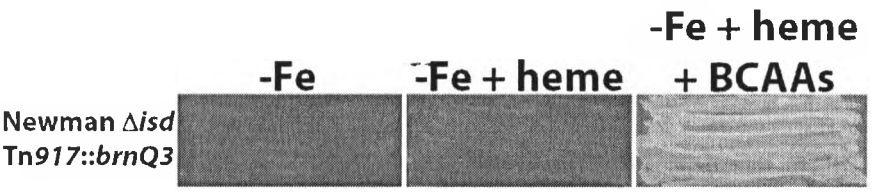
3.9. BCAAs Control Utilization of Heme as an Iron Source

As discussed above, the BrnQ3 protein has similarity to proven branched-chain amino acid transporters in other bacteria. Despite the presence of at least two other genes in *S. aureus* that may encode BCAAs transporters (i.e. BrnQ1 and BrnQ2), it was possible that the phenotype observed in strains containing Δ*isd* and *brnQ3* mutations was due not only to the absence of Isd proteins, but also to a deficiency in the uptake of BCAAs, and this may have downstream effects on the intake of heme. In agreement, we observed that incorporation of a mixture of exogenous ILE, LEU, VAL and THR (each at 5 mM) into growth media indeed complemented the heme growth defect displayed by Newman Δ*isd* Tn*917*::*brnQ3* (Fig. 14). This is the first demonstration that mutation of *brnQ3* affects BCAA uptake, and also the first demonstration that the uptake of BCAAs permits heme acquisition in *S. aureus*.

3.10. BrnQ3 is Essential for Full Virulence of *S. aureus*

To test the role of the Isd and BrnQ3 and concomitant heme uptake deficiency in *S. aureus* pathogenesis, cohorts of BALB/c mice were challenged intravenously with *S. aureus* Newman and isogenic *isd* and *brnQ3* mutants. Animals that were infected with

Figure 14. Incorporation of ILE, LEU, THR and VAL into Growth Media Complements the Heme Growth Defect of Newman Δ*isd brnQ3*::Tn*917*. Strain Newman Δ*isd brnQ3*::Tn*917* was streaked onto solid TMS media containing either addition of 10 μM EDDHA (left column labeled -Fe), addition of 10 μM EDDHA plus 2 μM heme (center column), or 10 μM EDDHA plus 2 μM heme + 5mM ILE, LEU, VAL, and THR. Plates were incubated for 24 hours at 37 °C.


-Fe
-Fe + heme
-Fe + heme
+ BCAAs
Newman Δisd
Tn917::brnQ3

Newman displayed signs of disease, including weight loss and significant numbers of bacteria in kidneys, liver and hearts (Fig. 15). More details on the clinical characteristics and bacterial load of each mouse organ can be found in Table 5. Interestingly, animals infected with Newman Δ*isd* showed an insignificant drop in the numbers of CFUs in the organs tested, in contrast to our own data (not shown) and recently published data (68) that demonstrate approximately 1-2 log drops in bacterial burden in organs taken from mice infected with Newman containing single gene mutations in *isdA*, *isdB* or *isdC*. Mice infected with Newman Δ*isd* had more abscesses than Newman WT, especially in the kidney. While the Newman *brnQ3* mutation did result in a significant drop in bacterial burden in hearts, it was more infective in the livers of the animals. Mice challenged with Newman Δ*isd brnQ3* were less moribund and lost significantly less weight than the other cohorts of mice, and had significantly less bacterial burden, relative to mice challenged with Newman, in all organs tested.

Figure 15. Comparison of % Weight Loss and Bacterial Load Recovered from Kidneys, Livers, and Spleens of Mice Infection with *S. aureus* Newman, Newman *Δisd*, Newman *brnQ3::Tn917*, Newman *Δisd brnQ3::Tn917*Newman Δ*isd brnQ3*::Tn*917* has Decreased Virulence in Mice. Cohorts of 10 mice were challenged intravenously with $5x10^6$ CFUs of the strains as indicated. After 4 days, mice were sacrificed, weighed, and organs aseptically removed, homogenized, serially diluted and drop plated for determination of CFU, as described in *Experimental Procedures*. Each symbol represents one mouse. Statistical significance with *P* value less than 0.0001 as determined by Student's unpaired *t* test.

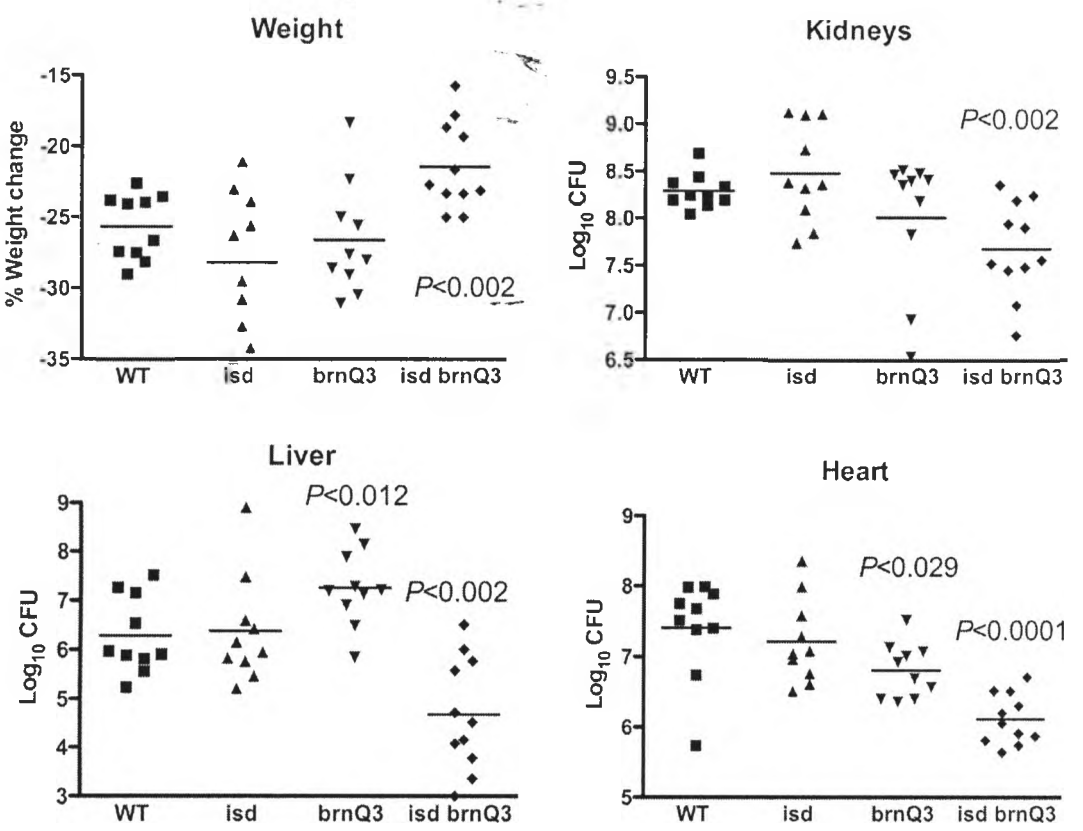

Weight
% Weight change
-15
-20
-25
-30
-35
P<0.002
WT
isd
brnQ3
isd brnQ3
Kidneys
Log10 CFU
9.5
9.0
8.5
8.0
7.5
7.0
6.5
P<0.002
WT
isd
brnQ3
isd brnQ3
Liver
Log10 CFU
9
8
7
6
5
4
3
P<0.012
P<0.002
WT
isd
brnQ3
isd brnQ3
Heart
Log10 CFU
9
8
7
6
5
P<0.029
P<0.0001
WT
isd
brnQ3
isd brnQ3

Table 5. Clinical characteristics and bacterial load of mice infected with *S. aureus* Newman, Newman *Δisd*, Newman *brnQ::Tn917*, Newman *Δisd brnQ::Tn917*

Infecting strain and Mouse number	% Weight Loss	Kidney (Log_{10} CFU)	Spleen (Log_{10} CFU)	Liver (Log_{10} CFU)	Heart (Log_{10} CFU)
1-Newman WT	23.89	8.24	5.85	7.15	7.98
2-Newman WT	23.63	8.13	5.59	6.53	6.74
3-Newman WT	24.14	8.33	0.00	7.51	7.38
4-Newman WT	27.54	8.19	4.60	5.56	7.99
5-Newman WT	24.02	8.19	6.23	5.88	7.51
6-Newman WT	29.05	8.68	4.26	5.91	7.75
7-Newman WT	26.67	8.04	4.80	5.23	5.73
8-Newman WT	22.67	8.24	6.00	5.97	7.40
9-Newman WT	28.18	8.37	0.00	7.26	7.89
10-Newman WT	27.46	8.43	5.53	5.81	7.68
Averages:[a]	**25.72 ± 2.28**	**8.28 ±0.18**	**4.29 ± 2.35**	**6.28 ± 0.78**	**7.40 ± 0.70**
1-Newman *Δisd*	34.21	8.73	4.80	8.91	7.99
2-Newman *Δisd*	29.53	8.32	6.21	5.21	6.96
3-Newman *Δisd*	21.08	8.38	5.65	5.95	7.08
4-Newman *Δisd*	32.67	9.10	0.00	6.15	7.28
5-Newman *Δisd*	26.29	9.09	5.18	6.43	7.58
6-Newman *Δisd*	23.03	8.09	5.36	6.59	6.61
7-Newman *Δisd*	35.00	9.12	0.00	7.48	8.35
8-Newman *Δisd*	30.81	7.84	6.19	5.83	7.04
9-Newman *Δisd*	25.63	7.74	3.78	5.46	6.51
10-Newman *Δisd*	23.91	8.36	3.15	5.76	6.76
Averages:[a]	**28.22 ± 4.91**	**8.48 ± 0.52**	**4.03 ± 2.33**	**6.38 ± 1.09**	**7.22 ± 0.60**
1-Newman *brnQ::Tn917*	18.37	7.83	5.83	7.89	6.36
2- Newman *brnQ::Tn917*	25.57	8.40	5.13	7.21	6.57
3- Newman *brnQ::Tn917*	25.00	8.18	5.49	7.20	6.69
4- Newman *brnQ::Tn917*	27.59	8.41	5.82	7.28	7.52
5- Newman *brnQ::Tn917*	28.57	8.48	3.56	6.91	6.40
6- Newman *brnQ::Tn917*	31.05	8.46	6.40	5.85	7.01
7- Newman *brnQ::Tn917*	22.34	6.93	4.32	8.14	7.07
8- Newman *brnQ::Tn917*	29.03	6.54	4.81	8.46	6.92
9- Newman *brnQ::Tn917*	28.00	8.51	5.89	6.49	7.13
10- Newman *brnQ::Tn917*	30.43	8.35	6.49	7.13	6.40
Averages:[a]	**26.60±3.90**	**8.01 ± 0.71**	**5.37 ± 0.93**	**7.26 ± 0.77**	**6.81 ± 0.39**
1-Newman *Δisd brnQ::Tn917*	19.32	7.08	2.70	3.36	6.20
2- Newman *Δisd brnQ::Tn917*	23.33	7.94	5.89	4.52	6.51
3- Newman *Δisd brnQ::Tn917*	23.33	7.90	6.23	5.58	5.81
4- Newman *Δisd brnQ::Tn917*	23.12	8.35	2.78	4.72	6.30
5- Newman *Δisd brnQ::Tn917*	22.70	7.45	3.85	4.08	5.91
6- Newman *Δisd brnQ::Tn917*	17.82	7.48	0.00	0.00	5.87
7- Newman *Δisd brnQ::Tn917*	21.62	7.56	2.70	3.00	5.74
8- Newman *Δisd brnQ::Tn917*	25.00	6.76	3.34	3.78	6.05
9- Newman *Δisd brnQ::Tn917*	15.76	8.19	3.98	5.77	6.71
10- Newman *Δisd brnQ::Tn917*	18.67	8.24	4.40	6.51	6.52
11- Newman *Δisd brnQ::Tn917*	25.00	7.52	5.18	6.00	5.64
Averages:[a]	**-21.33 ± 3.04**	**7.68 ± 0.50**	**3.73 ± 1.75**	**4.30 ± 1.82**	**6.11 ± 0.36**

[a] Averages are ± standard error of the mean

Chapter 4 - Discussion

It is vital for bacterial pathogens to obtain iron as it is a key factor to determining successful establishment of infection. Our laboratory as well as many others have shown in response to iron limitation, *S. aureus* engages a complex regulatory program involving a large repertoire of cytoplasmic, membrane-embedded, cell wall anchored and secreted proteins, that serve to alleviate iron restriction but also protect from iron and heme toxicity (1). *S. aureus* can acquire heme, abundant in the vertebrate host, to satisfy its requirement for iron. Paradoxically, heme is also toxic at elevated concentrations, especially for Gram-positive bacteria (1) and it is not surprising to find that mechanisms to alleviate heme toxicity exist in Gram-positive bacteria, including *S. aureus* and *C.diphtheriae* (15, 123, 139). One such mechanism is through the HssRS and HrtAB systems as reviewed in the Introduction of this thesis. *S. aureus*, among several other Gram-positive pathogens, expresses iron-regulated surface determinants (Isd), to acquire iron from host hemoglobin and heme. One of the key findings of this study is that *S. aureus* containing deletions of genes encoding IsdABCDEFH proteins can still acquire heme to satisfy its iron requirement for growth. Although the heme degrading enzyme IsdG is left intact, and assuming that the Isd system is the sole heme acquisition pathway for *S. aureus*, then technically heme-iron uptake should be completely abolished in such an *isd* mutant. Therefore, a Δ*isd* mutant (with deletions in all of the genes mentioned) should not be able to grow in iron-restricted media with heme provided as the sole iron source. In fact, we consistently observed that this mutant grew faster and to higher biomass than wildtype bacteria. This was constantly observed in different experiments such as *in-vitro* growth curve analysis, plate disk bioassay, or simply growing cells in liquid culture where heme is given as the sole iron source. Through various optimization assays, 10 μM of EDDHA was enough to chelate free iron

in the media that may be used by siderophores as this concentration did not promote growth of wildtype Newman cells. Our assays include 2 μM heme, a non-toxic concentration, but in these assays heme was the only biologically-available iron source and is the limiting nutrient; addition of $FeSO_4$ to the media allows growth to higher cell densities. It should be noted, that pertaining to plate disk bioassays, higher than usual concentrations of heme were used in order to see the growth phenotype. This is because the majority of the heme cannot be obtained by the cells or cannot properly diffuse into the solid agar as its chemical properties cause it to remain adhered to the disks. There can be two possible hypotheses for the phenomenon behind why Δ*isd* mutant exhibited greater growth than wild-type *S. aureus*. The first hypothesis is elimination of the cell wall anchored heme binding proteins allows quicker access of free heme to the membrane, where it can be captured by other membrane-localized heme transporters or simply through diffusion across the membrane since the molecular weight of heme is at the permeability limit of the membrane (~600 Da and see Introduction). Although *in-vitro* data have determined that heme can traffic between Isd proteins (94, 149), data on heme transfer reactions on the cell surface are lacking since experiments are performed with proteins that are not physically anchored onto a surface that would mimic the conditions of a cell wall.

The second hypothesis, which is slightly implied by recent work has uncovered a relatively slow k(off) rate of heme bound to IsdC (109), suggesting that *S. aureus* may use the Isd proteins to regulate or slow heme uptake into the cell so as to avoid potential intoxication. If this were the case, then it can also be implied that under normal circumstances and due to the lipophilicity of the porphyrin ring (see Introduction),

excess heme on the cell surface may insert, partially diffuse, or pass entirely through the lipid bilayer of the cell membrane. Therefore, it would seem that the placement of cell wall anchored heme-binding proteins serve as a buffer to sequester free heme on the surface and to re-direct heme import in an orderly fashion that would not lead to a toxic influx of heme into the cell. Therefore it should be expected that an Δ*isd* mutant would exhibit a lethal phenotype in the presence of even a low micromolar concentration of heme since there are no heme-binding proteins on the cell wall to serve as "heme buffers". However, the experiments did not demonstrate such lethality for an Δ*isd* mutant and so the results presented here appear to support the first hypothesis instead due to the observation of enhanced and continued growth of Δ*isd* mutant versus wild-type.

Strangely, if the first hypothesis holds true for an Δ*isd* mutant, then somehow the disordered influx of heme across the membrane does not lead to a toxic accumulation of heme (or iron) in the cytoplasm. There may be a couple of explanations for this. Perhaps the heme efflux systems are working at a higher capacity for an Δ*isd* mutant and therefore heme-iron homeostasis is maintained, which allows the mutant strain to continue to grow. The second explanation is that since the Δ*isd* mutant is already in an iron-starved condition, this would suggest that intracellular iron levels are also low. Therefore, the cells can now afford to take up a larger portion of heme-iron (and grow faster) without experiencing heme toxicity when a certain threshold of intracellular heme concentration has been reached. Nonetheless, it is also possible that a combination of both explanations can account for the unique growth phenotype of Δ*isd* mutant. Circumstantial evidence that, during growth, some heme remains bound to cell wall

heme binding proteins comes from our data in Figs. 9, 11 and 12, where we consistently find that the *isd* deletion strain (i.e. lacking IsdA, B, C and H) grows to higher biomass than wildtype strains in iron restricted heme-containing media. Therefore, we surmise that all heme provided to the *isd* mutant in our media is at least captured at the membrane and makes its way into the cell promoting growth to higher levels than can be achieved by wildtype cells in which some heme remains bound by Isd proteins within the cell wall. In addition to having the Isd proteins on the cell wall to regulate intake of heme, such proteins on the cell wall may also be an advantage to *S. aureus* since it has been shown that heme bound to proteins IsdA and IsdB provide resistance to killing by H_2O_2 (96).

It was perhaps not surprising to find that *S. aureus* possesses more than one mechanism for heme uptake as many other gram-positive bacteria such as *B. anthracis* use more than one method to obtain heme iron (see Introduction). Indeed, duplicity in iron acquisition mechanisms, owing to the importance of this metal for life, is generally found in all bacteria in which it has been studied. This would be advantageous to the organism in terms of potential interspecies competitions (where possessing a more efficient iron-acquisition machinery leads to greater fitness or adaptation to a certain environment) or potentially as a back-up iron acquisition strategy when the preferred system is interfered by an inhibitor or antibiotic. Not only can *S. aureus* acquire heme through more than one mechanism, but it also produces siderophores staphyloferrin A and staphyloferrin B and can utilize many others that itself does not produce (9). Although the identity of an additional transporter, or transporters, for heme remains undefined at this time and is part of our ongoing research efforts, our current data are in

agreement with the hypothesis that the expression of alternative heme transporter(s) is regulated by intracellular branched chain amino acids (BCAAs). This is based on the observation that supplementing the growth media of the *isd brnQ3* mutant with BCAAs ILE, LEU, VAL and THR alleviated the long lag phase in heme-containing media. Interestingly, BCAAs are known to act as effector molecules for CodY, a global transcriptional regulator highly conserved in low G+C Gram-positive bacteria that regulates gene expression, including virulence genes, in response to nutrient limitation (12, 22, 56, 60, 83, 84, 101, 115, 122). During nutrient deprivation, CodY derepresses a large number of genes and repression occurs only during exponential growth of cells when nutrients are abundant (22) Therefore, a decrease in BCAAs inside the cell is sensed by CodY to derepress genes controlling metabolic and cellular pathways (22). Therefore, genes that would be involved in biosynthesis of amino acids as well as transport systems for important nutrients would be activated. As in other Gram-positive bacteria, CodY is known to regulate the expression of many transport processes in *S. aureus*, including those for amino acids and oligopeptides, among others (83). The fact that BCAAs serve as effector molecules for CodY would suggest that the existing level of these amino acids can be a diagnostic indication of the cell's nutritional status. To bridge amino acid nutrition with the major theme of this thesis, which is heme transport, Wandersman and colleagues found that a dipeptide permease was involved in heme uptake in *E. coli* (76) and it remains to be defined whether similar permeases can influence uptake of heme in *S. aureus*. Therefore, it is tempting to speculate that CodY could be involved in regulating an oligopeptide transporter that has yet to be identified in *S. aureus* and that could allow heme utilization as an iron source (22, 76). Whether the characterized *E. coli* dipeptide permease is controlled by CodY or not is unknown. If

such a CodY-regulated transporter exists in *E. coli*, *S. aureus* or any other bacteria, then under conditions of iron or BCAA starvation, the genes encoding this transport system should be expressed and the transport system should be fully active to uptake either heme or amino acids/peptides depending on the nutritional requirement at the time of need.

In the absence of the duplicating effect of Isd function on growth on heme as an iron source, it was interesting to find such a drastic lag-phase phenotype associated with the mutation of *brnQ3*, since there are at least two additional BrnQ paralogs in *S. aureus* with approximately 55% total similarity to BrnQ3. However, the growth defect was complemented by introduction of *brnQ3* in trans (Fig. 11). Furthermore, the mutation was fully complemented with mM concentrations of BCAAs (isoleucine, leucine, valine, and threonine were provided) (Fig. 14), which indicates that mutation of *brnQ3* decreases internalization of BCAAs. This could indicate that BrnQ3 is the predominant BCAA importer and that BrnQ1 and BrnQ2 play lesser roles. In support of this idea, which remains to be proven but is actively being pursued in our ongoing studies, the *brnQ3* gene has been identified in several high-throughput screens for genes impacting on *S. aureus* virulence (11, 13, 33). Alternatively, BrnQ1 and BrnQ2 may be active in different growth conditions other than iron starvation for which BrnQ3 could be normally active. The reason why BrnQ3 may be specialized to function under low iron conditions and also for its high frequency of appearance as a virulence determinant candidate, may be due to the possible role of this branched-chain amino acid transporter as a means of an alternate heme acquisition system in *S. aureus* (and possibly in other bacteria). Perhaps during normal circumstances, the presence of the Isd system masks

the expression of an alternate heme uptake system in iron-restricted media. The increased lag-phase seen with the Newman *Δisd brnQ3::Tn197* double mutant may be because cells are not receiving the necessary amounts of BCAA that are required to make intermediate steps required by an alternative heme uptake system (115). However, the eventual restoration of growth may be explained by the *brnQ3* mutation in resulting in the derepression of CodY regulated genes (due to lack of a transporter for BCAAs and therefore an overall decrease in BCAA within the cell) that have a function in altering or activating the expression of secondary heme uptake systems (115).

As mentioned, large-scale screens haves identified that mutations in *brnQ3* can have an impact on virulence of *S. aureus*. We note that in our mouse challenge experiments, the *brnQ3* mutant was significantly attenuated in the heart but not other organs. Interestingly, this mutant had significantly higher counts in the liver than the other strains tested. The phenotype of hyper-virulence in the liver is reminiscent of that observed for a *S. aureus* HrtA mutant (139) that has recently been proposed to be due to *S. aureus* protein-mediated membrane-damage that increases expression of immunomodulatory proteins (4). Although speculative at this point, it is possible that the *brnQ*::Tn*917* mutant, which was used in the virulence experiment, expresses a mis-folded BrnQ3 protein in the membrane that results in a similar phenomenon as that observed by the Skaar group. This possibility is currently under investigation by other members of our laboratory. Furthermore, it is interesting that the accumulation of transposon-recombinant BrnQ protein inside the cell did not pose any toxicity to *S. aureus* and this is likely since the transposon mutant can still be complemented with a wild-type copy of brnQ in trans whilst the unnatural fusion protein continues to be

synthesized. Furthermore, it is clear that the combination of *isd* deletion and *brnQ3* mutation diminishes the virulence of *S. aureus* in several organs from our infection experiment. It is difficult to assess at this point if this is a result of the delayed ability to acquire host heme, or is the result of more pleiotropic effects associated with decreased capacity to import BCAAs. In addition, mice infected with Newman *Δisd* alone lost more weight when compared to mice infected with wildtype Newman cells. Also, kidneys from mice injected with Newman *Δisd* alone had more abscesses and more bacterial load than kidneys with wildtype Newman (Fig. 15 and Table 5.) The results from both weight loss and kidney abscess is in agreement with our *in-vitro* data; Newman *Δisd* consistently grew better and reached a higher final OD than did wildtype cells (Fig. 9). Furthermore, mice infected with Newman *Δisd brnQ3::Tn197* double mutant lost significantly less weight and had less bacterial load in the kidneys, livers, and hearts than mice injected with wildtype Newman cells (Fig. 15 and Table 5.). Again, this is in agreement with the results obtained from our *in-vitro* studies using the Newman *Δisd brnQ3::Tn197* double mutant (Fig 10). Further studies are required to better define the overall impact of these mutations on *S. aureus* biology.

The results obtained in this study prove fascinating for future researchers as it shows heme acquisition in *S. aureus* is much more complicated and can be intimately tied with additional nutritional networks that are part of the basic bacterial physiology. It appears that many intricate systems can contribute to heme uptake for we have yet to obtain an *S. aureus* mutant phenotype that is absolutely debilitated to grow on heme as the sole iron source. Furthermore, CodY may play an unexpected role as a key regulator in heme uptake and that this branch of nutritional acquisition may not have been

experimentally linked to CodY's realm of gene regulation. It will be interesting to see how a *codY* mutant behaves in the presence of heme as the sole iron source. As well, it will be interesting to investigate why a double mutant lacking both BrnQ3 and the Isd system displays the phenotype shown here. Also, we would like to determine what roles do BrnQ1 and BrnQ2 play in this story. Surely, more experiments need to be conducted in order to address the questions raised from this study. Most importantly, research in bacterial heme acquisition may now turn its attention towards better understanding the roles of branched-chain amino acid transporters and the global transcriptional regulator CodY in heme acquisition.

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