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Intracellular metalloporphyrin metabolism in *Staphylococcus aureus*

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Abstract The bacterial pathogen *Staphylococcus aureus* is responsible for a significant amount of human morbidity and mortality, and the ability of *S. aureus* to cause disease is absolutely dependent on the acquisition of iron from the host. The most abundant iron source to invading staphylococci is in the form of the porphyrin heme. *S. aureus* is capable of acquiring nutrient iron from heme and hemoproteins via two heme-acquisition systems, the iron-regulated surface determinant system (Isd) and the heme transport system (Hts). Heme acquisition through these systems is involved in staphylococcal pathogenesis suggesting that the intracellular fate of heme plays a significant role in the infectious process. The valuable heme molecule presents a paradox to invading bacteria because although heme is an abundant source of nutrient iron, the extreme reactivity of heme makes it toxic at high concentrations. Therefore, bacteria must regulate the levels of intracellular heme to avoid toxicity. Although the molecular mechanisms responsible for staphylococcal heme acquisition are beginning to emerge, the mechanisms by which *S. aureus* regulate intracellular heme homeostasis are largely unknown. In this review we describe three potential fates of host-derived heme acquired by *S. aureus* during infection: (i) degradation for use as a nutrient iron source, (ii) incorporation into bacterial heme-binding proteins for use as an enzyme cofactor, or (iii) efflux through a dedicated ABC-type transport system. We hypothesize that the ultimate fate of exogenously acquired heme in *S. aureus* is dependent upon the intracellular and extracellular availability of both iron and heme.

Keywords *Staphylococcus* · Heme · Isd · Metalloporphyrin · Bacteria

Since the acceptance of this manuscript, two papers have been published that are directly relevant to this review. The first describes IsdB as a hemoglobin receptor in S. aureus: J Bacteriol. 2006 Dec; 188(24): 8421–8429. The second identifies an IsdG-family member in a Gram negative bacterium: J Bacteriol. 2006 Sep; 188(18): 6476-6482.

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Introduction

The Gram positive bacterial pathogen *Staphylococcus aureus* causes a wide range of human diseases ranging from minor skin infections, such as folliculitis and impetigo, to more invasive diseases including endocarditis, sepsis, and toxic shock syndrome (Weems 2001). Importantly,

S. aureus is the leading cause of nosocomial disease and the incidence of multi-drug resistant isolates is increasing. In fact, up to 60% of hospital-acquired *S. aureus* isolates are methicillin-resistant and three cases of vancomycin-resistance have been reported in the United States to date (Todd 2006). This is especially alarming considering vancomycin is viewed as a last line of defense against multi-drug resistant *S. aureus*. These facts underscore the necessity of identifying novel targets for therapeutic intervention against this important human pathogen.

One potential method for combating *S. aureus* infection is to deny the bacteria nutrients required for survival. One such nutrient is iron, which is essential for bacterial growth and pathogenesis but scarcely available inside the human host (Bullen and Griffiths 1999; Braun 2001). Most bacterial cells need 10^5–10^6 iron atoms for growth due to a requirement for iron in numerous cellular processes including DNA synthesis, energy generation, and protection against reactive oxygen species (Bullen and Griffiths 1999; Braun 2001). However, 99.9% of iron in the human body is intracellular, and thus, not readily available to bacteria (Bullen and Griffiths 1999; Crichton 2001). Intracellular vertebrate iron is primarily a component of heme, or iron-protoporphyrin IX, which is typically bound to hemoglobin, the major hemoprotein within circulating erythrocytes (Crichton 2001). Heme is the reduced iron-containing porphyrin (Fe^{+2}), whereas the oxidized form (Fe^{+3}) is known as hemin. However, both are commonly referred to as ''heme'' so we will apply this convention throughout this review. Upon liberation from lysed erythrocytes, heme and hemoglobin are sequestered by hemopexin and haptoglobin in an effort to protect the host against toxicity caused by these reactive molecules. This process of heme scavenging by host proteins is extremely efficient, and under normal physiological conditions, free heme and hemoglobin are virtually undetectable in the serum (Bullen and Griffiths 1999). However, during invasive bacterial infections, hemolysin-mediated erythrocyte lysis likely leads to the local accumulation of appreciable amounts of heme and hemoglobin which can be used by the bacteria as a nutrient iron source. This idea is supported by the observation that hemopexin expression is increased upon bacterial infection (Klapper et al. 1972), potentially representing a response to the transient increase in free heme at the site of infection.

Upon release of hemoglobin from lysed erythrocytes, bacterial pathogens can utilize free hemoglobin or hemoglobin complexed to haptoglobin as nutrient iron sources (Wandersman and Stojiljkovic 2000; Heinrichs 2004). This process initiates with the direct binding of hemoproteins via specific bacterial receptors followed by removal and transport of the heme cofactor into the bacterial cell (Wandersman and Stojiljkovic 2000). Heme acquisition is required for *S. aureus* pathogenesis, and staphylococci preferentially acquire heme-iron over other physiologically relevant iron sources (Skaar et al. 2004b). The molecular machinery responsible for staphylococcal heme acquisition is encoded by two distinct systems, the iron-regulated surface determinant system (Isd), and the heme transport system (Hts) (Mazmanian et al. 2003; Skaar et al. 2004b).

The Isd heme import machinery is comprised of ten genes, each of which is regulated by the iron-dependent regulatory protein Fur (Mazmanian et al. 2003). Genes of the Isd system encode four cell wall anchored proteins (IsdABCH), a transpeptidase (SrtB), a membrane transport system (IsdDEF), and two cytoplasmic monooxygenases (IsdGI). Excluding SrtB, each protein component of the Isd system is capable of binding heme in vitro (Mazmanian et al. 2003; Mack et al. 2004). Additionally, IsdB and IsdH are responsible for the surface recognition of hemoglobin and hemoglobin-haptoglobin complexes by intact staphylococci (Dryla et al. 2003). Our model presumes that after the hemoprotein binds to its cognate surface receptor, the heme cofactor is removed from the hemoprotein by IsdA through an as-yet-uncharacterized mechanism. The heme is then transferred through the cell wall by IsdC and subsequently through the plasma membrane via the membrane transport system comprised of IsdDEF (Mazmanian et al. 2003). Heme can also transit into the cytoplasm through the membrane associated ABC-type transport system HtsABC, which is the more dominant heme transport

system *in vitro* (Skaar et al. 2004b). This implies that HtsABC, rather than IsdDEF, is the primary membrane heme transport system in *S. aureus* (Skaar et al. 2004b). The Hts system is composed of a lipoprotein (HtsA) and two permeases (HtsB and HtsC) which exhibit strong identity to permease components of the heme transport systems of *Yersinia pestis* (HmuU) and *Corynebacterium diphtheriae* (HmuU) (Thompson et al. 1999; Drazek et al. 2000). Heme acquisition by the Isd and Hts systems is a highly efficient process as demonstrated by the observation that staphylococci incubated with exogenous heme are saturated for heme uptake within 15 min (Mazmanian et al. 2003). The rapidity of heme uptake demonstrates the necessity of specialized molecular machinery capable of handling this valuable host iron source.

In addition to its value as a nutrient iron source, intact heme is a useful cofactor in numerous bacterial enzymes, such as cytochromes, catalases, and peroxidases. However, the reactivity of free heme presents a conundrum to pathogenic bacteria because heme is not tolerated well in its free form and therefore, excess internalized heme is toxic to invading bacteria. Keeping with this, bacterial pathogens have developed elaborate systems of managing internalized heme to prevent against heme-mediated toxicity while capitalizing on the value of heme as a nutrient source. In this regard, we have identified three potential fates of heme upon internalization by *S. aureus*: (i) the iron can be removed from the porphyrin ring by IsdG or IsdI for use as a nutrient source, (ii) the intact heme molecule can be complexed with membrane associated heme-binding proteins for use as an enzyme cofactor, or (iii) heme can be pumped out of the cell through a dedicated ABC-type efflux pump (Fig. 1). The ultimate fate of heme is presumably dependent upon the intracellular and extracellular availability of both iron and heme.

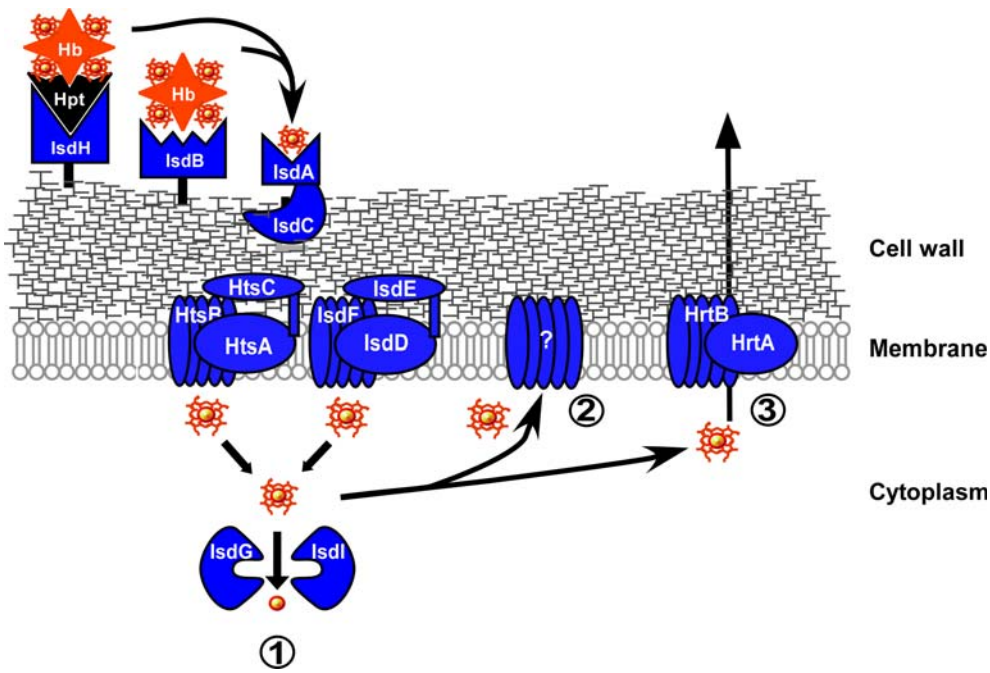

Fig. 1 The intracellular fate of heme. Once transported into the staphylococcal cytoplasm by the Isd or Hts system, there are three potential fates of heme: (1) Heme is degraded by the heme monooxygenases IsdG and IsdI to release free iron for use as a nutrient source, (2) Intact heme is complexed with membrane associated heme-binding factor for use as an enzyme cofactor, (3) HrtAB pumps excess heme or heme metabolites out of the cytoplasm to protect the bacteria from heme-mediated toxicity

Cytoplasmic heme degradation

When the intracellular iron concentration is low, bacteria can obtain iron for use in various metabolic pathways through the enzymatic degradation of exogenously acquired heme. Heme degrading enzymes have been identified in numerous pathogenic bacteria, including: *Corynebacterium sp*. (Schmitt 1997b), *Yersinia sp*. (Stojiljkovic and Hantke 1994), *Neisseria sp*. (Zhu et al. 2000b), *Pseudomonas aeruginosa* (Ratliff et al. 2001), *Escherichia coli* (Suits et al. 2005), *Bacillus anthracis* (Skaar et al. 2006) and *S. aureus* (Skaar et al. 2004a; Wu et al. 2005). Based on available structural data, bacterial heme degrading monooxygenases can be classified into three families, which we refer to as the HO-like family, the ChuS-family, and the IsdG-family of monooxygenases.

The first identified bacterial enzyme capable of degrading heme was HmuO from *C. diphtheria* (Schmitt 1997b). HmuO exhibits significant amino acid identity to vertebrate heme oxygenases, and consistent with this identity, HmuO cleaves heme to release α-biliverdin, carbon monoxide, and free iron (Wilks and Schmitt 1998). Structural analyses have revealed that HmuO is markedly similar to the structure of vertebrate HO-1, exhibiting a monomeric alpha-helical fold containing a conserved histidine residue at the N-terminus that is important for heme coordination (Schuller et al. 1999; Frankenberg-Dinkel 2004). Furthermore, HO-1 and HmuO both contain a single active site and GXXXG motif required for catalytic activity (Wilks and Schmitt 1998; Schuller et al. 1999). Based on these findings, we classify HmuO as a member of the HO-like family of bacterial heme monooxygenases. Additional members of this family have been identified in *Pseudomonas aeruginosa* (PigA) (Ratliff et al. 2001), *N. gononrrhoeae* (HemO) (Zhu et al. 2000b), and *N. meningitides* (HemO) (Zhu et al. 2000b).

A second enzyme family with reported heme degrading properties is represented by the ChuS protein from *E. coli* (Suits et al. 2005). ChuS shows no sequence or structural similarity to previously described heme oxygenases (HOs), however, orthologs of ChuS have been found in several other bacterial pathogens, including: ShuS in *Shigella* (Wilks 2001), HemS in *Yersinia* (Stojiljkovic and Hantke 1994), and PhuS in *Pseudomonas* (Lansky et al. 2006). In fact, the original description of HemS proposed that this enzyme encoded a protein with heme-degrading activity based on the increased sensitivity of a *hemS* mutant to excess heme (Stojiljkovic and Hantke 1994). Consistent with this, Suits et al. have reported that ChuS is a monomeric heme degrading enzyme (Suits et al. 2005). ChuS degrades heme in the presence of catalase and superoxide dismutase, demonstrating catalytic degradation of heme rather than non-enzymatic coupled oxidation (Suits et al. 2005). Structural analysis has shown that ChuS folds as a dimeric beta-barrel type protein comprised of a structural duplication in which both halves contain a predicted active site. Each half of the protein is independently capable of degrading heme, suggesting that the dual active sites are functional in the mature enzyme. In a conflicting report, Wilks et al. have proposed that ShuS (with 98% identity to ChuS) is a cytoplasmic heme shuttling protein rather than a heme oxygenase, because although it bound heme, the authors could not demonstrate enzymatic heme degradation in vitro (Wilks 2001). In addition, the authors reported that ShuS binds DNA, leading to the hypothesis that DNA binding by ShuS protects the cell from heme-dependent oxidative damage (Wilks 2001). However, a strain inactivated for *shuS* does not show increased heme-dependent sensitivity to oxidative stress as compared to wild type (Wyckoff et al. 2005). It has recently been reported that PhuS of *Pseudomonas* (with 41% identity to ChuS) is also a heme-trafficking protein (Lansky et al. 2006). PhuS facilitates the degradation of heme in vitro, however, contrary to the situation with ChuS, PhuS-dependent heme degradation does not occur in the presence of catalase. This result implies that PhuS-dependent heme degradation occurs through coupled oxidation and not enzymatic heme catalysis (Lansky et al. 2006). Based on these findings, the authors propose that PhuS acts as a transport protein that delivers heme to the heme oxygenase *pa*-HO (Lansky et al. 2006). More experiments are necessary to clarify the specific function and activity of this family of proteins; however, a role for ChuS-family

members in intracellular heme metabolism has been firmly established.

IsdG and IsdI, components of the Isd system discovered in *S. aureus*, are representative members of a third family of heme degrading enzymes which we have termed the IsdG-family of heme monooxygenases (Skaar et al. 2004a; Wu et al. 2005). IsdG and IsdI were originally investigated due to their genetic association with the Isd heme-transport system. Both IsdG and IsdI are iron-regulated cytoplasmically localized proteins that are 70% identical at the amino acid level. Pfam analysis places IsdG and IsdI in a family of monooxygenases responsible for the oxygenation of aromatic polyketides (Bateman et al. 2002; Wu et al. 2005). Based on these findings, it was predicted that IsdG and IsdI encode for iron-regulated heme-degrading monooxygenases in the staphylococcal cytoplasm. This hypothesis was confirmed upon the demonstration that IsdG and IsdI bind and degrade heme in the presence of reductant or reductase. This reaction releases free iron and occurs in the presence of catalase, ruling out the possibility that heme-degradation by IsdG or IsdI is occurring through a non-enzymatic coupled oxidation reaction (Skaar et al. 2004a). Taken together, these results confirmed that IsdG and IsdI represent the first identified members of a novel family of heme degrading enzymes.

IsdG-family members have exclusively been identified in Gram-positive bacteria, including: *S. aureus, S. epidermidis, B. anthracis*, and *Listeria monocytogenes* (Skaar and Schneewind 2004). We have demonstrated functional conservation of IsdG-family members across genera through investigations into the *B. anthracis* IsdG (BaIsdG) (Skaar et al. 2006). BaIsdG is 35% identical to the staphylococcal IsdG-family enzymes, and BaIsdG is the only identifiable IsdG-family member in the *B. anthracis* genome. BaIsdG enzymatically degrades heme to release free iron, and this activity is required to protect *B. anthracis* against heme-mediated toxicity (Skaar et al. 2006). This observation implies that IsdG family members represent an evolutionarily conserved mechanism utilized by Gram positive pathogens to protect against heme-mediated toxicity and obtain iron from host heme during infection.

The IsdG-family members are structurally very different from the HO-like and ChuS families of heme oxygenases in that they fold as a homodimeric beta-barrel type structure with two separate active sites (Wu et al. 2005). Moreover, mutational analyses revealed that the IsdG-family members degrade heme through the action of a catalytic NWH triad present in the active site of each monomer of the mature enzyme (Wu et al. 2005). The functional ramifications of two active sites is not known, however, it is possible that dual sites increases the efficiency of heme degradation, or alternatively, allows the enzyme to utilize different electron donors. Interestingly, IsdG and IsdI exhibit a striking structural similarity to ActVA-ORF6, a monooxygenase belonging to the same family of enzymes as IsdG and IsdI as predicted by Pfam analysis (Bateman et al. 2002; Sciara et al. 2003). ActVA-ORF6 is a tailoring enzyme responsible for the oxidation of aromatic polyketides in *Streptomyces coelicolor* (Sciara et al. 2003). Taken together, these structural findings suggest that convergent evolution has provided bacterial pathogens with distinct enzyme families capable of releasing iron from the macrocyclic conjunction of heme. On the other hand, divergent evolution appears to have altered the substrate specificity of IsdG and ActVA-ORF6 enzymes, likely due to the drastically different environments inhabited by staphylococci and streptomyces.

Until recently, all heme oxygenases were reported to degrade heme to one of four isomers of the blue-green molecule biliverdin (BV IX): BV IXα, IXβ, IXγ, or IXδ (Frankenberg-Dinkel 2004). However, a novel heme-degradation pathway was recently described in the blood-sucking insect *Rhodnius prolixus*, in which heme is chemically modified before oxidative cleavage of the tetrapyrrole and the resulting product is a dicysteinyl-BV IXγ (Paiva-Silva et al. 2006). We have found that the major heme degradation product of IsdG and IsdI does not exhibit the blue-green color consistent with the typical HO reaction product biliverdin, but rather is a light yellow (unpublished observation). Mass spectral analyses of isolated reaction products from both IsdG and IsdI-catalyzed reactions identified a

single molecule with a mass of 599 Da, supporting the colorimetric observation that the IsdG and IsdI major heme degradation product is not biliverdin, which has a mass of 582 Da in the same analysis. Tandem MS has revealed that the products of IsdG and IsdI-mediated heme degradation are identical and represent a novel heme degradation product (unpublished observation). Based on these observations, members of the IsdG family of heme monooxygenases evidently cleave the tetrapyrrole in a novel way, producing a slightly different product than either BV IX or the newly identified dicysteinyl-BV IXγ. We are currently undertaking further analyses to definitively identify the structure of this novel heme degradation product and to elucidate the exact catalytic mechanism of this class of heme monooxygenases. Identifying this novel heme degradation product will have important ramifications for staphylococcal biology as heme metabolism plays a vital role in the pathogenesis of *S. aureus* infections (Skaar et al. 2004b).

Exogenous heme as an enzyme cofactor

Although a valuable nutrient iron source, intact heme is potentially an important molecule to invading bacteria. This presumption is based on the requirement for heme as a cofactor in numerous enzymes involved in energy generation and protection against reactive oxygen species (Thony-Meyer 1997; Braun 2001). In this regard, heme is required in the bacterial plasma membrane as a cofactor for cytochromes of the electron transport chain (Thony-Meyer 1997). When iron is abundant in the environment, it is possible that bacteria use exogenously acquired heme as an enzyme cofactor, as it is thermodynamically favorable to import host molecules rather than synthesize a porphyrin ring de novo. This molecular hijacking hypothesis is supported by our observation that exogenous heme is acquired and segregated intact to the staphylococcal membrane when non-heme iron sources are concurrently available (Skaar et al. 2004b). Furthermore, using a radiolabeled heme molecule, it has been shown that heme added exogenously to growing cultures of *B. subtilis* leads to uptake and incorporation of heme into four distinct c-type cytochromes (Schiott et al. 1997). The molecular mechanisms responsible for trafficking heme to proteins of the Gram positive membrane have not been evaluated in detail. The machinery involved in this process is predicted to include a number of activities including systems responsible for heme and apocytochrome transport across the membrane, chaperones to assist in apocytochrome folding, and heme lyases responsible for incorporating heme into mature cytochromes.

A piece of the staphylococcal cytochrome synthesis puzzle was solved by the identification of a mutation in the *ctaA* gene which was found to inhibit the ability of *S. aureus* to survive prolonged starvation (Clements et al. 1999). CtaA is a heme O monooxygenase that converts heme O to heme A (Svensson and Hederstedt 1994), and therefore, inactivation of *ctaA* prevents the formation of heme A-containing cytochromes, which subsequently leads to an uncoupling of electron transport. This uncoupling causes a commensurate increase in oxidative stress when the bacteria are recovering from starvation conditions (Clements et al. 1999). These results demonstrate that heme is required in the membrane as a cofactor in cytochromes and is important for preventing the formation of toxic oxidizing products. Additional experiments are necessary to identify the specific factors that bind host-derived heme in the membrane and to elucidate the benefit afforded to bacterial pathogens by these molecular hijacking reactions.

Non-iron metalloporphyrins

Non-iron metalloporphyrins are comprised of the same tetrapyrrole backbone structure as heme with a substitution for the coordinated central metal ion. These molecules became of particular interest to bacteriologists upon the publication of a seminal paper by Igor Stojiljkovic and colleagues that demonstrated a potent antibacterial effect of non-iron metalloporphyrins against pathogenic bacteria, including several multi-drug

resistant clinical isolates of *S. aureus* (Stojiljkovic et al. 1999). It was hypothesized that these heme-like molecules act as ''molecular Trojan horses'' that enter bacteria through heme transport systems where they then exert their antibacterial affect in the cytoplasm or membrane (Stojiljkovic et al. 1999). The potent antibacterial effect of metalloporphyrins suggests that these molecules target a vital cellular process inside the bacterium. In turn, this suggests that the intracellular targets of metalloporphyrins may represent novel therapeutic options against bacterial infection. Keeping with this, we have investigated the intracellular fate of exogenously provided metalloporphyrins chelated with gallium, cobalt, manganese and zinc. As an initial measure of the fate of metalloporphyrin analogues inside *S. aureus*, we combined non-iron metalloporphyrins with purified IsdG and IsdI in vitro and analyzed degradation spectrophotometrically. IsdG and IsdI are both capable of binding all metalloporphyrins tested, as evident by the presence of a Soret band, indicative of porphyrin binding. However, IsdG and IsdI are not able to degrade non-iron containing metalloporphyrins in the presence of an electron donor (unpublished data). These data suggest that the toxic effect of metalloporphyrins on *S. aureus* is not due to release of the metal ion upon tetrapyrrole cleavage, but rather, due to some effect of the intact non-iron metalloporphyrin.

Previous investigations suggest that anaerobically grown *S. aureus* are resistant to metalloporphyrin toxicity (Stojiljkovic et al. 1999), implying that the mechanism of metalloporphyrin toxicity is related to aerobic respiration. In bacteria, the molecular machinery that carries out aerobic respiration is typically localized to the membrane and dependent on the presence of heme binding cytochromes (von Wachenfeldt and Hederstedt 1992; Thony-Meyer 1997). Based on this, we tracked metalloporphyrin metabolism in *S. aureus* in an effort to identify the subcellular fate of these compounds. Bacteria grown in gallium-protoporphyrin IX (Ga-PPIX) were fractionated and the intracellular localization of Ga-PPIX was determined using inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS). Because we have found that Ga-PPIX is not degraded by IsdG or IsdI, it can be assumed that the localization of the gallium ion reflects the localization of the intact metalloporphyrin. Furthermore, gallium is not typically present at detectable levels inside staphylococcal cells so any gallium detected in these cells represents acquired metalloporphyrin. In these preliminary experiments greater than fifty percent of the total cellular gallium was found in the plasma membrane fraction, supporting the hypothesis that metalloporphyrins are transported to and stored in the plasma membrane, where they likely exert their toxic effect (unpublished data). Unlike iron, which commonly exists in the +2 or +3 oxidation states, gallium can only exist as Ga^{+3}. Therefore, Ga-PPIX does not have the same oxidation potential as heme, which could render membrane-bound cytochromes unable to transfer electrons, resulting in the overproduction of oxidative radicals and observed toxicity. Interestingly, exposure to exogenous metalloporphyrin leads to a more potent antimicrobial effect than inactivation of the heme synthesis genes *hemB* or *ctaA* (von Eiff et al. 1997; Clements et al. 1999). More specifically, *S. aureus* strains inactivated for *hemB* are predicted to be unable to form mature cytochromes, yet they are capable of moderate growth rates on solid medium (von Eiff et al. 1997). In contrast, exposure to less than 1 μg ml^{-1} of gallium protoporphyrin IX abrogates staphylococcal growth in liquid media (unpublished data). This fact suggests that the intracellular toxicity of Ga-PPIX is more complicated than simply blocking aerobic respiration through cytochrome inactivation.

Heme efflux

As reviewed above, it is well established that heme and hemoproteins can serve as iron sources to a variety of pathogenic bacteria. This is due to the fact that many bacterial pathogens encode efficient heme uptake systems that allow the bacteria to bind and traffic heme-iron into the cytoplasm. Although heme (Fe-metalloporphyrin) is a valuable nutrient source to invading bacteria, the efficiency of heme-uptake systems can overpower the heme degradation machinery,

leading to the accumulation of toxic levels of heme or heme-metabolites. In fact, high intracellular concentrations of heme can result in oxidative damage due to its ability to catalyze peroxidase and oxidase reactions. These reactions lead to the generation of reactive oxygen species which subsequently damage DNA and proteins (Everse and Hsia 1997). In the past several years it has become evident that heme can be toxic to a variety of bacteria including *S. aureus, Streptococcus faecalis, B. cereus, N. meningitides, Mycobacterium smegmatis, Y. enterocolitica, S. dysenteriae*, and *C. diphtheria* (Ladan et al. 1993; Stojiljkovic et al. 1999; Bibb et al. 2005; Rasmussen et al. 2005; Wyckoff et al. 2005). Thus, for infecting bacteria, the utility of heme as a nutrient source must be balanced against its toxicity at high concentrations.

The avoidance of heme-mediated toxicity is particularly important during situations where pathogens are in contact with high levels of exogenous heme, such as might be encountered during blood-borne infections. The means by which bacterial pathogens avoid the toxic effects of intracellular heme accumulation is not well understood, however, research into the Gram negative genus *Neisseria* has revealed multiple mechanisms by which this bacterium avoids heme-mediated toxicity. These include heme degradation through the heme oxygenase HemO (see above) (Zhu et al. 2000a; Zhu et al. 2000b), and heme occlusion through the PilQ pore *(pilQ1)* (Chen et al. 2004). In addition, disruption of the gene of hydrophobic tolerance (*ght*) increases the sensitivity of *Neisseria* to heme, although the mechanism by which Ght protects against heme-mediated toxicity has not been defined (Rasmussen et al. 2005). Finally, disruption of the neisserial multiple transferable resistance (*mtr*) efflux system also increases sensitivity to heme-mediated toxicity (Bozja et al. 2004). This effect is likely due to the inability of the mutant bacteria to ''pump out'' the excess heme or heme-metabolites, again resulting in an increased concentration of intracellular heme and/or heme metabolites.

The mechanisms by which Gram positive bacteria avoid heme-mediated toxicity are less clear. Recently, it was shown that disruption of a *C. diphtheriae* heme-dependent transduction system *chrAS* results in increased sensitivity to heme-mediated toxicity (Bibb et al. 2005). The *ChrAS* system has been shown to sense heme and subsequently induce expression of *hmuO*, the *C. diphtheria* heme oxygenase (Schmitt 1999; Bibb et al. 2005). In contrast to the effects described above with mutants of the neisserial *hemO*, the increased heme-mediated toxicity observed in the *chrAS* mutant is independent of *hmuO* (Bibb et al. 2005). In fact, *hmuO* mutants and wild-type strains are equally sensitive to heme-mediated toxicity (Bibb et al. 2005). These data suggest that the ChrAS system is also involved in regulating the expression of other genes that are required for the protection against heme-mediated toxicity. Another example of heme oxygenase-mediated protection against heme toxicity was demonstrated upon inactivation of *B. anthracis isdG*, which led to a significant increase in heme toxicity (Skaar et al. 2006). Based on these findings, it is clear that pathogenic bacteria have evolved different mechanisms to avoid the toxic effects of heme, allowing them to use heme as a nutrient source while avoiding the detrimental effects of cytoplasmic heme accumulation.

A primary focus of our laboratory is investigating how *S. aureus* avoid heme-mediated toxicity. We have shown that *S. aureus* can grow using heme and hemoproteins as the sole iron source (Skaar et al. 2004b), suggesting that *S. aureus* is able to capitalize on the nutrient-iron value of these molecules while avoiding heme-mediated toxicity. Keeping with this, *C. diphtheria* (Schmitt 1997a; Schmitt 1999) and *Bordetella sp*. (Kirby et al. 2001; Vanderpool and Armstrong 2003) are able to sense heme and subsequently induce the expression of systems involved in heme acquisition and metabolism. In light of these findings, we sought to test the ability of *S. aureus* to alter protein expression in response to changes in intracellular heme status. In this regard we have performed differential expression analysis using two-dimensional difference gel electrophoresis (2D-DIGE), which identified 21 proteins that respond exclusively to the presence of heme, irrespectively of changes in iron availability (Friedman et al. 2006). These experiments

revealed that the protein exhibiting the most dramatic increase (45-fold) in response to heme is a hypothetical-uncharacterized ABC-type transporter that we have named the heme regulated transport system (Hrt) (Friedman et al. 2006). Importantly, this transporter is conserved across many pathogenic bacteria, including *B. anthracis* and *L. monocytogenes* and BLAST analyses have revealed that the Hrt system exhibits low similarity to antibacterial-peptide and lipoprotein exporters (Altschul et al. 1990). Further analysis of the localization of this gene in the *S. aureus* chromosome indicates that it is in a bicistronic operon containing an ABC-type transporter (*hrtA*) and a transporter permease (*hrtB*).

We hypothesize that the Hrt system prevents heme-mediated toxicity through the regulated expression of an efflux transporter that pumps excess heme and/or heme-metabolites out of the cell. This assumption is based on the observation that HrtA is over-expressed when *S. aureus* is grown in heme-containing medium, and HrtAB has low level identity to small molecule efflux pumps. To test this hypothesis and to determine the contribution of the Hrt system in vivo, we have generated *hrtAB* mutant *S. aureus* strains. Our preliminary studies indicate that the Hrt system is required for *S. aureus* growth in high heme concentrations, suggesting that the Hrt system is involved in avoidance of heme-mediated toxicity, potentially by exporting excess heme and/or heme-metabolites out of the cell (unpublished data). These findings highlight the importance of the Hrt system to staphylococcal heme metabolism. Further investigations into the mechanism and function of heme-mediated induction of Hrt and Hrt-mediated heme transport are currently underway in our laboratory.

Summary

Heme acquisition is a process that is vital to infection by numerous bacterial pathogens (Genco and Dixon 2001; Stojiljkovic and Perkins-Balding 2002; Skaar et al. 2004b). Traditionally, the uptake of heme has been considered a method by which bacterial pathogens acquire nutrient iron during infection. This presumption is supported by the requirement for heme uptake systems in multiple infection models and the significant iron requirement of virtually all bacterial pathogens (Genco and Dixon 2001; Stojiljkovic and Perkins-Balding 2002; Skaar et al. 2004b). Keeping with this, heme degradation machinery has been identified in numerous bacteria that infect vertebrates (Frankenberg-Dinkel 2004). The enzymatic degradation of heme is modeled to be an important process during pathogenesis; however, this contention has not yet been supported by animal models of infection. Although in vitro data support a role for heme degradation machinery in bacterial physiology, it is probable that the fate of heme inside the bacteria is multifaceted. In this regard, a small body of evidence is emerging that supports the molecular hijacking hypothesis which proposes that heme acquired from the host can be incorporated intact as an enzyme cofactor in respiratory proteins of the bacterial membrane (Schiott et al. 1997; Skaar et al. 2004b). Furthermore, we have identified a *S. aureus* heme-inducible efflux pump that presumably transports excess heme or heme metabolites out of the bacterial cytoplasm to prevent against heme-mediated toxicity. It is possible that the molecular decision to degrade, incorporate, or expel heme is dependent on the level of iron and heme available to the invading bacterium.

Our model envisions that upon entry into the host, *S. aureus* encounters an environment devoid of free iron. This iron starvation will lead to the Fur-dependent activation of the Isd and Hts systems (Mazmanian et al. 2003; Skaar et al. 2004b). Upon hemolysin-mediated erythrocyte lysis, free hemoglobin and hemoglobin-haptoglobin are bound by the surface proteins of the Isd system and heme is internalized through the membrane associated transport systems HtsABC and IsdDEF (Mazmanian et al. 2003; Skaar et al. 2004b). Initially, cytoplasmic heme may represent the only abundantly available iron source to staphylococci. Therefore, IsdG and IsdI degrade heme to release the iron in order to satisfy the nutrient iron requirement of the bacteria (Skaar et al. 2004a). As additional iron sources become available, or heme-derived iron is accumulated in

cytoplasmic bacterial ferritins, it is possible that heme is no longer degraded but is instead utilized in its intact form as a cofactor for bacterial proteins, including membrane-associated cytochromes. This would prevent *S. aureus* from expending the energy and resources required to synthesize the complex porphyrin ring of heme. However, it is conceivable that the efficiency of heme uptake can overpower this machinery, leading to the accumulation of high levels of cytoplasmic heme. Due to the reactivity of heme, cytoplasmic heme accumulation facilitates the formation of toxic oxygen radicals leading to cytotoxicity. This would be particularly detrimental to staphylococci that have encountered the oxidative burst of the host neutrophil, the primary immune cell that responds to staphylococcal infections (Foster 2005). In this regard, we have identified the HrtAB system as a novel heme responsive transporter that we predict is involved in efflux of heme or heme metabolites. In support of this model, the HrtAB system increases expression upon exposure to host neutrophils (Voyich et al. 2005). Although additional experiments are needed to test the role of HrtAB in protecting *S. aureus* against heme-mediated toxicity, this system might be a mechanism by which *S. aureus* prevents cytoplasmic reactive oxygen species build-up through heme efflux.

The specialized heme-uptake systems of bacteria are expressed during infection in order to acquire iron from host heme and hemoproteins (Wandersman and Stojiljkovic 2000; Heinrichs 2004; Skaar and Schneewind 2004). These systems can be exploited to import toxic heme-like molecules and in this regard, non-iron metalloporphyrins are potential chemotherapeutic agents against *S. aureus* infection. In fact, porphyrins and metalloporphyrins have been used safely in the clinic for several years as tumor therapy and in clinical imaging (Bozja et al. 2004). In terms of antibacterial effects, it was previously demonstrated that a single dose of deuteroporphyrin IX mixed with hemin was able to eradicate *S. aureus* burn wound infection with a killing efficiency of 99.97% (Orenstein et al. 1997). Additionally, Stojiljkovic et al. demonstrated that primary human fibroblasts treated with 100 μg ml^{-1} gallium-protoporphyrin IX were equally as viable as untreated cells, and this concentration is 100-fold greater than what we have found is necessary to inhibit *S. aureus* growth in vitro (Stojiljkovic et al. 1999; unpublished data). Moreover, it has also been shown that Ga-PPIX is also highly efficient at inhibiting *N. gonorrhoeae* and *H. ducreyi* growth (Bozja et al. 2004). In light of the increasing identification of multi-drug resistant strains of *S. aureus* it is paramount that we develop new therapeutics against this pathogen. Metalloporphyrins, specifically Ga-PPIX, have been shown to be potent antimicrobial agents against a variety of pathogens, including multi-drug resistant *S. aureus* (Stojiljkovic et al. 1999; Bozja et al. 2004; unpublished data), and preliminary data indicates that Ga-PPIX may represent a very safe and effective microbicide.

Our model for the fate of exogenously acquired intracellular heme presented above leaves many unanswered questions. First, how does *S. aureus* sense the intracellular and extracellular availability of both iron and heme in order to determine the best use for host-derived heme? Proper metabolism of this reactive nutrient is essential to ensure pathogen survival inside the host. Second, what is the mechanism by which heme is degraded by the IsdG-family of heme monooxygenases? Determining the molecular structure of the degradation products of the IsdG and IsdI catalyzed reactions is paramount to establishing a mechanism for heme degradation by this newly identified family of enzymes. Third, what factor(s) in the bacterial membrane complex exogenously acquired heme? We have shown that exogenously acquired heme is preferentially sorted to the staphylococcal membrane when additional iron sources are present (Skaar et al. 2004b), and previous data from *B. subtilis* demonstrate that exogenously-provided heme may be used as a cofactor in c-type cytochromes (Schiott et al. 1997). Based on a role of heme acquisition in pathogenesis (Skaar et al. 2004b), determining the fate of heme in the staphylococcal membrane may lead to the discovery of novel targets for therapeutic intervention against *S. aureus* infection. Fourth, what is the intracellular target of the toxic non-iron metalloporphyrin Ga-PPIX? It is possible that Ga-PPIX targets the same factor(s) in the

membrane that binds heme, and due to its limited oxidation potential, leads to an uncoupling of electron transport. However, our preliminary studies discussed in this review suggest that there are additional targets of this toxic molecule that have yet to be identified. Finally, what is the role of the HrtAB efflux system in protection against heme-mediated toxicity? An initial step toward answering this question will be the identification of the specific molecules that HrtAB transports. Elucidating the pathways involved in staphylococcal heme metabolism is critically important in the development of novel therapeutics, as heme metabolism has a vital role in the pathogenesis of *S. aureus* infections (Skaar et al. 2004b).

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