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Molecular mechanisms of *Staphylococcus aureus* iron acquisition

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Abstract

The unique redox potential of iron is ideal for use as a cofactor in diverse biochemical reactions. Iron is therefore vital for the growth and proliferation of nearly all organisms, including pathogenic bacteria. Vertebrates sequester excess iron within proteins in order to alleviate toxicity and restrict the amount of free iron available for invading pathogens. Restricting the growth of infectious microorganisms by sequestering essential nutrients is referred to as nutritional immunity. In order to circumvent nutritional immunity bacterial pathogens have evolved elegant systems that allow for the acquisition of iron during infection. The Gram-positive extracellular pathogen *Staphylococcus aureus* is a commensal organism that can cause severe disease when it gains access to underlying tissues. Iron acquisition is required for *S. aureus* colonization and subsequent pathogenesis. Herein we review the strategies *S. aureus* employs to obtain iron through the production of siderophores and the consumption of host heme.

Introduction

Staphylococcus aureus are Gram-positive non-motile cocci, clinically distinguishable by a golden hue and the ability to clot vertebrate blood. *S. aureus* is often found as a part of the skin microflora and innocuously colonizes the nares of a third of the world's population (42, 55). Upon breaching the epithelium this extracellular pathogen can cause severe ailments including bacteremia, pneumonia, osteomyelitis, endocarditis, and septic shock (40). Moreover, *S. aureus* develops resistance to antibiotics at a remarkable pace presenting a significant clinical challenge. Methicillin resistant *S. aureus* (MRSA) has recently become a serious problem in the clinical setting, highlighted by the fact that mortality due to MRSA infection has surpassed HIV-associated mortality in the United States (11). The decreasing efficacy of available antibiotics underscores the need to increase our understanding of the fundamental processes that promote *S. aureus* pathogenesis, as these processes could represent targets for novel therapeutics.

In the late 1800s Alexander Ogston discovered *S. aureus* cocci in pus isolated from an abscess (1), establishing the formation of tissue abscesses as a pathological hallmark of *S. aureus* infection. Within the abscess, *S. aureus* is confronted with a robust host immune response and an environment devoid of essential nutrients (13, 17). Nutrient iron is required for *S. aureus* growth and persistence within abscesses and hence must be acquired during infection (13, 71, 80, 82). Most vertebrate iron is utilized as a cofactor in biochemical reactions that occur intracellularly. This intracellular pool of iron is generally not available to extracellular pathogens such as *S. aureus*. Moreover, the amount of free iron found within

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the serum is negligible, as it is almost always complexed to high-affinity iron binding proteins. This process of iron sequestration by the host, also referred to as nutritional immunity, inhibits the growth of invading microorganisms (9, 84). In response to this severe iron limitation, *S. aureus* has evolved sophisticated strategies to obtain iron required to proliferate within vertebrates. This review seeks to provide a comprehensive analysis of the pathways *S. aureus* utilizes to obtain iron during infection.

Iron is sequestered within vertebrates

S. aureus is a commensal organism that can inflict life-threatening damage upon its host if it is able to gain access to underlying tissues. The ability of *S. aureus* to colonize nearly every major vertebrate organ underscores the considerable public health threat posed by this organism. *S. aureus* is the number one cause of heart and skin infections, the number one cause of soft tissue infections, the leading cause of hospital acquired infections, and a primary cause of bacterial pneumonia (7, 9, 28, 35, 47, 84). Each organ presents a unique challenge to colonization. Factors such as oxygen tension, organ-specific immune responses, and the availability of nutrients influence the outcome of staphylococcal pathogenesis.

Iron acquisition has been referred to as the “critical determinant” deciding the outcome of the host-pathogen interaction (84). Greater than 90% of the iron in mammals resides intracellularly and is therefore not a viable source of iron for extracellular pathogens unless it can be liberated from host cells (24). Extracellular iron is bound by high affinity iron-binding proteins such as transferrin, found in the serum, and lactoferrin, found in the lymph and mucosal secretions. These glycoproteins have a high affinity for free iron. *S. aureus* indirectly steals iron from lactoferrin or transferrin through the production of siderophores. Siderophores are secreted small molecules that have an extremely high affinity for iron and out-compete host iron-binding proteins. Siderophore-iron complexes are recognized by cognate receptors on the bacterial surface permitting the theft of iron from lactoferrin or transferrin.

In addition to being bound by proteins, iron is also complexed to the tetrapyrrole ring of heme. Heme represents 80% of iron within the host and is the preferred iron source of *S. aureus* (71). The most abundant hemoprotein within vertebrates is hemoglobin which binds four molecules of heme and is contained with circulating erythrocytes. In order to access this rich source of iron, *S. aureus* lyses erythrocytes through the secretion of hemolysins resulting in the liberation of hemoglobin. The host counters the displacement of hemoglobin through the action of the high-affinity hemoglobin binding protein haptoglobin. Haptoglobin is plentiful within the serum and becomes more abundant during inflammation (56). The hemoglobin-haptoglobin complex is one of the strongest noncovalent interactions reported in serum ensuring that the complex does not dissociate until the proteins and heme are recycled in the liver (37). Despite the strength of the hemoglobin-haptoglobin complex, *S. aureus* is capable of binding this complex and utilizing it as a source of iron (78).

Damage to erythrocytes also results in the release of free heme from liberated hemoglobin. To prevent bacterial access to this iron source, hemopexin binds heme with high affinity and traffics it to the liver where it is endocytosed by hepatocytes and cleared from the serum (77). This interaction favors the host as *S. aureus* cannot use the hemopexin-heme complex as a source of iron (78). *In vitro* studies have validated heme, hemoglobin, and haptoglobin-hemoglobin as sources of iron that support *S. aureus* proliferation. Scanning electron microscopy has established that erythrocytes are present with abscesses, confirming that *S. aureus* has abundant access to this critical nutrient source during tissue colonization (13). Iron homeostasis in the host is strictly regulated to ensure that iron is available for essential biochemical reactions while simultaneously preventing iron-associated toxicity and bacterial

growth. Animal models have confirmed that disrupting host iron homeostasis increases the severity of *S. aureus* infections (31). Moreover, *S. aureus* infections in the healthcare setting are often associated with patients that have alterations in their iron status. Liver transplantation can result in iron overload, as the liver is the primary source of iron in the human body (24). The mortality rate in liver transplant patients that suffer from *S. aureus* bacteremia is over 80% (69). It is clear that disruptions in iron homeostasis are not only detrimental to the host, but also lead to increased *S. aureus* disease due to the promotion of bacterial replication during infection. The following sections provide a detailed overview of our current understanding of the mechanisms *S. aureus* employs to acquire iron during infection.

S. aureus responds to iron starvation through the activity of the transcriptional regulator Fur

S. aureus responds to the iron-restricted environment of the host by dramatically altering its protein expression profile. This change in protein expression is mediated by the iron-dependent *f*erric *u*ptake *r*egulator (*fur*) (29, 80). In the presence of iron, Fur binds a consensus DNA sequence known as the *fur* box, found upstream of *fur*-regulated genes. When iron becomes limiting, Fur releases from the DNA, alleviating Fur-mediated transcriptional repression (5). This results in transcriptional repression of the Fur regulon when *S. aureus* is iron replete and de-repression when *S. aureus* is iron starved. Hence, *S. aureus fur*-deficient strains exhibit a gene expression profile that mirrors that of iron starved organisms. *In vivo* imaging of *S. aureus* infected mice has demonstrated that Fur-regulated genes are expressed in heart and kidneys abscesses, suggesting that staphylococci are starved for iron in these organs (60, 63). A comparison of cytoplasmic protein profiles between wild type *S. aureus* and an isogenic *fur* mutant found twenty staphylococcal proteins that are more abundant in the absence of Fur, suggesting that these proteins are negatively regulated by Fur (29). In addition to iron acquisition systems, this analysis revealed an increase in glycolytic and fermentative enzymes, indicating that *S. aureus* modulates its metabolism in order to adapt to the iron-starved environment of the host. Under iron-limiting conditions *S. aureus* increases fermentative metabolism, resulting in the production of lactate. Secretion of lactate lowers the pH of the microenvironment and the affinity of transferrin for iron (29). These data demonstrate that *S. aureus* alters the host environment in a way that promotes the release of iron from host proteins presumably increasing iron availability.

Fur regulates the expression of staphylococcal virulence factors that are involved in attachment to host cells, biofilm formation, and manipulation of host wound healing (3, 12, 36, 38). Fur also regulates the expression of secreted cytolytic and immunomodulatory toxins, which play a profound role in decreasing the host immune response to favor bacterial survival (54). In *fur* mutants the cytolytic toxins are more abundantly expressed while immunomodulatory toxins are decreased compared to wild type cells. This altered exoprotein profile leads to an increase in susceptibility to neutrophil killing and consequently, reduced virulence in a murine pneumonia model of infection (79). These findings highlight the importance of Fur to staphylococcal pathogenesis.

S. aureus siderophore production

Extracellular iron complexed to host proteins, such as transferrin and lactoferrin, are targets for the iron scavenging activity of siderophores. Siderophores are small molecules that are secreted by bacteria and have an exceptionally high affinity for iron. *S. aureus* produces two distinct siderophores named staphyloferrin A and staphyloferrin B that share many properties (Fig. 1A). Bacterial siderophore biosynthesis proceeds through two different

pathways, the non-ribosomal peptide synthetase pathway and the non-ribosomal peptide synthetase independent pathway (NIS). Both staphyloferrin A and staphyloferrin B are synthesized via the NIS pathway and are part of the carboxylate family of siderophores. The genes involved in the biosynthesis and import of both staphyloferrin A and B are regulated by Fur and are therefore maximally expressed in iron-limiting environments (29, 45). These siderophores have been synthesized *in vitro* and the chemically synthesized siderophores restore the iron scavenging defects of mutants unable to produce the respective siderophore. The presence of a third *S. aureus* siderophore named aureochelin has been suggested but this molecule has not been characterized (19). Supernatants isolated from mutants unable to synthesize both staphyloferrin A and staphyloferrin B are unable to support the growth of wild type *S. aureus* in an iron-depleted environment (6). This finding highlights the importance of staphyloferrin A and B to *S. aureus* iron acquisition and calls into question the relevance and/or existence of aureochelin. *In vitro* studies have demonstrated that siderophore-mediated iron acquisition is the dominant mechanism by which *S. aureus* steals iron from transferrin, suggesting that *S. aureus* does not produce a transferrin receptor involved in iron acquisition (58).

Staphyloferrin A

The importance of staphyloferrin A to *S. aureus* iron acquisition was demonstrated by the discovery that mutants unable to produce staphyloferrin B sustain limited growth in serum (6). A bioinformatic screen looking for homologues of known NIS synthetases in *S. aureus* identified the genes that encode for staphyloferrin A production, the *sfnABCD* operon (6, 18). Genetic and biochemical approaches subsequently confirmed the assignment of *sfnABCD* as encoding for staphyloferrin A synthesis (6, 18). Inactivation of *sfnABCD* does not attenuate growth in serum, but inactivation of *sfnABCD* in a mutant unable to produce staphyloferrin B severely impairs *S. aureus* growth in serum (6). Biochemical experiments revealed that staphyloferrin A has a mass of 479 Daltons and is composed of a molecule of D–ornithine that links together two molecules of citrate via amide bonds (41). Mixing citrate, D-ornithine, ATP, SnfB and SnfD is sufficient for the *in vitro* synthesis of staphyloferrin A (18). Consistent with the structure of staphyloferrin A, addition of D-ornithine to *S. aureus* growth media results in the increased production of staphyloferrin A (51).

ABC transporters specific for siderophores are often found in close proximity to the genes responsible for siderophore production. Genes encoding the ABC transporter HtsABC are found adjacent to the *sfn* operon; however HtsBC has been reported to transport heme (Fig. 1A). In a series of elegant genetic experiments, Beasley *et al.* determined that the HtsABC transporter is also required for staphyloferrin A import (Fig. 1A)(6). These results suggest that HtsABC might be a promiscuous system involved in the transport of multiple iron sources.

The crystal structure of HtsA, the lipoprotein receptor of staphyloferrin A, supports its involvement in siderophore transport (6, 34). This structure represents the first siderophore receptor to be structurally characterized from a Gram-positive bacterium. The conformational change that highlights HtsA staphyloferrin A binding leads to an unprecedented ligand entrapment (34). Given the structural features of HtsA-staphyloferrin A binding it is unlikely that heme is a ligand for HtsA. This suggests that an alternative lipoprotein acts as a receptor for heme transport through HtsBC although this supposition remains to be experimentally verified.

Staphyloferrin B

The structure of staphyloferrin B is distinct from that of staphyloferrin A (Fig. 1A). Staphyloferrin B is an alpha-hydroxycarboxylate siderophore composed of L-2,3-diaminopropionic acid (Dap), 1,2-diaminoethane (Dae), and α-ketoglutaric acid with a molecular mass of 448 Daltons. The synthesis of staphyloferrin B is encoded by the *sbnABCDEFGHI* operon (14). *In vitro* synthesis of staphyloferrin B requires only the NIS synthetases SbnC, SnbE, SbnF and the decarboxylase SbnH. These proteins synthesize staphyloferrin B when mixed with ATP, magnesium, Dap, citrate, Dae and α-ketoglutaric acid (14). Inactivation of *sbnE* leads to a decrease in bacterial load in the kidneys of systemically infected mice, underscoring the importance of staphyloferrin B-mediated iron acquisition to pathogenesis (20). Staphyloferrin B import is mediated by the *s*taphylococcal *i*ron *r*egulated transporter (s*irABC*) (21). SirBC is predicted to be the membrane permease that supports the transport of staphyloferrin B into the cytoplasm. SirA is the lipoprotein receptor component of the staphyloferrin B import system and SirA undergoes a conformational change leading to the enclosure of staphyloferrin B in the binding pocket, in a manner analogous to HtsA (33). The fact that staphyloferrin A and B bind their cognate lipoprotein receptors with dissociation constants in the low nanomolar range underscores the exquisite evolution of this organism to obtain iron from its host during infection.

As staphyloferrin A and B are likely the only siderophores produced by *S. aureus* it is possible that these molecules perform unique roles during infection. Staphyloferrin A and B may scavenge iron from different host proteins or reach maximal iron-binding capacity at different sites of colonization. Alternatively, the metabolic pathways that supply the precursors for the biosynthesis of each siderophore may be favored at specific sites of colonization. Future work will refine the role of each siderophore during infection.

Transport of siderophores across the staphylococcal membrane

Transport of molecules across the membrane requires energy and therefore ABC transporters are typically associated with an ATPase. One unusual feature of *sirABC* and *htsABC* is that neither operon encodes a putative ATPase (72). The *f*erric *h*ydroxymate *u*ptake operon *fhuCBG* encodes a putative ATPase named *fhuC* (also referred to as *fhuA*). FhuC is the ATPase necessary for transport of staphyloferrin A and B (6, 72). This finding was established by the observation that growth of an *fhuCBG* mutant is inhibited in an iron-deplete environment and cannot be restored by the addition of supernatants containing either staphyloferrin A or B. Growth of this mutant can be complemented by *fhuC* provided *in trans* and supplementation with supernatant containing either staphyloferrin A or B (6). Therefore, inhibiting FhuC could have debilitating effects on the ability of *S. aureus* to procure iron during infection, establishing FhuC as a promising therapeutic target.

Despite the fact that *S. aureus* has not been demonstrated to produce hydroxymate-type siderophores, this organism has the capacity to utilize these siderophores as a source of iron. The ability to scavenge hydroxymate siderophores produced by other bacteria, also known as xenosiderophores, may allow *S. aureus* to establish residence amongst the microbiome. The uptake of xenosiderophores by *S. aureus* is also dependent upon *fhuCBG* (65). The lipoprotein receptors FhuD1 and FhuD2 are required for *fhuCBG*-mediated xenosiderophore uptake (Fig. 1A). The two genes encoding FhuD1 and FhuD2 (*fhuD1* and *fhuD2*) are located within distinct loci in the *S. aureus* genome (Fig. 1C). Unlike SirA and HtsA, FhuD1 and FhuD2 undergo only a modest conformational change upon siderophore binding (66, 67). This feature of the lipoprotein receptors probably reduces the affinity for any one siderophore, but facilitates a broad spectrum binding ability for many xenosiderophores. *S. aureus* can therefore out-compete other bacteria by stealing xenosiderophores while

retaining staphyloferrin A and B-mediated iron uptake. This feature likely contributes to the observation that *S. aureus* is a significant clinical problem for patients that have been administered siderophore-based therapy to treat iron-overload disease. For example, *S. aureus* is the leading cause of bacterial infection in thalassemic patients that have been treated with the siderophore desferrioxamine to reduce iron overload after a blood transfusion (8, 62). Since FhuD1 and FhuD2 increase the variety of iron sources that can be utilized by *S. aureus*, inhibiting these receptors could reduce the competitive advantage of *S. aureus* in bacterial communities and decrease the degree to which this pathogen is found as part of the normal flora. Considering that most hospital-acquired staphylococcal infections originate from the patients normal flora, this decolonization strategy would have significant clinical impact (83).

Heme uptake in staphylococci

Heme represents the primary reservoir of iron within vertebrates and *S. aureus* can fulfill its iron requirement by obtaining iron from heme (24). In fact, *S. aureus* preferentially imports and utilizes heme-iron when grown in the presence of both transferrin and heme or hemoglobin, suggesting that heme-iron is the preferred source of iron during infection (71, 78). Hemoglobin is the most abundant hemoprotein in vertebrates making this protein an attractive source of iron for *S. aureus*. The ability to lyse erythrocytes through the secretion of hemolysins promotes the release of hemoglobin from this intracellular reservoir. In this regard, *S. aureus* can utilize intact erythrocytes as sole iron source *in vitro* (78). These experiments suggest that *S. aureus* encodes systems dedicated to the acquisition of heme-iron bound to erythrocyte hemoglobin.

S. aureus heme and hemoglobin receptors

Gram-positive bacteria are shielded from the environment by a thick cell wall, composed of the disaccharide *N*-acetylmuramic acid-(β1-4)-*N*-acetylglucosamine (MurNAc-GlcNAc) and the cell wall tetrapeptide (L-Ala-D-isoGln-L-Lys-D-Ala). Chains of MurNAc-GlcNAc are bound to the cell wall peptide via an amide bond between MurNAc and the L-Ala. In *S. aureus*, the glycan chains and the cell wall tetrapeptide are cross-linked via a pentaglycine crossbridge producing a continuous layer of peptidoglycan (30) (23). This network of peptidoglycan protects the cytoplasmic membrane from environmental insult and is the region of the bacterium that directly interfaces with the host. Sortase A (SrtA) is a transpeptidase that covalently anchors proteins to the cell wall of *S. aureus* (48–50). SrtA substrates are characterized by a hydrophobic domain, followed by an LPXTG motif and a charged tail located at the C-terminus of the protein. Surface localized proteins are linked to the cell wall via SrtA-mediated cleavage of the LPXTG domain between the threonine and glycine, and a subsequent transpeptidation between the modified LPXTG domain and the glycine peptide of an immature peptidoglycan subunit (for review of *S. aureus* SrtA see ref. 53). *S. aureus srtA* mutants are severely attenuated in a murine abscess model of infection (14, 49).

The thickness of the cell wall presents a considerable barrier to the import of nutrients such as heme-iron. Heme was the first molecule for which a mechanism of import through the Gram-positive cell wall was elucidated. Much of what is currently known about heme import was initiated by a genome wide search for proteins homologous to SrtA. This search uncovered a second sortase encoded within *S. aureus* named sortase B (SrtB). SrtB is also a transpeptidase; however this enzyme anchors proteins that contain an NPTQN motif in place of LPXTG. The genomic region surrounding *srtB* contains several features suggestive of a heme utilization system that has since been named the *i*ron regulated *s*urface *d*eterminant (Isd) system. The *S. aureus* Isd system is encoded within five operons: *isdA*, *isdB*,

isdCDEFsrtBisdG, *isdH*, *orfXisdI* (Fig. 1C). A consensus *fur* box is located upstream of each of these operons and therefore all of these genes are iron-regulated (49, 63). IsdA, IsdB, and IsdH each contain an LPXTG motif and hence are covalently anchored to the cell wall by SrtA. IsdC contains an NPQTN motif and is anchored to the cell wall by SrtB. IsdDEF is a membrane localized ABC transporter complex, suggesting a role in transporting iron-containing molecules across the cytoplasmic membrane (48). IsdA, IsdB, IsdC, IsdD, IsdE, IsdG, and IsdI bind heme (48, 70). Heme uptake into *S. aureus* requires SrtA, SrtB, IsdA, and IsdE (48). The current model for Isd-mediated heme import proposes that IsdA, IsdB, and IsdH are surface-exposed hemoprotein receptors that pass heme to IsdC. IsdC then transports heme through the cell wall to the membrane localized IsdDEF ABC transport system (Fig. 1B).

The transfer of heme between the Isd heme receptors to IsdE has recently been described *in vitro*. IsdA, IsdB, IsdH, and IsdC contain NEAr iron Transporter (NEAT) domains. NEAT domains are conserved stretches of 125 amino acids found within proteins that neighbor putative iron transporters (2). Each NEAT domain binds one heme molecule within a groove that coordinates binding to the heme-iron via a tyrosine residue (32, 68). IsdA and IsdC each contain one NEAT domain, while IsdB contains two NEAT domains. Heme is transferred unidirectionally from NEAT domain 2 of IsdB to the NEAT domains of either IsdA or IsdC. Heme is also transferred in a unidirectional manner from the NEAT domain of IsdA to the NEAT domain of IsdC (46). Transfer of heme through the cell wall-localized Isd proteins IsdA, IsdB, or IsdC to the membrane-localized heme receptor IsdE was confirmed *in vitro* using magnetic circular dichroism (MCD) and electrospray ionization mass spectrometry (ESI-MS). The NEAT domain of IsdC can transfer heme to IsdE. Heme is not transferred between IsdA and IsdE (52). These *in vitro* studies provide a structural basis for heme-binding and transport through the cell wall that can be used to develop competitive inhibitors that disrupt this process.

In vivo heme is complexed to proteins. Hemoglobin represents an abundant reservoir of heme that is targeted by the *S. aureus* hemoglobin receptor IsdB (78). The haptoglobin-hemoglobin complex is also a viable source of iron for *S. aureus* and this complex is bound by IsdH (25, 27, 78) (Fig 1. B). IsdH contains three NEAT domains (25). Structural analysis of the IsdH NEAT domains revealed that NEAT domain 1 binds both hemoglobin and haptoglobin. NEAT domain 2 binds hemoglobin with nanomolar affinities and NEAT domain 3 weakly binds heme. Heme can be transferred in a bidirectional fashion from NEAT domain 3 of IsdH to NEAT domain 2 of IsdB, and in a unidirectional fashion to the NEAT domains of IsdA, IsdC, and IsdE (52, 86).These results suggest a model whereby IsdH strips heme from the haptoglobin-hemoglobin complex through the sequential activity of the NEAT domains and transfers heme to IsdB, IsdA, IsdC or IsdE (Fig. 1B) (26, 59).

The *in vitro* experiments defining heme transfer via the NEAT domains of IsdH, IsdB, and IsdA implies that these proteins interact in such a way that allows for the transfer of heme between the proteins *in vivo*. Indeed IsdA and IsdB are colocalized at the cell surface in an iron-dependent fashion (60). Mild iron starvation results in the visualization of discreet IsdA and IsdB foci, while more severe iron starvation leads to the nearly circumferential distribution of both proteins. Recent findings suggest that subcellular protein localization in Gram-positive cocci can be dictated by a YSIRK/GS motif encoded within the N-terminus of the protein (10, 22). The signal sequence of IsdB contains a YSIRK/GS motif while IsdA does not. Visualization of newly synthesized IsdB using gold-labeled IsdB antibodies and electron miscopy revealed that IsdB is found specifically localized to the sites of cell division. IsdA localization is more ubiquitous but a significant portion of IsdA is also found at the site of cell division (60). Despite the more uniform nature of IsdA localization, its contact with IsdB has been detected using immuno-coprecipitation highlighting the strength

of this interaction. Hemoglobin binding to the cell surface also colocalizes with IsdB at discrete iron-regulated foci. In keeping with the role of IsdB and IsdH as hemoprotein receptors, inactivation of *isdB* or *IsdH* reduces hemoglobin binding to *S. aureus*. In fact, mutating *isdH* or *isdA* in an *isdB* background severely abrogates the hemoglobin binding activity of the cell. These results highlight the cooperative nature of hemoglobin binding between these proteins and provide support for the *in vitro* observations that heme can be transferred between IsdH, IsdB, and IsdA.

Heme transport into the cytoplasm

Compared to our understanding of the transport of heme through the cell wall, much less is known about the molecular details involved in transporting heme across the membrane. The current model of IsdDEF function is that the IsdE lipoprotein receives heme from IsdC. IsdE then passes heme to IsdF, the ABC permease, which transports heme through the membrane using energy provided by the ATP hydrolyzing activity of IsdD. This model is supported by experiments showing IsdE and IsdD bind heme and apo-IsdE receives heme from the NEAT domain of IsdC (32, 48, 52). However, inactivation of *isdDEF* impairs, but does not abolish, *S. aureus* growth on heme as the sole iron source. This implies that an additional membrane transporter(s) imports heme-iron. A homology search for alternative ABC-type iron transporters revealed a previously uncharacterized *fur*-regulated transport system that was named *hts* (*h*eme *t*ransport *s*ystem). The *htsABC* system, like *isdDEF*, comprises a membrane-associated lipoprotein (HtsA) and two putative ABC transporters (HtsB and HtsC). HtsB and HtsC have significant homology to HmuU, the heme transport permease of *Corynebacterium diphtheriae*. Inactivation of *htsB* and *htsC* reduces the ability of *S. aureus* to import heme-iron, which results in a decrease in bacterial burden in the liver and kidneys of mice infected with these mutants (71). The binding pocket of HtsA is a large basic patch of positively-charged amino acids, making it difficult to envision that HtsA binds heme, a hydrophobic molecule (34). A plausible explanation for these two apparently conflicting findings is that HtsBC could interact with IsdE to transport heme and with HtsA to transport staphyloferrin A. HtsABC does not encode an ATPase, and FhuC was been implicated as the ATPase involved in siderophore transport [65]. The protein that provides the energy needed for HtsBC to transport heme across the membrane is unknown. Inactivation of *isdE* together with *htsA* leads to a significant reduction in virulence demonstrating the importance of these transporters to *S. aureus* survival in the host (47).

Release of iron from heme

In order to utilize heme as a source of iron, bacterial pathogens must have mechanisms of opening the macrocyclic conjunction of heme to release the iron atom. *S. aureus* encodes two cytoplasmic heme-degrading proteins within the Isd system named IsdG and IsdI. *isdG* is co-transcribed along with the other genes in the *isdCDEFsrtBisdG* operon (70). *isdI* is an intrachromosomal paralogue of *isdG* that is also Fur-regulated and is encoded in a bicistronic operon with an open reading frame of unknown function (70). The amino acid sequences of IsdG and IsdI share 64% identity and the crystal structures of IsdG and IsdI can be superimposed with a root mean square deviation of 1.0 Å, underscoring the significant similarity between these proteins (85). IsdG and IsdI were the first identified members of a family of heme degrading enzymes that bears their name, the IsdG-family of heme oxygenases.

The degradation of heme is a reaction that occurs in both eukaryotes and bacteria. Before the discovery of the IsdG-family of heme oxygenases, all characterized heme degradation reactions resulted in the production of biliverdin, carbon monoxide, and iron. In vertebrates, biliverdin is then further reduced to the potent antioxidant bilirubin by biliverdin reductase

(75). Three major features distinguish the IsdG-family of heme oxygenases from the classical heme oxygenase family. First, IsdG and IsdI are structurally distinct from the classical heme oxygenases (85). Second, the binding of heme to IsdG or IsdI results in the severe distortion of the planarity of heme to the point where it appears ruffled (44). This represents the highest degree of heme distortion for any known heme-binding protein and plays a significant role in the third distinctive feature of IsdG-family members: IsdG and IsdI degrade heme to a novel chromophore. Heme degradation catalyzed by the IsdG-family of heme oxygenases results in the production of a mixture of β- and σ-isomers of oxo-bilirubin, molecules collectively referred to as staphylobilin (Fig. 1B) (64). The heme degradation reactions occur *in vitro* in the presence of an electron donor, such as NADPH-cytochrome P450 (70). The electron donor used to catalyze IsdG-family-mediated heme degradation *in vivo* is unknown. *isdGI* mutants are attenuated in the murine model of systemic infection implicating heme degradation and staphylobilin formation as being important for pathogenesis (63).

The physiological significance of encoding two seemingly identical enzymes has been the subject of considerable research and has revealed subtle differences in the regulation of IsdG and IsdI. Expression of both *isdG* and *isdI* is transcriptionally regulated by Fur (63). IsdG stability is also dependent on the presence of heme such that IsdG is degraded in the absence of heme while IsdI is not (63). These results suggest that *S. aureus* can tailor the amount of heme degradation in order to fulfill the nutrient iron requirement of this organism. Targeting IsdG-family enzymes has significant clinical potential due to the requirement for these enzymes during colonization and the absence of this enzyme family within humans.

Despite the fact that the primary function of heme uptake systems is to satisfy the nutrient iron need of *S. aureus*, appreciable heme is brought into the cell during iron replete conditions. In this case, *isdG* and *isdI* are not expressed and heme is instead distributed intact to the bacterial membrane (71). It is likely that this exogenously acquired heme is destined for proteins involved in respiration since heme is an essential cofactor for proteins involved in the transfer of electrons. Consistent with this model, the respiratory pathways of some Gram positive pathogens rely entirely on the ability of the bacteria to scavenge heme. Taken together, these results imply that *S. aureus* differentially utilizes heme depending upon its metabolic needs. In conditions of iron starvation, exogenously acquired heme is degraded to release free iron. In contrast, when iron is abundant, heme is acquired and utilized intact to populate cytochromes of the electron transport chain. This reduces the requirement for endogenous heme synthesis and in effect decreases the metabolic burden on the bacterium. How iron levels within the host impact the subcellular fate of heme within *S. aureus* remains to be uncovered.

The response of *S. aureus* to heme toxicity

Heme is used as a cofactor in many biological systems due the redox potential of the encircled iron atom. However, this redox active molecule can also be toxic at high levels. This creates a paradox for microorganisms that utilize heme as a source of iron. *S. aureus* resolves this paradox by adapting to heme toxicity, as evidenced by its resistance to lethal levels of heme when first exposed to sub-lethal concentrations of heme (81). A mechanistic explanation for this adaptation was provided by the observation that *S. aureus* exposed to sub-lethal concentrations of heme up-regulates an ABC transport system 45-fold compared to untreated controls (29). Based on the heme responsiveness of this system, it has been named the *h*eme-*r*egulated *t*ransporter (*hrtAB*) (Fig. 2). The ATPase of this system (*hrtA*) is encoded in a bicistronic operon with the predicted permease (*hrtB*), and both are required for adaptation to heme-mediated toxicity. The ATPase activity of HrtA is influenced by various physiochemical conditions including: the concentration of ATP, temperature, pH, and metal

cofactors. Mutants of *hrtA* that are unable to hydrolyze ATP are unable to adapt to heme toxicity. These findings mechanistically link the ATPase activity of HrtA with the alleviation of heme toxicity (73).

While the ATPase activity of HrtA has been characterized, the permease activity of HrtB has yet to be confirmed. Moreover, the substrate of HrtAB and the mechanism by which HrtAB detoxifies heme are unknown. The current model predicts that HrtAB acts as an efflux pump that expels a toxic metabolite that accumulates as a result of heme exposure. HrtB expression in the absence of HrtA results in a decrease in the integrity of the membrane and an altered protein secretion profile (4). This phenomenon has a dramatic effect on the virulence of *S. aureus* (discussed below).

The up-regulation of HrtA in response to heme is dependent on a two-component system known as the *h*eme *s*ensor *s*ystem (*hssRS)* (Fig. 2). Bacterial two-component systems are typically comprised of a response regulator and a histidine kinase. These systems are critical for the proper physiological response to environmental cues. In the case of HssRS, the environmental cue is heme toxicity and the physiological response is the up-regulation of *hrtAB*. Heme exposure activates the histidine kinase, HssS, resulting in autophosphorylation. HssS then transphophorylates HssR, the response regulator component of the system. Phosphorylated HssR binds to a direct repeat sequence within the *hrtAB* promoter in order to induce transcription of *hrtAB* (74) (Fig. 2). Expression of *hrtAB* is dependent on HssRS and inactivation of either *hssS* or *hssR* results in an inability to adapt to heme toxicity. Despite the fact that heme triggers HssRS-dependent *hrtAB* activation, the ligand that induces the initial autophosphorylation of HssS is unknown. Identification of the ligand for HssRS will provide much needed information regarding the mechanisms by which heme promotes cellular toxicity, a finding which will have broad biological implications.

The role of heme acquisition during infection

Considering the requirement for iron during infection, the Isd system plays a major role during pathogenesis. In addition to binding heme, IsdA is capable of binding a wide variety of host proteins, promotes adherence to cells in tissue culture, and acts in concert with IsdB to promote resistance to neutrophil killing (16, 57). Because IsdA binds multiple host proteins, the specific contribution of IsdA-mediated heme-binding to infection has been difficult to assess. Inactivation of *isdA* leads to decreased ability to colonize human skin and a decrease in bacterial load in murine kidneys five days post systemic infection (14, 15). These results suggest that IsdA is important for the colonization and persistence of *S. aureus* in these two host environments. IsdA is expressed in the hearts and livers of infected animals, but *isdA* mutants display wild type levels of colonization in both of these organs. Heme transport via IsdA may be overshadowed in this infection model because *in vitro* data have demonstrated the NEAT domains of IsdH and IsdB can bind heme and transfer it directly to IsdC or IsdE (52). Similar to the *isdA* mutant, inactivation of *isdC* leads to only a modest decrease in bacterial loads in infected kidneys after five days of infection (14). In light of the *in vitro* evidence demonstrating that heme can be passed directly from IsdH and IsdB to the heme lipoprotein receptor IsdE it remains possible that heme transport across the cell wall could occur via this route *in vivo* (Fig. 1B).

IsdB appears to be the lynchpin of heme acquisition *in vivo. S. aureus* inactivated for *isdB* display a log decrease in the number of bacteria recovered from the spleen and kidneys following systemic infection of mice (60). IsdB expression is prominent in the heart of infected animals and colonization of the heart by the *isdB* mutant is reduced by two orders of magnitude (60). This latter result suggests that hemoglobin recognition is particularly

important to cardiac colonization. Inactivation of *isdH* alone does not affect bacterial burden in this model implicating IsdB as the critical hemoprotein receptor during systemic infection. The importance of IsdB to *S. aureus* infection combined with the surface exposed localization of the protein establishes IsdB as a potential vaccine candidate. Mice immunized with purified IsdB exhibit greater survival after subsequent challenge with *S. aureus* than mock-immunized mice (43). Full protection is observed when the IsdB vaccine includes the cell wall anchored proteins IsdA, SdrD, and SdrE (76). Moreover, antibodies against IsdA and IsdB rescue animals from lethal staphylococcal challenge and inhibit abscess formation (39). Future studies will focus on determining the efficacy of this vaccination strategy in human subjects.

Animal models of infection are limited due to the fact that virulence factors expressed by human pathogens have evolved to bind human-specific molecules. Advances in the genetic manipulation of mice have made it possible to engineer “humanized” mice that express human versions of proteins that are targeted by bacterial virulence factors. This technology has been used to investigate the interaction between *S. aureus* and human hemoglobin. There is a considerable amount of divergence between the surface exposed amino acids of human and murine hemoglobin. These amino acids likely represent the binding platform for the *S. aureus* hemoglobin receptor IsdB. This is supported by the observation that the affinity of IsdB for human hemoglobin is significantly higher than the affinity for mouse hemoglobin. Moreover, human hemoglobin is more readily utilized as an iron source by *S. aureus* in culture. In keeping with this, the burden of *S. aureus* is increased by an order of magnitude in the hearts and livers of mice expressing a human hemoglobin transgene following systemic infection (61). This increase in virulence is dependent on IsdB, as *S. aureus* mutants inactivated for *isdB* proliferate to similar levels in both wild type mice and mice expressing human hemoglobin. These results establish the human hemoglobin-expressing mice as a humanized mouse model that more accurately represents the nutrient environment encountered by *S. aureus* during infection. This infection model will be valuable for determining the contribution of hemoglobin-iron acquisition to colonization of a variety of infection sites, and in the search for inhibitors specific for the human-hemoglobin-IsdB interaction.

The last step to obtaining iron from heme is mediated by the heme oxygenases IsdG and IsdI. These two enzymes are differentially regulated at the posttranslational level through proteolytic degradation of IsdG in the absence of heme (63). A clue to the physiological importance of this differential regulation comes from the organic-specific colonization profile of *isdG* and *isdI* mutants. Inactivation of *isdG* alone or in combination with *isdI* results in a decrease in bacterial burden in both the heart and kidneys of infected animals. Inactivation of *isdI* leads to a decrease in bacterial burden only in the heart (63). The finding that both IsdI and IsdG are independently required for full virulence suggests that these enzymes have distinct roles during vertebrate colonization. In addition, the requirement for each of these enzymes during systemic infection establishes the heme degradation machinery as a target for therapeutic intervention.

Heme toxicity and infection

S. aureus strains inactivated for individual components of the Isd system are unable to satisfy their nutrient iron need and are therefore attenuated for growth within vertebrates. Conversely, an inability to cope with heme stress through inactivation of *hssR* and *hrtA* results in a hypervirulent phenotype. In a murine abscess model of infection *hssR* or *hrtA* mutants exhibit more overt symptoms of infection and the number of abscesses in the liver is elevated compared to mice infected with wild type *S. aureus* (81). The hypervirulence of *ΔhrtA* is specific to the liver and is due to increased expression of immunomodulatory

proteins in these mutant strains [98]. This results in an impairment in neutrophil migration and activation which promotes bacterial survival. The increased secretion of immunomodulatory proteins that leads to the hypervirulent phenotype is due to a loss of membrane integrity in the *hrtA* mutant (4). The mechanism leading to the hypervirulence of the *hssR* mutant has yet to be elucidated. The *ΔhrtA* phenotype highlights the delicate balance of the host-pathogen interaction and raises questions regarding the mechanism by which *S. aureus* senses and responds to membrane damage induced by the host immune response. The observation that orthologues of *hssRS* and *hrtAB* are conserved in many Gram-positive pathogens that come in contact with vertebrate blood provides further support that the ability to sense heme-dependent toxicity is important for pathogenesis.

Concluding remarks

Nutritional immunity protects the host from invading microorganisms by sequestering iron and other essential nutrients. To combat this immune strategy *S. aureus* utilizes a multifaceted approach that targets the most abundant iron reservoirs within the host. The efficiency with which *S. aureus* is able to overcome nutritional immunity is clinically relevant given the significant public health threat posed by this pathogen. The successful treatment of staphylococcal infections with antibiotics is proving to be difficult due to the prevalence of antibiotic resistant strains. Therefore, it is necessary to explore alternative strategies to combat this pathogen that is becoming resistant to all clinically relevant antibiotics.

The importance of iron acquisition systems to *S. aureus* virulence suggests these systems are viable targets for therapeutic intervention. *S. aureus* siderophores steal iron from host proteins while the Isd system consumes host heme and releases iron. Both pathways are expressed within the iron-limited host environment. The functional redundancy built into staphylococcal iron acquisition systems guarantees the pathogen obtains enough iron to successfully colonize a variety of diverse niches within the host. Colonization is decreased but not abolished when either pathway is inactivated, suggesting that both siderophore-iron and heme-iron are critical to pathogenesis. The finding that *S. aureus* grown in iron-replete conditions transports exogenous heme to the membrane intact implies that *S. aureus* has alternative uses for heme. Clearly there is much to be learned regarding the nutrient uptake and processing pathways that allow this pathogen to proliferate during infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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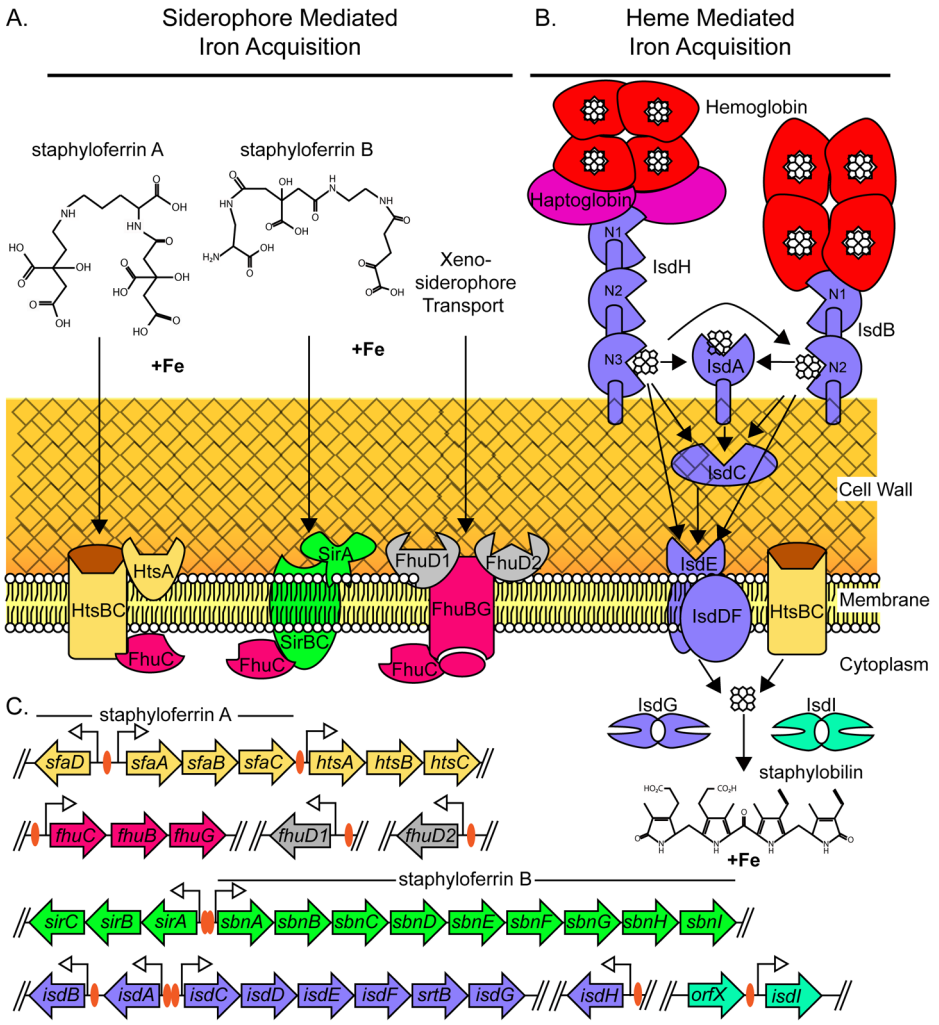

Figure 1. A model of the *Staphylococcus aureus* iron acquisition pathways

A. *S. aureus* produces two siderophores, staphyloferrin A and staphyloferrin B. Staphyloferrin A import is mediated by the HtsA lipoprotein and HtsBC permease. The SirA lipoprotein is the receptor for staphyloferrin B and the SirBC permease mediates the translocation of staphyloferrin B across the membrane. *S. aureus* imports xenosiderophores produced by other bacteria through the binding activity of FhuD1 and FhuD2 receptor lipoproteins and the FhuBG permease. The energy needed for siderophore uptake is provided by the FhuC ATPase. **B.** Heme acquisition is mediated by the Isd system. IsdH binds hemoglobin-haptoglobin and IsdB binds hemoglobin. Heme is passed through the NEAT domains of IsdH (N1-N3) IsdB (N1-N2), IsdA, and IsdC. Heme can also be passed from IsdH or IsdB directly to the IsdE heme-receptor lipoprotein. Heme transport across the membrane occurs through either the IsdDF or HtsBC permeases. Once in the cytoplasm heme is a substrate for the heme-degrading enzymes IsdG and IsdI. *S. aureus* degradation of heme leads to the release of iron and the production of staphylobilin. **C.** Genetic loci involved in *S. aureus* iron acquisition pathways. The *sfa* operon encodes the genes required for staphyloferrin A biosynthesis, while the genes within the *sbn* operon encode the staphyloferrin B synthesis genes. Promoter regions containing a consensus *fur* box are indicated with an orange oval. Genes are not drawn to scale.

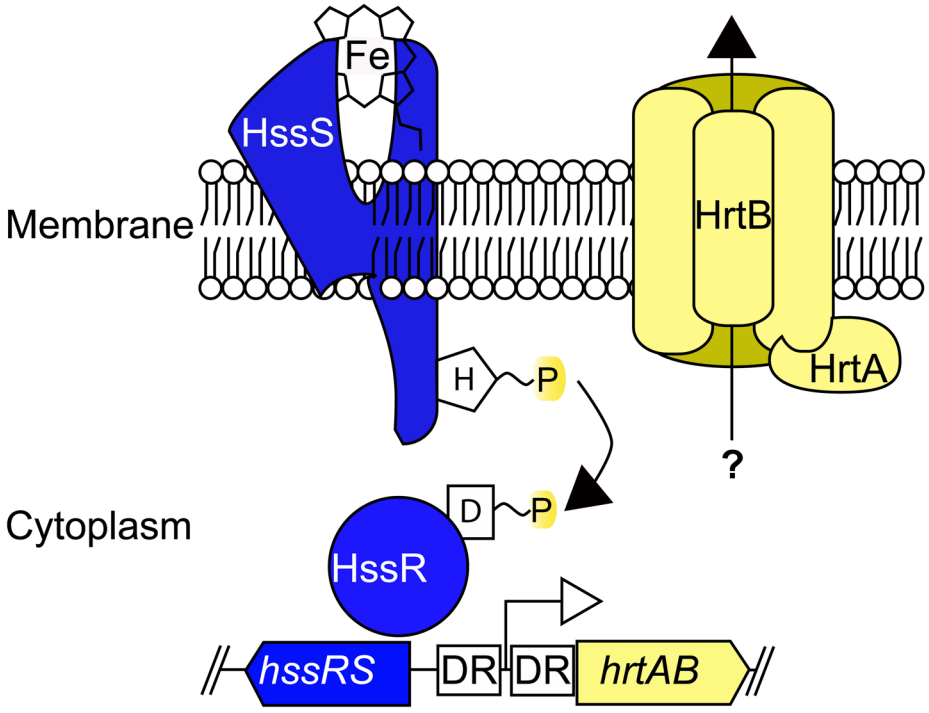

Figure 2. Sensing and alleviation of heme-associated toxicity

HssS senses exposure to heme through an unknown mechanism. This results in the autophosphorylation of HssS which is followed by a phosphorelay between histidine 279 of HssS and aspartate 52 of HssR. Phosphorylated HssR binds to a direct repeat sequence (DR) found within the *hrtAB* promoter region resulting in the expression of those genes. The HrtA ATPase and the HrtB permease form an ABC-type transport system that alleviates heme-mediated toxicity through an unknown mechanism.