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The Heme Sensor System of *Staphylococcus aureus*

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Abstract

The important human pathogen *Staphylococcus aureus* is able to satisfy its nutrient iron requirement by acquiring heme from host hemoglobin in the context of infection. However, heme acquisition exposes *S. aureus* to heme toxicity. In order to detect the presence of toxic levels of exogenous heme, *S. aureus* is able to sense heme through the heme sensing system (HssRS) two-component system. Upon sensing heme, HssRS directly regulates the expression of the heme-regulated ABC transporter HrtAB, which alleviates heme toxicity. Importantly, the inability to sense or respond to heme alters the virulence of *S. aureus*, highlighting the importance of heme sensing and detoxification to staphylococcal pathogenesis. Furthermore, potential orthologues of the Hss and Hrt systems are found in many species of Gram-positive bacteria, a possible indication that heme stress is a challenge faced by bacteria whose habitats include host tissues rich in heme.

Staphylococcus aureus Pathogenesis

Staphylococcus aureus is a Gram-positive bacterial pathogen that is responsible for a tremendous amount of morbidity and mortality [1]. *S. aureus* is the most frequent causative agent of hospital-acquired infections and community-acquired infections that necessitate admission to emergency rooms in the USA [2]. Antibiotic-resistant strains of *S. aureus* such as community-acquired methicillin-resistant *S. aureus* (CA-MRSA) are increasingly prevalent, even among otherwise healthy individuals [3, 4]. This point is underscored by the fact that MRSA infections are now responsible for more US deaths annually than the human immunodeficiency virus [3, 5]. Taken together, these facts have galvanized efforts aimed at identifying novel therapeutic targets against *S. aureus*. One area that has received significant attention in this regard is the identification of factors that allow *S. aureus* to adapt to and survive within the hostile host environment.

A commensal of the skin and anterior nares, *S. aureus* enters tissues of the host when epithelial barriers become compromised [6–8]. Following tissue invasion, *S. aureus* is able to cause infection of virtually any anatomical site leading to diseases ranging from skin and soft tissue infections to endocarditis, osteomyelitis, pneumonia, meningitis, and septicemia. Regardless of the site of infection, *S. aureus* invariably encounters host blood during infection. Blood is the bearer of cells of the immune system that are important for combating

staphylococcal infection, as well as the carrier of nutrients that invasive bacteria must acquire in order to colonize and proliferate within host tissues [9–11].

Staphylococcal Heme Metabolism

One nutrient that *S. aureus* must acquire in the context of infection is iron. Iron is required for a number of cellular processes including nucleotide biosynthesis, aerobic respiration, and resistance to reactive oxygen species [12, 13]. Within host tissues, free iron is actively maintained at concentrations that are too low to support the growth of *S. aureus* and most other bacterial pathogens [12]. Nonetheless, host tissues are rich in other forms of iron, such as iron in the context of the porphyrin heme. Heme is the cofactor for hemoglobin within erythrocytes and myoglobin within myocytes and therefore is highly abundant within the host [12, 14]. In order to satisfy its nutrient iron requirement during infection, *S. aureus* expresses a set of cell wall-anchored, membrane-associated, and cytoplasmic proteins that are devoted to the acquisition of iron from heme associated with hemoglobin [9, 10]. Designated the iron-regulated surface determinants (Isd) and the heme transport system (Hts), these factors bind hemoglobin, remove the cofactor heme, traffic heme across the cell wall, import heme into the cytoplasm, and degrade heme to release free iron to satisfy nutrient needs [9, 10, 15–17]. Mutation of Isd or Hts family members attenuates the virulence of *S. aureus*, highlighting the importance of heme acquisition to staphylococcal pathogenesis [17, 18].

The Adaptive Response of *S. aureus* to Heme

Conceptually, heme acquisition on the part of a bacterial pathogen raises an interesting paradox. Although a number of bacterial pathogens express systems dedicated to the acquisition of heme during infection, heme is a toxic molecule that is capable of generating reactive oxygen species and damaging cells. Therefore, biological systems that acquire, synthesize, or associate with heme must also possess systems devoted to the avoidance of heme toxicity [10, 19–22]. Given the significant association of *S. aureus* with blood during infection and the efficiency of the staphylococcal heme uptake machinery, it is likely that *S. aureus* expresses systems that protect this bacterium from the toxic side effects of heme acquisition during infection.

An initial indication that *S. aureus* may express a heme detoxification system(s) came from experiments designed to test whether *S. aureus* is sensitive to the toxic effects of heme and whether the bacterium is able to respond accordingly [23]. These experiments revealed two important observations. First, *S. aureus* is indeed sensitive to heme toxicity. When *S. aureus* is subcultured into medium containing a high heme concentration, no bacterial proliferation occurs as measured by growth curve analysis due to a bactericidal activity that heme exerts on *S. aureus* [23]. Second, *S. aureus* is able to sense the presence of exogenous heme and adapt to growth in its presence. This can be demonstrated by pre-exposing *S. aureus* to a sub-toxic concentration of heme, then subculturing the bacteria into medium containing an otherwise toxic heme concentration and monitoring bacterial proliferation. When staphylococci are pre-exposed to heme, appreciable growth occurs and cells are more resistant to the bactericidal activity of heme [23]. These results suggest that *S. aureus* is

capable of sensing and adapting to the presence of exogenous heme, presumably through a coordinated change in the expression of a heme detoxification system.

The Heme Regulated Transporter (HrtAB)

To test whether *S. aureus* expresses a heme detoxification system in response to growth in heme, a proteomics-based technique known as two-dimensional difference gel electrophoresis (2D-DIGE) [24, 25] was utilized to search for proteins that change in abundance when *S. aureus* is exposed to heme. 2D-DIGE allows a quantitative comparison to be made of the abundance of individual proteins in a complex sample across multiple conditions. Following quantitative comparison of proteins between samples, individual proteins that change in abundance in a given condition can be identified by mass spectrometry.

Using 2D-DIGE, 21 proteins that display alterations in abundance when *S. aureus* is grown in high heme were identified [26]. Most of these proteins exhibit modest 2-to 5-fold changes in expression, however a single protein is increased in abundance an impressive 45-fold when *S. aureus* is grown in heme. This protein was identified by mass spectrometry as the ATPase component of an uncharacterized staphylococcal ABC transport system [23, 26]. Ubiquitous amongst bacteria and eukaryotic organisms, ABC transporters consist of an integral membrane permease and a peripherally associated ATPase that together couple the cleavage of ATP to the translocation of small molecules, ions, and proteins across the cell membrane [27–29]. In order to reflect the heme-responsive expression pattern of the ATPase component, this particular ABC transporter was named the heme-regulated ABC transporter (*hrtAB*; *hrtA*, ATPase; *hrtB*, permease) [23, 26]. *HrtA* and *HrtB* are predicted to be co-transcribed, an indication that they work together to form a functional transport system.

To test whether HrtAB plays a role in the previously described adaptive response of *S. aureus* to heme toxicity, mutants that lack the members of the *hrtAB* operon were generated [23]. These mutants were tested for the ability to adapt to growth in an otherwise toxic heme concentration by pre-exposure to heme and subculture into medium containing a high level of heme. These experiments revealed that mutants that lack either *hrtA* or *hrtB* fail to adapt to heme toxicity [23]. Furthermore, these mutants are highly sensitive to the bactericidal effect of heme on staphylococci. These findings indicate that HrtAB is critical for the ability of *S. aureus* to adapt to and proliferate in toxic concentrations of heme. Together with the heme-regulated expression of HrtAB, these data point to a model in which *S. aureus* senses heme and upregulates the expression of HrtAB, which results in relief from heme toxicity [23]. Given the predicted function of HrtAB as an ABC transporter, it was initially hypothesized that HrtAB exports excess heme that may accumulate in the staphylococcal cytoplasm due to the efficiency of heme uptake by the Isd and Hts systems. However, it is equally likely that HrtAB does not export heme itself from the cytoplasm, but exports byproducts of heme toxicity or heme metabolites that accumulate in bacterial cells upon heme acquisition, and thus alleviates heme toxicity through this mechanism. In support of this later contention, mass spectrometry-based tracking experiments using isotopically labeled heme have not revealed a role for HrtAB in staphylococcal heme efflux [unpubl. data].

During systemic infections, *S. aureus* is intimately associated with vertebrate blood, the most abundant source of heme in the host. Furthermore, the staphylococcal Isd and Hts systems have evolved to rapidly and efficiently acquire heme as a nutrient iron source. To cope with the toxicity inherent to the rapid intracellular amassing of heme, *S. aureus* relies on the heme-dependent upregulation of HrtAB to avoid heme toxicity [9, 10, 23, 30]. In keeping with this, it would appear likely that a mutant strain of *S. aureus* with impaired resistance to heme toxicity would have a diminished ability to cause disease as such a strain might succumb to heme toxicity during infection. However, *S. aureus hrtA* displays a remarkable phenotype in a murine abscess model of systemic infection. Rather than being attenuated in its ability to infect mice and cause disease, *S. aureus hrtA* exhibits increased virulence [23]. Mice infected with *S. aureus hrtA* develop significant signs of disease early after infection and survive for a reduced length of time compared to mice infected with wild-type *S. aureus*. This increased virulence was further manifested upon autopsy, which revealed that infection with *S. aureus hrtA* leads to considerable liver abscess formation and increased bacterial burden in this organ. Surprisingly, this hypervirulence appears to be liver-specific as examination of other organs in the same infection model does not reveal differences in virulence between *S. aureus hrtA* and wild type.

A potential mechanistic explanation for the liver-specific hypervirulence of *S. aureus hrtA* surfaced during comparison of the immune response to *S. aureus* wild type and *hrtA*. Cell populations in the livers of mice infected with wild type and *hrtA* were subjected to FACS-based analysis. These experiments revealed no appreciable strain-specific differences in the immune response to staphylococci colonizing the kidneys and spleens of infected mice. Furthermore, examination of infected livers did not reveal any differences in the levels of a number of cells of the innate immune system including natural killer cells, dendritic cells, and invariant natural killer T cells [23]. However, significant differences between livers from mice infected with wild type and *hrtA* were observed in terms of the number of granulocytes and phagocytes present at the site of infection [23]. Specifically, livers of mice infected with *hrtA* harbor half the number of CD11b⁺/CD11c⁻ phagocytes and fewer CD11b⁺/Ly6G⁺ granulocytes than those from mice infected with wild type [23]. This is consistent with histological analysis of both sets of livers that revealed fewer polymorphonuclear cells present in tissue infected with *hrtA*. These results suggest that *S. aureus hrtA* either interferes with the recruitment of phagocytes and granulocytes to the site of infection or that *hrtA* exhibits an increased ability to kill these cell types as they migrate toward invading staphylococci.

S. aureus is notorious for its ability to combat cells of the immune system through the secretion of effector molecules that interfere with leukocyte or lymphocyte function [31]. One possible explanation for the increased ability of *S. aureus hrtA* to inhibit neutrophil recruitment to the site of infection is that when this heme-sensitive strain is under heme stress, it alters the quantity or the types of effector molecules that it secretes, thereby altering host cell function or survival. In order to test whether *S. aureus hrtA* exhibits a heme-dependent alteration in the amount or types of secreted effector molecules, microarray and secreted protein profile experiments were performed on *S. aureus hrtA* grown in the presence and absence of heme. These experiments revealed that upon exposure to heme, *S. aureus hrtA* dramatically increases the expression and secretion of a number of

immunomodulatory proteins that interfere with the recruitment of phagocytes to the site of infection [23, 30]. The secreted effector response of *S. aureus hrtA* to heme stress seems to specifically inhibit the recruitment of host immune cells to the site of infection rather than increase the lysis of these cells, as the expression of a number of cytolytic exotoxins in this strain is repressed [30]. This secreted protein response correlates with the observation that fewer neutrophils and granulocytes are present in abscesses formed by *S. aureus hrtA*, providing a possible explanation for the increased virulence of this strain [23]. These data are all consistent with a model in which *S. aureus hrtA* encounters host heme during infection, which induces heme stress and causes this strain to secrete effector molecules that interfere with host phagocyte recruitment and function [23, 30]. This enables *S. aureus hrtA* to form larger abscesses and reach higher levels than wild-type *S. aureus* in the liver.

It is unclear why the virulence of *hrtA* is perturbed only in the livers of infected mice and not in other organs; however, it is possible that *S. aureus* encounters greater heme stress when infecting the murine liver than the kidney or spleen. In this regard, it can be envisioned that hemoglobin catalysis in the liver following normal erythrocyte turnover as a result of intravascular hemolysis increases the amount of heme available for interaction with invading staphylococci. It should be pointed out that the heme stress experienced by *S. aureus* within the host must be at a level significant enough to alter virulence gene expression but not so dramatic as to negatively impact growth of *S. aureus hrtA* during infection. The fact that *S. aureus hrtA* specifically increases the expression of immunomodulatory virulence factors in response to heme stress raises the tantalizing possibility that regulatory circuits exist within staphylococci that respond to stress and are capable of activating a secreted protein response that specifically targets immune cell recruitment [30].

The ABC transporter HrtAB has been demonstrated to be critical for the ability of *S. aureus* to proliferate in otherwise toxic heme levels [23, 26, 30]. Perturbation of this system also increases the virulence of this important human pathogen [23]. However, the original observation of a heme-dependent increase in HrtAB expression that led to the discovery of this system is a particularly important finding, suggesting the presence of a regulatory system that responds to heme and alters gene expression accordingly [26]. In order to identify potential candidates for an HrtAB-regulating system, the genomic context surrounding *hrtAB* in the *S. aureus* chromosome was investigated for genes encoding potential regulatory systems. This simple genomics-based approach was conceptually feasible because bacterial regulatory systems are often genomically co-localized with their cognate target genes [32].

Interrogation of the *S. aureus* chromosome for potential HrtAB-regulating systems revealed the presence of two open reading frames immediately adjacent to *hrtAB* that are predicted to be transcribed in the opposite direction from *hrtAB* [23]. Importantly, these genes are annotated as members of a bacterial two-component system (TCS). This TCS is predicted to be composed of a membrane-localized histidine kinase and a cytoplasmic response regulator with a DNA-binding domain. In general, membrane-localized histidine kinases recognize a particular environmental signal, which is thought to induce a conformational change in the protein, leading to autophosphorylation on a conserved histidine residue within the cytoplasmic domain of the histidine kinase [33]. Response regulators catalyze

transphosphorylation from the histidine kinase they recognize to a response regulator aspartic acid residue, an event that increases the ability of these proteins to activate the transcription of target genes [34]. Interestingly, ABC transporter-TCS clusters have only been found in the Gram-positive Firmicutes and are generally thought to be involved in resistance to bacteriocins, which are antibacterial compounds made by one bacterial strain to inhibit the growth of other surrounding bacteria [32]. It is thought that bacteria that synthesize a particular bacteriocin encode a TCS that recognizes this compound, controlling a resistance response. However, another ABC transporter-TCS cluster in *S. aureus* (the GraRS/ApsRS TCS and the VraFG ABC transporter) has recently been found to be involved in the resistance of this organism to the toxicity of certain cell wall-active antibiotics as well as antimicrobial peptides [35–37]. These studies suggest that ABC transporter-TCS clusters may not be exclusively devoted to bacteriocin resistance, but may be involved in the resistance to toxic host compounds or antimicrobial agents.

The Heme Sensor System (HssRS)

Given the heme-regulated expression pattern of HrtAB and the fact that a TCS is found co-localized with *hrtAB* on the *S. aureus* chromosome, a model was envisioned in which this TCS responds to heme, thereby inducing the expression of HrtAB and initiating the *S. aureus* heme detoxification response. To test whether the *hrtAB*-proximal TCS is involved in the response of *S. aureus* to heme toxicity, mutant strains lacking either the histidine kinase or the response regulator gene were generated. As with *S. aureus hrtA*, inactivation of either the response regulator or histidine kinase leads to an inability to adapt to heme toxicity or proliferate in high heme concentrations [23]. Furthermore, a strain of *S. aureus* lacking the *hrtA*-proximal response regulator displays an increased bacterial burden in the murine liver upon infection, a similar phenotype observed for *S. aureus hrtA* [23]. This suggests that this TCS functions along with HrtAB to control the heme toxicity response of *S. aureus* during infection. This TCS was therefore designated the *heme sensor system* (*hssRS*; *hssR*, response regulator; *hssS*, histidine kinase) [23].

The fact that HssRS is required for the adaptation of *S. aureus* to heme toxicity as well as the predicted function of HssRS as a sensor system that regulates gene expression together imply that HssRS directly controls transcription of *hrtAB*. Three experiments were carried out to test this hypothesis. First, the level of *HrtAB* transcript was measured by RT-PCR in wild-type and *S. aureus hssR* grown in the presence or absence of heme. In wild-type *S. aureus*, heme induces an increase in the level of *HrtAB* transcript, suggesting that the heme-regulated expression of HrtAB occurs at the level of gene expression [23]. This heme-dependent increase in the *HrtAB* transcript is not observed in a *S. aureus* strain lacking the *hssR* gene, implying that the HssRS TCS is critical for the ability of *S. aureus* to increase the expression of *HrtAB* in response to heme. Second, a plasmid-based reporter construct was generated to indirectly monitor HrtAB expression by fusing the *hrtAB* promoter to the coding sequence for the XylE reporter enzyme. Wild-type *S. aureus* harboring this *hrtAB* promoter-*xylE* fusion exhibits a significant heme-dependent increase in reporter activity, further indicating that the heme-dependent expression of HrtAB occurs through an increase in synthesis of the *HrtAB* transcript driven by the *hrtAB* promoter [23, 38]. Furthermore, no heme-dependent increase in reporter activity occurs in *S. aureus hssR* or *hssS*,

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establishing the importance of the HssRS TCS in the regulation of HrtAB expression in response to heme [23, 38]. Third, 2D-DIGE was utilized to determine the abundance of all of the *S. aureus* cytoplasmic proteins in wild type and *hssR* grown in the presence and absence of heme [38]. This experiment revealed two important findings. First, HrtA is increased in abundance in response to heme in wild type but not in *hssR*, further establishing the importance of HssRS in the regulation of HrtAB expression [38]. Second, HrtA is the only cytoplasmic protein that depends on *hssR* for a heme-dependent increase in abundance [38]. This suggests that the HssRS TCS may specifically regulate the expression of HrtAB rather than a set of genes [38].

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These experiments demonstrate a dependence on HssRS for the response of *S. aureus* to heme toxicity through the regulation of HrtAB expression. However, they do not delineate the signaling events in *S. aureus* that connect HssRS to HrtAB expression. A series of experiments was therefore carried out to elucidate the mechanism by which HssRS regulates HrtAB expression in response to heme. Potential residues required for HssS-HssR signaling were identified by aligning the amino acid sequence of the predicted HssS cytoplasmic domain with the cytoplasmic domains from a number of known and characterized TCS histidine kinases. These experiments revealed a highly conserved histidine residue in HssS (His249) that aligns with the histidine residue known to be phosphorylated in response to signal recognition in other histidine kinases [38]. Similar alignments with HssR revealed a conserved aspartic acid residue (Asp52) that aligns with those residues known to undergo phosphorylation in other response regulators [38]. Based on the identification of these residues, it was postulated that upon signal recognition, HssS catalyzes autophosphorylation at His249 and that HssR subsequently catalyzes transphosphorylation to Asp52, activating the latter protein. To test this hypothetical signal transduction pathway, recombinant HssS and HssR as well as their predicted phosphotransfer site mutants were purified and tested for in vitro signaling. While HssS undergoes time-dependent autophosphorylation in these experiments, HssS mutated at its conserved histidine residue fails to undergo phosphorylation [38]. When HssR is added to phosphorylated HssS, HssR is able to catalyze transphosphorylation, indicating that these proteins are capable of phosphotransfer in vitro [38]. Furthermore, HssR mutated at its predicted phosphorylation site fails to catalyze transphosphorylation from HssS [38]. These experiments suggest that HssS undergoes autophosphorylation at His249 and that HssR catalyzes transphosphorylation to Asp52. However, these experiments were all carried out in vitro and therefore do not fully establish the importance of these signaling events in the functioning of HssRS in *S. aureus*.

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To this end, the HssS and HssR mutants that fail to undergo signaling in vitro were tested for functionality in vivo. This was accomplished by creating plasmids containing a full-length copy of either wild-type HssS or HssS mutated at His249, which is required for in vitro autophosphorylation. Strains harboring these plasmids were tested for the ability to rescue the heme-sensitive phenotype of *hssS* by growth curve analyses. Importantly, the plasmid bearing wild-type HssS rescues the growth of *S. aureus* lacking the *hssS* gene while the plasmid containing the mutant form of HssS that fails to undergo in vitro autophosphorylation is unable to rescue *hssS* [38]. These differences occur in spite of significant expression of both proteins. Furthermore, a similar result is seen with HssR: while wild-type HssR rescues *S. aureus* lacking the *hssR* gene, HssR mutated at the aspartic

acid residue required for in vitro phosphotransfer is unable to complement *hssR* even though both proteins are expressed by *S. aureus* [38]. Cumulatively, these results suggest that HssS-HssR signaling is essential for the ability of *S. aureus* to respond to the toxicity of heme.

Given the observation that HssRS is required for an increase in transcription of *hrtAB* driven by the *hrtAB* promoter and the fact that HssR is modeled to contain a DNA-binding domain, it was predicted that HssR binds directly to the *HrtAB* promoter upon phosphorylation. To test this prediction, recombinant HssR was phosphorylated in vitro and added to magnetic beads coated with DNA corresponding to the *hrtAB* promoter. Proteins that associate with the *hrtAB* promoter are precipitated along with the magnetic beads used in this assay and can then be eluted from the beads and analyzed by immunoblot analysis. It was found that phosphorylated HssR is able to bind to such beads coated with *hrtAB* promoter DNA [38]. Importantly, phosphorylated HssR does not associate with beads coated with *hrtAB* intragenic DNA and non-phosphorylated HssR does not bind to the *hrtAB* promoter in vitro [38]. Furthermore, HssR mutated at its phosphorylation residue is not able to bind to the *hrtAB* promoter [38]. These experiments suggest that upon phosphorylation, HssR binds directly to the *hrtAB* promoter. To test the in vivo relevance of these observations, pull-down experiments using DNA-coated magnetic beads similar to those described above were performed using cytoplasmic extracts from *S. aureus* grown in the presence and absence of heme. These experiments revealed that HssR binds to the *hrtAB* promoter when *S. aureus* encounters heme, and that no binding of HssR to the *hrtAB* promoter occurs when *S. aureus* is grown in medium lacking heme [38]. Together, these results suggest that HssS senses heme and initiates a signaling pathway that leads to HssR phosphorylation and binding of phosphorylated HssR to the *hrtAB* promoter.

Response regulators such as HssR that contain DNA-binding domains usually exhibit specificity for certain sites within the promoters of the genes they regulate. Such sites often consist of short direct repeat sequences to which the effector domain of a response regulator is thought to associate through a head-to-tail dimerization mechanism [34]. Accordingly, the *hrtAB* promoter was interrogated for direct repeat sequences as a means of identifying potential HssR-binding sites. These analyses revealed a perfect direct repeat sequence upstream of the *hrtAB* coding sequence (GTTCATATT(N₂)GTTCATATT) [38]. To test whether this direct repeat is involved in the response of the *hrtAB* promoter to heme-dependent activation by HssRS, the *hrtAB* promoter-*xyIE* fusion reporter construct was subjected to mutational analyses. Truncation of the *hrtAB* promoter up to this direct repeat fails to alter activity of the *hrtAB* promoter, but truncation of half or all of this repeat completely eliminates *hrtAB* promoter heme responsiveness [38]. Furthermore, mutation of four residues within the *hrtAB* promoter that are conserved in the *hrtAB* promoters of other species of staphylococci also eliminates the response of this promoter to heme [38]. This suggests that the *hrtAB* promoter direct repeat is required for the ability of this promoter to respond to heme through HssRS. In light of the fact that HssR binds directly to the *hrtAB* promoter, in vitro promoter-binding experiments were carried out to test whether HssR binds to this direct repeat. To this end, HssR was eluted from the *hrtAB* promoter with increasing concentrations of double-stranded oligonucleotides corresponding to either the wild-type *hrtAB* promoter direct repeat or a pseudo-direct repeat mutated at the four residues required

for the functioning of the *hrtAB* promoter in vivo. While the intact direct repeat elutes HssR, the mutated direct repeat fails to elute HssR from the *hrtAB* promoter [38]. These results suggest that upon phosphorylation, HssR binds to the direct repeat within the *hrtAB* promoter.

Together, the known features of HssS-HssR signaling and the mechanism by which these events impinge on the *hrtAB* promoter to regulate expression of HrtAB are shown in schematic form in figure 1. Nonetheless, a number of aspects of the HssRS and HrtAB systems remain to be elucidated. First, the transport substrate for HrtAB is unknown. Originally, it was proposed that HrtAB removes excess heme from the staphylococcal cytoplasm in order to alleviate heme toxicity [23, 30]. However, it is equally possible that a toxic molecule accumulates in *S. aureus* upon intracellular amassing of exogenously acquired heme. It can also be envisioned that breakdown of heme by *S. aureus* results in the buildup of toxic heme metabolites that must be exported from the staphylococcal cytoplasm. More studies focused on the function of HrtAB need to be carried in order to understand the mechanism of heme toxicity in *S. aureus* and the means by which HrtAB alleviates heme toxicity.

Another aspect of HssRS/HrtAB function that is not fully understood is the nature of the signal that is directly sensed by the histidine kinase HssS. Although HssS may sense heme by direct binding, HssS also could sense one of the toxic effects that heme has on the cell. Genomics-based approaches have not revealed a potential heme-binding domain in HssS, and the predicted sensing domain of this kinase has little sequence similarity to any protein with a known function [23]. Furthermore, no amino acids that typically engage in axial coordination of the iron atom of heme are conserved in the putative sensing domain of all of the potential HssS orthologues [unpubl. observations]. Identification of the ligand for HssS will lay the groundwork for a mechanistic understanding of how this histidine kinase is activated upon heme exposure. This would represent a significant advance for understanding HssS function and the mechanism employed by *S. aureus* to regulate the tight balance between heme acquisition and heme toxicity.

HssS represents one of the few histidine kinases that respond to a molecular marker of vertebrate tissue and one of two histidine kinases known to respond to heme. Importantly, other TCS that recognize heme are found in the Gram-positive pathogen *Corynebacterium diphtheriae*, a bacterium that is not closely related to *S. aureus* [39–42]. In *C. diphtheriae*, the TCS ChrAS and HrrAS are capable of responding to heme or hemoproteins [41]. However, in addition to the absence of any significant sequence identity between the staphylococcal and corynebacterial systems, two major aspects of ChrAS and HrrAS distinguish them from the *S. aureus* HssRS system. First, ChrAS and HrrAS both regulate expression of the *C. diphtheriae* heme oxygenase HmuO, while HssRS does not appear to regulate expression of the *S. aureus* heme oxygenases [38, 41, 42]. HmuO is capable of catabolizing heme and is therefore a critical factor required for heme metabolism in *C. diphtheriae* [43]. In *S. aureus*, IsdG and IsdI are iron-regulated heme oxygenases that also break down heme [10, 16, 44]. However, 2D-DIGE experiments have failed to reveal a role for HssRS in the regulation of IsdG/I expression, indicating that HssRS does not regulate these heme-degrading enzymes in *S. aureus* [38]. Second, despite the fact that HssRS from

S. aureus and ChrAS/HrrAS from *C. diphtheriae* both respond to heme, the histidine kinase HssS and the kinases ChrS/HrrS differ dramatically in terms of the predicted structure of their signal-sensing domains. While HssS is predicted to have an N-terminal periplasmic sensing domain flanked by two transmembrane helices, ChrS/HrrS are predicted to have sensing domains composed of five transmembrane helices flanked by short loops [23, 42]. This difference in predicted structure suggests that HssS may sense heme through a mechanism that is different from that of ChrS/HrrS or that these TCS might sense different effects that heme has on the Gram-positive bacterial cell. Interestingly, *C. diphtheriae* strains lacking the *chrAS* genes exhibit elevated heme sensitivity that is not dependent on reduced HmuO expression [39]. This suggests the presence of a ChrAS-regulated heme-detoxification system in *C. diphtheriae* [39]. It is tempting to speculate that *C. diphtheriae* encodes a system functionally analogous to the *S. aureus* HrtAB system that protects this organism from heme toxicity.

The ability of bacterial pathogens to sense and respond to heme is not limited to Gram-positive organisms. The Gram-negative mammalian pathogen *Bordetella pertussis* also encodes a heme sensing system [45–51]. In *B. pertussis*, the heme receptor BhuR binds to heme, transducing signals into the periplasm that activate the cytoplasmic membrane protein RhuR [46, 48, 50]. RhuR alters the activity of the cytoplasmic protein RhuI, which regulates the expression of the *B. pertussis* heme uptake system BhuRSTUV [46, 48, 50]. Through this mechanism, *B. pertussis* is able to sense exogenous heme and increase the expression of systems required for heme uptake from the host. Thus the *B. pertussis* RhuIR/BhuR system differs from the HssRS system of *S. aureus* in that the former is devoted to the sensing of host heme for the purposes of heme acquisition, whereas the latter is required for sensing host heme to avoid heme toxicity [23, 38, 45–51]. Furthermore, HssRS is a TCS, while RhuI is an extracytoplasmic sigma factor and RhuR is a regulator of RhuI activity. Thus, the mechanism by which heme is sensed by these systems as well as the details of signaling are likely distinct. It appears that the pathogens *S. aureus*, *C. diphtheriae* and *B. pertussis* are all able to sense host heme and transduce signals that alter gene expression, but that these events occur through distinct mechanisms and for different reasons.

Evolutionary Implications of Heme Sensing in Gram-Positive Bacteria

A role for the TCS HssRS and the heme-regulated ABC transporter HrtAB has been established in the ability of *S. aureus* to avoid heme toxicity as well as in the pathogenesis of this organism [23, 38]. Interestingly, a number of distinct Gram-positive bacteria also encode potential orthologues of these systems (fig. 2) [23]. Most of these bacteria are either pathogens or saprotrophs (microorganisms that break down tissues of dead animals) and thus are likely to encounter host heme. For example, *Bacillus anthracis*, the cause of anthrax infection, is a bacterium that has a significant bloodstream component throughout the late stages of systemic infection [52]. *B. anthracis* also is capable of inducing hemolysis and has been shown to acquire iron from heme, potentially exposing this organism to heme toxicity during infection [53–56]. Accordingly, *B. anthracis* encodes potential HssRS/HrtAB orthologues (fig. 2) and has been shown to adapt to heme toxicity [23]. Although the putative *B. anthracis* Hss and Hrt systems have not been experimentally proven to be functional in this organism or orthologous to the corresponding systems in *S. aureus*, these

initial observations suggest that *B. anthracis* must respond to heme toxicity at some point throughout its life cycle. Potential Hss and Hrt genes are also found in the pathogenic or saprotrophic Listeriae such as *L. monocytogenes* and *L. innocua* as well as other Bacilli including *B. cereus* and *B. thuringiensis* (fig. 2). *Exiguobacterium sibiricum*, an extremophile isolated from Siberian permafrost, also encodes potential Hss/Hrt systems (fig. 2). The details of the life cycle of this organism are currently unknown, but the fact that it encodes potential Hss/Hrt systems suggests that it may experience exogenous heme exposure in its natural habitat. Importantly, Hss and Hrt genes appear to be absent from the genomes of a number of non-pathogenic and non-saprotrophic Gram-positive bacteria including *B. subtilis* and *B. licheniformis*, a possible indication that Hss and Hrt are only required for the fitness of bacteria that encounter exogenous heme throughout their life cycles. The fact that Hss and Hrt genes are widespread throughout Gram-positive bacteria that associate with heme-containing tissues suggests that the ability of these organisms to overcome heme toxicity has been critical for their capacity to occupy different niches, whether pathogenic or saprotrophic. Furthermore, the fact that genes encoding Hss and Hrt are present in a number of human pathogens establishes the members of these systems as potential targets for therapeutic agents that could interfere with bacterial pathogenesis through the perturbation of heme metabolism. This idea is supported by the alteration in virulence observed upon inactivation of the staphylococcal Hrt systems.

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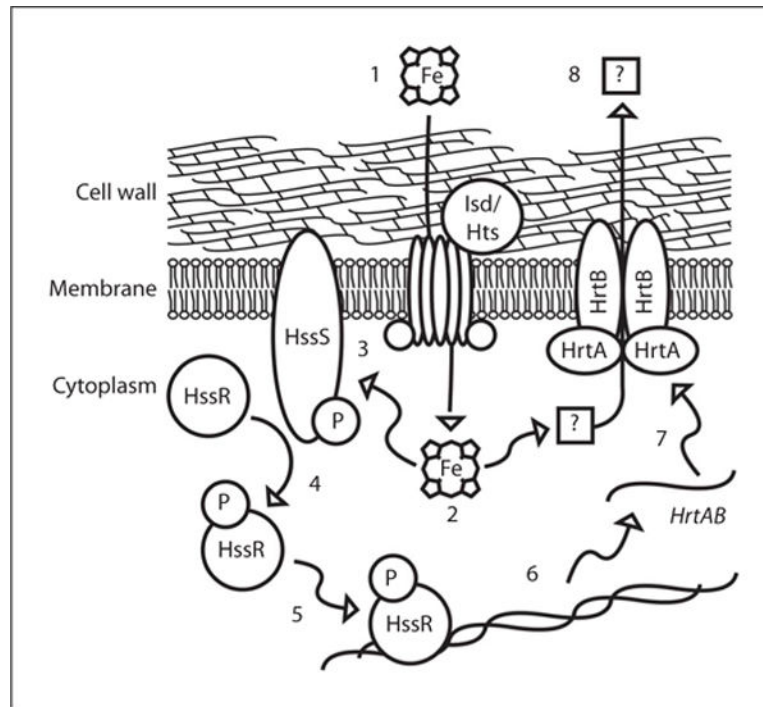


Fig. 1. Signaling events that connect heme sensing by the HssRS TCS to upregulation of HrtAB and heme detoxification: (1) Heme is imported into the staphylococcal cytoplasm through the actions of the Isd and Hts systems. (2) Heme activates the histidine kinase HssS directly through a receptor-ligand interaction, or indirectly through the heme-mediated accumulation of toxic metabolites. (3) Activation of HssS results in autophosphorylation and (4) transphosphorylation of HssR. (5) Phosphorylated HssR binds to a direct repeat sequence within the *hrtAB* promoter. (6) This increases the transcription of the *hrtAB* genes by recruitment of RNA polymerase. (7) Newly synthesized HrtAB alleviates heme toxicity (8) through the export of heme or an unknown molecule that accumulates in the *S. aureus* cytoplasm upon heme exposure.

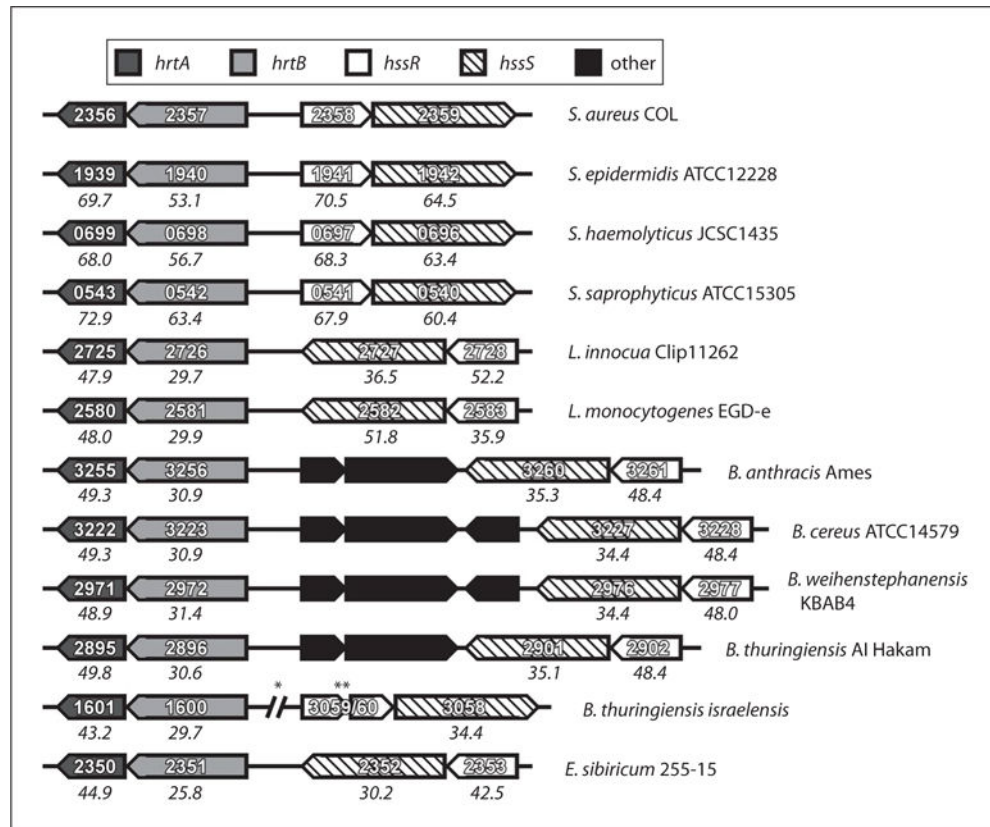


Fig. 2. Conservation of HssRS and HrtAB across Gram-positive bacteria. Sequenced bacterial genomes were interrogated for potential orthologues of *S. aureus* *hssRS* and *hrtAB* genes by BLAST analyses. The indicated bacterial strains were found to encode potential Hss/Hrt systems. Boxes indicate open reading frames and point in the direction of transcription. Dark grey boxes: HrtA; light grey boxes: HrtB, empty boxes: HssR, boxes with diagonal lines: HssS; black boxes: non-Hss/Hrt open reading frames (other genes). Numbers within boxes indicate the annotated gene number. Numbers below boxes are percent similarities with respect to the corresponding *S. aureus* COL Hss/Hrt gene. Percent similarities were calculated at the amino acid level using a Lipman-Pearson Protein Alignment (gap penalty = 4, gap length penalty = 12). Single asterisk denotes large separation between genes; double asterisk denotes that this potential HssR orthologue is split into two open reading frames in the genome of this species.