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Heme Synthesis and Acquisition in Bacterial Pathogens

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Abstract

Bacterial pathogens require the iron-containing cofactor heme to cause disease. Heme is essential to the function of hemoproteins, which are involved in energy generation by the electron transport chain, detoxification of host immune effectors, and other processes. During infection, bacterial pathogens must synthesize heme or acquire heme from the host; however, host heme is sequestered in high-affinity hemoproteins. Pathogens have evolved elaborate strategies to acquire heme from host sources, particularly hemoglobin, and both heme acquisition and synthesis are important for pathogenesis. Paradoxically, excess heme is toxic to bacteria and pathogens must rely on heme detoxification strategies. Heme is a key nutrient in the struggle for survival between host and pathogen, and its study has offered significant insight into the molecular mechanisms of bacterial pathogenesis.

Keywords

iron uptake; hemoglobin; Isd system; heme toxicity; NEAT domain

Introduction

Heme and iron are essential for life

The tetrapyrrole cofactor heme is important for the cellular processes of most organisms and essential to many lifeforms across domains of life. Heme, a porphyrin ring complexed with iron, serves as a redox active moiety required for the function of many cellular proteins. Heme functions as an electron shuttle in enzymes of the electron transport chain and is required for cellular respiration. Additionally, cells rely on heme for the function of many widely conserved enzymes including catalase, nitric oxide synthase, hemoglobin (Hb), and others. Heme is also an important molecule involved in diverse cellular processes including signaling, gas sensing, microRNA processing, and cellular differentiation [1–4]. Thus, nearly all organisms must satisfy the requirement for heme through either synthesis or acquisition.

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Heme coordinates an iron atom at its center which is vital for heme's electron transfer abilities and redox activity. Like heme, iron is nearly universally required for life, and only a few exceptions have been identified [5,6]. As an inorganic cofactor, iron can act alone or in iron–sulfur clusters as a prosthetic moiety for members of the oxidoreductase, nitrogenase, hydrogenase, dehydrogenase, and hydratase enzyme families [7–12]. Therefore, organisms have evolved elaborate strategies to acquire, store, and regulate intracellular iron for heme-dependent and other iron-dependent enzymes.

Nutritional immunity limits host iron availability

Nutritional immunity, a concept articulated primarily by Eugene Weinberg in the 1970s, describes the processes by which humans and other organisms sequester iron to limit acquisition by bacterial pathogens [13,14]. Nutritional immunity has since been expanded to include the host processes that manipulate local levels of manganese, zinc, and other transition metals in order to metal starve or intoxicate the invading pathogens (reviewed previously in Refs. [15,16–18]). The limited access of pathogens to metals serves as an antimicrobial strategy and limits bacterial replication. For instance, free iron rarely exists in the mammalian host. The solubility of ferric iron in aerobic solution is exceedingly low, and high-affinity iron-binding proteins, including transferrin, lactoferrin, albumin, and ferritin, sequester iron extracellularly and intracellularly. Iron-binding proteins function to transport iron, protect host cells from iron-mediated oxidative damage, and keep iron from pathogens. However, bacterial pathogens have developed exquisite tactics to overcome iron limitation and elaborate high-affinity iron receptors and chelators. In this regard, an evolutionary arms race has developed at the host–pathogen interface involving host iron-binding proteins and the mechanisms bacteria encode to steal iron.

Heme is an important host iron source

Heme makes up the greatest reservoir of iron in the host and serves as an iron source for many bacterial pathogens. Humans and other metazoa synthesize heme through a variety of steps in the mitochondria and cytosol. This pathway, called the Shemin or four-carbon pathway, begins with the condensation of glycine and succinyl-CoA to form the committed precursor δ-aminolevulinic acid (ALA) [19–21]. A series of enzymes produces protoporphyrin IX from ALA and iron is inserted, forming protoheme IX. For the sake of simplicity in this review, heme will refer to ferrous and ferric iron forms of protoheme IX. Heme is then bound by hemoproteins to serve a variety of intracellular and extracellular tasks. Catalase, peroxidase, and myeloperoxidase rely on heme to catalyze the hydrolysis of peroxide molecules. Energy generation by the electron transport chain relies on heme-dependent *c*- and *b*-type cytochromes of the ubiquinol–ferricytochrome *c* oxidoreductase (Complex III) family [22,23]. Hemoproteins involved in tissue oxygen homeostasis include myoglobin and neuroglobin. Perhaps the most well-known hemoprotein is the oxygen transporter Hb. Its abundance and location in erythrocytes make Hb a rich heme source for pathogens. Hb contains about two-thirds of the body's iron, and a single erythrocyte contains more than 280 million molecules of Hb [15,24]. Bacterial pathogens have evolved high-affinity Hb-binding proteins for the acquisition of heme, and these proteins will be described below.

Owing in part to the reactive nature of heme-iron, free heme and Hb are toxic to the human host and bacterial pathogens alike [25,26]. To prevent excess heme toxicity, eukaryotic heme synthesis is highly regulated and heme homeostasis and sequestration are well orchestrated. When Hb is released from erythrocytes or otherwise exists extracellularly, it is rapidly bound by haptoglobin (Hp) [27]. The abundance of cell-free Hb is thought to be very low in healthy adults, but a variety of genetic disorders, infections, and other disease states can increase the concentration of free Hb [28]. Free Hb and its modified forms, in the presence of reactive oxygen species, exhibit cytotoxic effects toward endothelial cells [29]. However, the relevance of these *in vivo* studies is unclear, and a comprehensive understanding of concentrations to achieve Hb toxicity in healthy humans has not been achieved [25]. On the other hand, in the absence of infection-free heme that has been liberated from its hemoprotein likely only exists transiently in serum or in cells. In serum, heme is immediately bound by the highly abundant albumin ($k_d \approx 10$ nM) then transferred to hemopexin ($k_d < 1$ pM) [30]. The heme is delivered to cells expressing the hemopexin receptor; these cells then degrade the heme using heme oxygenases [30]. The rapid sequestration and degradation of free heme in the blood is vital to the survival of erythrocytes, as heme in the presence of reactive oxygen species exhibits cytotoxicity and lipid peroxidation at micromolar concentrations [31,32]. During infection of host heme- and Hb-replete niches, bacterial pathogens experience heme toxicity and encode systems to protect from heme toxicity as well [33]. Therefore, heme is at the center of a complex interplay between host and pathogen for survival.

Bacterial Heme Synthesis

Divergent heme synthesis pathways in Gramnegative and Gram-positive organisms

While both humans and bacteria share the early heme precursor ALA, most bacteria (Alphaproteo-bacteria are the exception), archaea, and plants synthesize ALA from charged glutamyl-tRNA^{Glu} *via* the “C5 pathway” (Fig. 1) [21,34,35]. The gluta-myl-tRNA reductase HemA produces the highly reactive intermediate glutamate-1-semialdehyde, which HemL, a glutamate-1-semialdehyde amino-mutase, converts to ALA [36,37]. The three steps, from ALA to uroporphyrinogen, are well conserved and thought to be the evolutionary core of heme biosynthesis. ALA dehydratase (also called porphobilinogen synthase; annotated as HemB) is responsible for the condensation of two ALA to porphobilinogen (PBG) [38]. The linear tetrapyrrole hydroxymethylbilane (HMB) is produced by a head-to-tail condensation and deamination of four PBG molecules, catalyzed by HMB synthase (alternatively called PBG deaminase, annotated as HemC) [39,40]. Under physiological conditions, HMB will spontaneously cyclize to form the uroporphyrinogen I isomer, a biosynthetic deadend. Therefore, most bacteria utilize uroporphyrinogen III synthase (HemD) to catalyze the cyclization of HMB through a *spiro*-intermediate to form uroporphyrinogen III [41].

Uroporphyrinogen III can be utilized for the synthesis of several tetrapyrrole-based cofactors. Uroporphyrinogen III decarboxylase (HemE) decarboxylates the four acetate side chains to methyl groups, producing coproporphyrinogen III, the next step in heme synthesis [42]. Additionally, uroporphyrinogen III can be shunted from heme synthesis and converted to precorrin-2 to synthesize vitamin B12, coenzyme F430, and siroheme [43]. The Ahb

enzymes of some archaea and sulfur-reducing bacteria can convert siroheme (produced from uroporphyrinogen) to heme [44,45]. The contribution of the Ahb alternative heme pathway has not been demonstrated in bacterial pathogens.

In Gram-negative organisms, as well as eukaryotes, coproporphyrinogen III is converted to proto-porphyrinogen IX by coproporphyrinogen III oxidase. This step is the first of the terminal three steps in the classical heme synthesis pathway (in blue in Fig. 1) and is catalyzed by oxygen dependent HemF or by oxygen independent HemN [46,47]. Protoporphyrinogen IX is subsequently oxidized to form proto-porphyrin IX, by a six-electron oxidation catalyzed by one of three protoporphyrinogen oxidase enzymes. HemG, in Gammaproteobacteria and some Alphaproteobactera and Deltaproteobacteria, uses the respiratory chain as its electron acceptor and is not dependent on oxygen [48]. HemJ is poorly characterized but represents the most common protoporphyrinogen oxidase among Alphaproteobactera and Deltaproteobacteria [49]. The third protoporphyrinogen oxidase is HemY, an FAD- and oxygen-dependent protoporphyrinogen oxidase found in some Proteobacteria as well as eukaryotes [50]. The final step of the classical pathway is the insertion of ferrous iron by protoporphyrin ferrochelatase (HemH) to form protoheme IX, called heme [51]. From ALA to heme, the steps of the classical synthesis pathway are shared by eukaryotes and Gram-negative bacteria.

The terminal steps of the classical pathway were considered universally conserved for all heme synthesizing organisms. However, just in the last few years, the terminal steps of heme synthesis in the Gram-positive phyla Firmicutes and Actinobacteria have been described with genomic and biochemical analysis and termed the non-canonical or transitional pathway [34,52]. Very few HemF or HemN coproporphyrinogen oxidases can be identified in Gram-positive genomes; instead it has been realized that the annotated HemY in these organisms functions as a coproporphyrinogen oxidase to form coproporphyrin III [34,53]. The Gram-positive HemH, a coproporphyrin ferrochelatase, inserts ferrous iron to form coproheme [52]. Finally, coproheme is decarboxylated by HemQ, an enzyme unique to members of the Firmicutes and Actinobacteria to form protoheme IX [54–57]. It is now clear that Grampositive organisms utilize a unique series of terminal steps to synthesize heme (in green in Fig. 1).

Regulation of heme synthesis

Despite the vital role of heme to bacterial physiology, the regulation of heme biosynthesis has not been well studied outside of a few model organisms. In bacteria, regulation has been recognized to occur largely at two steps, abundance of the initial enzyme HemA and transcription of the coproporphyrinogen oxidase enzymes. Regulation of HemA is typically heme-dependent, indicating that bacteria reduce synthesis of heme and all intermediates in heme-replete conditions. This process has been extensively studied in *Escherichia coli* and *Salmonella enterica* serovar. Typhimurium. The addition of heme to cell extracts of *E. coli* reduces total HemA activity, without inhibiting the activity of the purified enzyme [58,59]. This was explained by the observation that excess heme results in the proteolytic degradation of HemA in *Salmonella*, suggesting that HemA might bind excess heme [60]. The Clp and Lon proteases are responsible for this reduction in HemA levels [61].

Furthermore, mutations in HemA have been described that render HemA resistant to heme- and protease-mediated degradation, indicating that HemA binds excess heme, and holo-HemA but not apo-HemA is a substrate for proteolytic degradation [62,63]. In this manner, cellular levels of heme can regulate the first step of heme synthesis and limit the unnecessary synthesis of heme intermediates as well as the consumption of iron. Recent metabolic engineering efforts to enhance ALA production in *E. coli* suggest that protoporphyrin IX post-translationally inhibits HemB, an additional example of feedback inhibition [64]. It is likely that for many organisms, heme and terminal heme intermediates can have post-translational regulatory effects on heme synthesis enzymes. Like *Salmonella* and *E. coli,* the Gram-positive bacterium *Bacillus subtilis* regulates levels of HemA. While a mechanistic explanation has not been described, the membrane protein HemX post-transcriptionally regulates HemA abundance in *B. subtilis* [38,65]. Homologs of *B. subtilis* HemX exist in multiple Gram-positive pathogens; however, the function of HemX and HemA regulation has yet to be detailed.

In addition to the regulation of HemA enzyme levels, the transcription of *hemA* is also a point of control for heme biosynthesis. Two promoters exist upstream of *hemA* in the Gram-negative pathogen *Pseudomonas aeruginosa*, and these promoters contain binding sites for the regulators Anr (oxygen sensing), Dnr (redox regulator), IHF (integration host factor), and NarL (nitrate regulator) [66,67]. Therefore, *hemA* expression is induced in the presence of oxygen or when oxygen is lacking but an alternative electron acceptor such as nitrate is present for utilization of heme-dependent respiration. In *B. subtilis*, *hemEHY* is induced anaerobically and *hemAXCBL* is induced by peroxide through de-repression of PerR [38,68]. As in *B. subtilis*, PerR has been implicated as a regulator of the *hemEHY* operon in *Staphylococcus aureus*; yet recent work has demonstrated that major differences exist between *B. subtilis* and *S. aureus* PerR orthologs, and therefore, it is difficult to conclude that PerR plays a role in *S. aureus* heme synthesis [69,70]. *Corynebacterium diphtheriae*, a member of the Actinobacteria phylum, encodes two heme-responsive two-component systems (TCS). The response regulator HrrA directly binds the promoters of *hemA*, *hemE*, and *hemH* to repress their transcription in heme-replete conditions [71]. Similarly, ChrA can repress transcription of *hemA* in heme replete conditions [72,73]. These data suggest that in *C. diphtheriae*, heme utilization is preferred over synthesis when exogenous heme is available. Together, these examples point to the transcriptional and post-translational control of HemA as a central step in heme synthesis regulation.

The expression of coproporphyrinogen oxidase genes is the second major point of heme synthesis regulation. In several species, *hemF* and *hemN* are regulated by different oxygen- or anaerobic-responsive regulators to ensure proper expression of oxygen-dependent or oxygen-independent coproporphyrinogen oxidases. OxyR, a global regulator in *E. coli*, is responsible for the induction of oxygen-dependent *hemF* expression in hydrogen peroxide stress. It has been suggested that the [Fe-S] cluster in oxygen-independent HemN is vulnerable to peroxide damage, so HemF is produced to take the place of HemN [74]. In *B. subtilis*, the transcription of coproporphyrinogen III oxidases *hemN* and *hemZ* (a second coproporphyrinogen oxidase, not to be confused with oxygen-dependent HemY) is induced anaerobically by the regulatory cascade of ResDE, Fnr, and YwiD to replace the oxygen-dependent HemY [75–78]. Similarly, *Pseudomonas hemF* and *hemN* are expressed

anaerobically under the control of Anr and Dnr, while Anr induces the expression of only *hemN* aerobically [79]. It has been suggested, but not validated, that the expression of oxygen-dependent *hemF* in oxygen limited conditions by Anr and Dnr serves to consume residual oxygen during the transition to anaerobiosis, which would protect other anaerobically induced oxygen-sensitive proteins [79]. Thus, oxygen is a key regulatory of expression of coproporphyrinogen oxidase genes.

Contribution of heme synthesis to pathogenesis

With a few notable exceptions including *Bartonella hensaela*, *Enterococcus faecalis*, *Haemophilus influenzae*, and *Streptococcus* spp., most human pathogens encode complete heme biosynthetic pathways [80–83]. However, the contribution of heme synthesis to the pathogenesis of bacterial pathogens is largely understudied. For *S. aureus*, whose reliance on heme acquisition during infection has been well established, it is now clear that heme biosynthesis is vital to cause disease in murine models of infection [84–86]. Inactivation of *hemA*, which renders *S. aureus* heme deficient, causes the small-colony variant (SCV) phenotype [87]. During systemic infection, this mutant is highly defective at colonizing the murine heart and liver relative to wildtype *S. aureus* [87]. A mutant lacking *hemB*, also a heme-deficient SCV, demonstrates reduced colonization and bone destruction in a murine model of osteomyelitis [88,89]. These data demonstrate that for *S. aureus*, heme acquisition is insufficient to support organ colonization and therefore heme biosynthesis is critical to pathogenesis. Importantly, the SCV phenotype is encountered clinically. Despite their reduced virulence, SCVs are generally more resistant to antibiotics and oxidative stress, more equipped to evade the immune system by living intracellularly, and are likely the etiological agent of persistent staphylococcal infections [89–92] (reviewed in Ref. [93]).

Less evidence for the role of heme synthesis during infection is available for other pathogens. For the intracellular pathogen *Brucella abortus*, *hemH* is required for virulence in a murine model of brucellosis [94]. Therefore, like *S. aureus*, host heme utilization is insufficient and synthesis is required for full virulence. In addition to *B. abortus* and *S. aureus*, the advent of whole genome *in vivo* analysis of mutants using techniques such as transposon-sequencing and signature tagged mutagenesis has highlighted the role of heme synthesis. In these infections, genes with marked mutations that are recovered at a lower frequency from the infected tissue relative to growth *in vitro* are considered important to infection. These types of experiments have demonstrated a role for different heme synthesis genes during infection. Transposon mutantsdisrupted in *hemY* were found to be defective for *P. aeruginosa* colonization of the murine gastrointestinal tract [95]. *hemN* was found to be important for *Yersinia pestis* infection of deep tissue [96]. Transposon mutants lacking *hemE* in *Acinetobacter baumannii* were less effective at colonizing the murine lung [97]. Finally, *hemG* was found to be important for *Listeria mono-cytogenes* oral infection [98]. Based on these trans-poson library infections, heme synthesis is vital to the fitness of a variety of pathogens.

Current challenges and opportunities

The divergence between the terminal steps of Gram-positive heme synthesis and the classical pathway utilized by Gram-negative organisms as well as humans presents the

opportunity for targeted small molecule interventions to inhibit or activate Gram-positive heme synthesis. The terminal Gram-positive enzymes HemQ, which exists only in Actinobacteria and Firmicutes, as well as HemY and HemH, which recognize different substrates than the eukaryotic host enzymes, present three potential targets. Small molecules have been described that modulate heme synthesis *in vivo*, while *in vitro* inhibitors of *S. aureus* HemY have recently been reported, suggesting that Gram-positive heme synthesis is an attractive drug target [52,99,100].

Outside of a few model pathogens, very little is understood regarding the regulation of heme synthesis, particularly during pathogenesis. Regulation is a central question in understanding the role of heme synthesis in infection. Considering that in some niches host heme is available and can reach toxic levels, pathogens with the capacity to both steal and synthesize heme must regulate both pathways. For *S. aureus*, in which heme synthesis and acquisition are vital during infection, the regulation of heme synthesis is unknown. This is despite the observation over half a century ago that the rate of staphylococcal heme synthesis is modulated by exogenous heme [101]. For other pathogens, the contribution of heme synthesis to disease is still unclear, but whole-genome *in vivo* fitness experiments like transposon-sequencing suggest many bacterial pathogens rely on heme biosynthesis to cause disease, and this field of research provides ample opportunity for further exploration.

Gram-Positive Heme Acquisition Strategies

Bacterial pathogens utilize a variety of heme acquisition strategies during infection, ranging from surface receptors to secreted proteins that bind either heme or hemoproteins. Heme acquired from the host is used fully intact or degraded to liberate heme-iron and both processes are important during bacterial pathogenesis. Gram-positive pathogens, including *S. aureus, Bacillus anthracis*, and *C. diphtheriae* rely on heme acquisition during infection. The heme uptake pathways of these three pathogens will be presented as models for theGram-positive processes, along with the regulation of the pathway and evidence for the role of heme uptake during pathogenesis.

The *S. aureus* Isd paradigm

The *I*ron-regulated *s*urface *d*eterminant system (Isd), first described in *S. aureus*, is the paradigm for Gram-positive heme acquisition [102]. During infection, *S. aureus* utilizes the leukocidins HlgAB and LukED to lyse erythrocytes and liberate Hb into the bloodstream [103]. This results in accessible free heme, heme bound by hemopexin (Hx), free Hb (Hb), and Hb bound by Hp to form the Hp–Hbcomplex. The Isd system enables utilization of free heme, or heme bound to Hb and Hp–Hb complexes. Isd proteins bind heme and Hb at the cell wall surface with conserved *nea*r *t*ransporter (NEAT) domains. The NEAT domains are 120–125 aa domains that constitute a conserved eight-stranded β-sandwich fold [104,105]. Heme is bound in a hydrophobic pocket with critical coordination by tyrosine residues in a YXXXY motif. These NEAT-containing surface proteins (IsdB, IsdH, IsdA in *S. aureus*) shuttle heme to NEAT-containing IsdC. IsdC transfers heme to the membrane-associated transporter IsdDEF for transit across the membrane. To access host heme and hemoproteins, IsdB, IsdH, and IsdA are covalently attached to the peptidoglycan by the standard Sortase A

cysteine transpeptidase [106]. IsdB contains two NEAT domains, NEAT1 (N1) binds Hb and Hb–Hp, but not Hp and N2 binds heme; as such IsdB is believed to be the primary Hb-binding protein [85,107,108]. IsdH contains three NEAT domains, N1 and N2 bind both Hb and Hp, andN3bindsheme [109,110]. IsdA, which is partially surface exposed, contains a single heme-binding NEAT domain [102]. The current model (Fig. 2), supported by strong structural evidence, suggests that IsdB-N1 binds Hb, and IsdB-N2 extracts heme [111]. Similarly, IsdHN1 and N2 bind Hb and Hp, and IsdH-N3 extracts the heme. The heme is then transferred either directly to IsdC or shuttled *via* IsdA to IsdC.

S. aureus encodes an iron-regulated Sortase B (SrtB) for which IsdC is the only substrate, and SrtB attaches IsdC to peptidoglycan in such a way that IsdC is not surface exposed but rather buried in the cell wall, which is 15–30 nm thick [112,113]. This organization allows heme transferred from surface Isd proteins to pass through the cell wall to the membrane by IsdC's single heme-binding NEAT domain. IsdC alone transfers heme to the IsdE of the IsdDEF transporter [114]. At the membrane, IsdDEF transit heme across the membrane and into the cytosol.

Upon import, heme is incorporated into staphylococcal proteins or degraded. Exogenous heme accumulates in the membrane and is also capable of complementing the growth of heme-deficient mutants [84]. Alternatively, the heme oxygenases IsdG and IsdI degrade heme to release iron [115] (reviewed in Ref. [116]). IsdG and IsdI are structurally similar and are the first described members of the Isd heme oxygenase family, which catabolizes heme to staphylobilin instead of biliverdin [117–119]. IsdG and IsdI are required for growth using heme as a sole iron source and are expressed during infection [115,120].

The widely conserved ferric uptake regulator (Fur) is the principle regulator of the expression of heme acquisition systems in *S. aureus.* In iron-deplete conditions, Fur no longer represses its regulon, allowing the transcription of the *isdB*, *isdA*, *isdC-DEFsrtBisdG,* and *isdI* loci [102]. During infection of iron-deplete niches, the heme acquisition system and associated iron-liberating heme oxygenases are expressed. Further regulation of the heme oxygenases exists; IsdG abundance increases in the presence of heme and IsdG half-life is increased when heme-bound [120]. Also, the Clp proteases have a role in Hb acquisition by modulating IsdB levels [121]. Additional heme-dependent regulation likely exists but has not been described.

Isd-mediated heme acquisition is vital to the virulence of *S. aureus*. Heme is the preferred iron-source during systemic infection, in part because a heme-responsive transcriptional regulator activates iron siderophore synthesis only when heme-iron is unavailable [84,122]. The role of the Isd system has been extensively demonstrated in murine infection models. Mutants lacking components of the Isd system are highly defective inpathogenesis, highlighting the importance of heme acquisition to staphylo-coccal disease [84–86,108,120,123,124].

Isd-dependent heme uptake by *B. anthracis*

B. anthracis encodes a heme uptake system that shares the core of the *S. aureus* Isd, but with additional unique proteins. *B. anthracis* encodes two secreted hemophores termed IsdX1 and

IsdX2 [125]. These are the first described Gram-positive hemophores and bind heme, Hb, and methemoglobin [125– 129]. IsdX1 contains one NEAT domain, while IsdX2 contains five NEAT domains; both are secreted past the cell wall as they lack sortase signals or membrane spanning domains [125]. *B. anthracis* also encodes other NEAT contain proteins; Hal contains a single NEAT domain and leucine-rich repeats, which extract heme from Hb [130]. Unlike IsdX1/2, Hal is sortase anchored to the cell wall [131]. A second, recently described NEAT protein is BslK, which is non-covalently attached to the cell wall and transfers heme to IsdC [132]. The current proposed model (Fig. 2) is that IsdX1 is secreted, binds heme, and transfers heme to wall-anchored IsdC. IsdX2 can bind free heme, accept heme from IsdX1, and transfer heme to IsdC. The multiple NEAT domains ofIsdX2 have been proposed to be important for these multiple functions, and it has been suggested that IsdX2 can serve as a heme storage protein. IsdDEF transports heme across the membrane for utilization by IsdG, an orthologue of the *S. aureus* heme oxygenase [133]. The diversity of heme and Hb-binding proteins relative to *S. aureus* may be the result of the greater variety of environmental niches that germinant and sporulent *B. anthracis* inhabits.

The role of *B. anthracis* heme acquisition during infection is not clear. A guinea pig infection model demonstrated that *ΔisdCX1X2* was as virulent as wild type, yet these proteins are expressed during infection [134]. Also, a mutant of *B. anthracis* lacking Hal demonstrated reduced virulence in a model of inhalational anthrax [135]. It is likely that the IsdX1/X2 hemophores, BslK, and Hal are partially redundant, and a mutant lacking all four proteins would be highly defective in causing anthrax.

In addition to *S. aureus* and *B. anthracis*, many other pathogens have evolved NEAT-containing heme acquisition systems, including *Staphylococcus lugdunensis, L. monocytogenes,* and *Streptococcus pyogenes* [136–143]. The conservation of NEAT-mediated heme uptake highlights the contribution of host heme to bacterial infection.

C. diphtheriae heme uptake

C. diphtheriae utilizes non-NEAT-mediated heme uptake systems for heme-iron acquisition, termed HmuTUV, HtaABC, and ChtABC/CirA. The Hmu (*he*min-*u*ptake) system was the first heme acquisition system described in Gram-positive organisms. The associated heme oxygenase, HmuO, was discovered and described first, and then HmuTUV was discovered for the ability of a plasmid encoding *hmuTUV* to complement a *Corynebacterium ulcerans* strain that cannot grow on Hb as a sole iron source [144,145]. Sequence analysis suggests that HmuTUV acts asan ABC transporter that shuttles heme across the cell membrane [146]. It was later discovered that an additional gene is encoded within the *hmuTUV* operon, termed *htaA* (*h*eme-*t*ransport *a*ssociated) [147]. Adjacent to this locus are the genes *htaB* and *htaC*. Unlike the sortase anchoring of other Gram-positive uptake systems, HtaA and HtaB contain N-terminal secretion signals as well as C-terminal intermembrane domains. This results in surface exposure of HtaA and HtaB, which both bind heme. Interestingly, a portion of HtaA is secreted and not anchored to the cell envelope. HtaA isolated from cell culture is unable to complement the growth of an *htaA* mutant, suggesting that surface bound HtaA may serve as a heme receptor and secreted HtaA may serve as a hemophore [147,148]. However, heme transfer between HtaA molecules and further description of the

function of HtaA on the surface have not been reported. In addition to heme, HtaA can acquire heme from Hb and transfer heme to HtaB, suggesting a heme shuttle from HtaA toHtaB toHmuT; HmuT isa surface-anchored lipoprotein, which then transfers heme to the cognate ABC transporter HmuUV [148]. While the Isd NEAT domains rely on tyrosine alone as the axial ligand for heme binding, HmuT relies on an N-terminal histidine and a C-terminal tyrosine to coordinate heme [149].

Inactivation of the Hmu/Hta systems does not completely eliminate growth with heme as a sole iron source, suggesting the involvement of an additional heme uptake system [147]. This led to the characterization of the ChtAB and CirAChrC operons, which are regulated by iron levels *via* DtxR. DtxR is the Diphtheria Toxin regulator which activates the expression of Diphtheria Toxin as well as HmuTUV and HtaABC [150,151]. ChtAB and ChtC appear to be the result of gene duplication of HtaAB, as all three groups of proteins have sequence similarity, N-terminal secretion signals, and C-terminal transmembrane domains, and contain the same heme-binding domain [152]. Like HtaAB, ChtAB and CirAChtC are surface exposed and ChtAB and ChtC bind heme and Hb. It appears that these heme-binding proteins serve redundant functions, and as such, a mutant lacking both HtaB and ChtB is deficient at utilizing Hb as an iron source [152]. Recently, it has been shown that ChtA and ChtC are both capable of binding Hp–Hb for heme extraction, and acquisition of heme from Hp–Hb requires HtaA [153]. The current model (Fig. 2) for Hp–Hb heme acquisition involves binding of Hp–Hb by a combination of HtaA and ChtA or ChtC, heme extraction either actively or passively, and transfer to HtaB, HmuT, and HmuUV [153].

Gram-Negative Heme Acquisition Strategies

The outer membrane of the cellular envelope of Gram-negative organisms presents an additional barrier to heme acquisition. Therefore, Gram-negative heme uptake systems consist of outer-membrane receptors that either bind heme and hemoproteins directly, or bind heme-bound secreted hemophores. Heme then transits the periplasm and is brought into the cell *via* ABC transporters at the inner membrane. The versatile opportunistic pathogen *P. aeruginosa* encodes direct heme uptake and hemophore systems at the outer membrane, *H. influenzae* uses a hemophore uptake system, and *Neisseria meningitidis* uses a unique bipartite receptor for heme acquisition from host hemoproteins . These pathogens are presented as models for Gram-negative heme uptake systems.

P. aeruginosa

P. aeruginosa encodes direct and indirect systems for heme uptake. The Phu (P*seudomonas h*eme *u*ptake) consists of a TonB-dependent PhuR which binds heme and transports it to the periplasm. PhuR activity is representative of Gram-negative TonB-dependent outer-membrane receptors. These β-barrel proteins bind substrates (often iron containing molecules) with high affinity, and rely on proton motive force and TonB for transport across the outer membrane [154]. TonB is an inner-membrane protein with a substantial periplasmic portion for direct interaction with periplasmic domains of the outer membrane proteins. Upon PhuR translocation of heme into the periplasm, the soluble periplasmic

protein PhuT binds heme and brings it to PhuUV, an ABC transporter at the inner membrane.

In addition, HasA/HasR (*h*eme *a*ssimilation *s*ystem) is utilized for heme uptake. HasA is a secreted hemophore that binds heme and transfers it to a second TonB-dependent transporter, HasR. Like other Gram-negative heme-binding motifs, HasA coordinates heme using histidine and tyrosine residues with picomolar affinity. Data from the orthologous HasA hemophore of *Serratia marcescens* suggest that HasA binds Hb and extracts heme, then HasA transfers heme to HasR [155,156]. The present model (Fig. 3) for these two heme uptake systems suggests that Phu is the principle heme acquisition system but full heme utilization requires HasA/HasR. HasA/HasR may be more relevant as a heme sensing system; under low heme conditions, the inner-membrane HasS binds the sigma factor inhibitor HasI. When heme is available, HasS instead binds HasR, and HasI is free to recruit RNA polymerase to activate the transcription of *hasAR*, *hasSI*, *phuSTUV*, and *phuR* [157]. The *P. aeruginosa* heme uptake system PhuSTUV/PhuR is regulated by Fur in addition to the HasI sigma factor detailed above. Recently, small regulatory RNAs have been described that impact *phuS* mRNA levels, suggesting another layer of heme-responsive regulation [158,159]

In contrast to many other organisms, *Pseudomonas* encodes a soluble cytoplasmic heme-binding protein that is not a heme oxygenase. This protein, PhuS, transfers heme to the heme-oxygenase HemO for iron liberation. PhuS, unlike many hemoproteins, binds ferric-iron heme and subsequently transfers it to HemOunder iron-deplete conditions [160]. The dissociation constant of the heme–PhuS–HemO complex is in the nanomolar range, suggesting that PhuS transfers heme to HemO specifically and not to the second *Pseudomonas* heme oxygense, BphO [160]. While the PhuS heme transfer has not been described completely, PhuS has been shown to bind heme as a monomer utilizing one of two histidine residues (His209 and His212), and a third binding site exists when PhuS is in dimeric form [161]. Further *in vitro* characterization and structural analysis has led to a model whereby heme coordination occurs primarily at the His212 ligand and induces a conformational change required for interaction with HemO [162,163]. Additionally, *in vitro* heme oxygenase activity has been attributed to PhuS; however, the *in vivo* relevance of this function is unclear as no biliverdin-β (the product of HemO heme catabolism) is detected in a mutant lacking *hemO* [164,165].

A recent clinical evaluation of genetic changes to *P. aeruginosa* during infection of cystic fibrosis lungs revealed the importance of heme acquisition during infection [166]. Long-term infection led to the selection of mutations in the promoters of the *phuSTUWV* and *phuR* loci, resulting in greater Phu expression. These changes to *phu* transcription confer a growth advantage enabling the utilization of heme from Hb as the sole iron source and suggest that heme is an important iron source during chronic *Pseudomonas* infection. The infections also selected for mutants that demonstrate enhanced expression of the *feo* ferrous-iron acquisition genes, indicating that ferrous iron is also a source of bioavailable iron. These clinical data confirm experimental findings suggesting that *P. aeruginosa* heme acquisition contributes to chronic infection.

H. influenzae

H. influenzae is a notable exception to the other pathogens outlined here, as it is incapable of synthesizing heme and therefore requires heme uptake for aerobic respiration [167]. It is capable of acquiring heme from diverse host sources (Fig. 3), including hemopexin, free heme, albumin-bound heme, myoglobin, and Hb; the variety of heme sources is in accordance with its absolute reliance on exogenous heme [168]. *H. influenzae* has evolved a variety of heme uptake systems important for growth *in vitro* using various host heme sources. While some systems are well described, less is known about others, and a global understanding of the utilization of these heme uptake systems during infection is lacking.

The HxuCBA system, described primarily in *H. influenzae* type B, is capable of heme acquisition from free heme and heme-hemopexin (Hx). HxuA is a secreted hemophore that is released from the outer membrane by its transporter HxuB [169–171]. HxuA exhibits no heme-binding motif but rather demonstrates high-affinity binding specifically to Hx with little distinction between apo- and holo-Hx [172]. HxuC is a TonB-dependent transporter that binds heme after release from the Hx–heme–HxuA complex and imports it into the periplasm [173]. Additionally, HxuC is capable of acquiring heme from serum albumin (Alb) independent of HxuA [174]. HpbA is another heme acquisition protein identified in nontypeable and type B *H. influenzae*. A lipoprotein, HbpA is important for growth using Hb, Hp–Hb, and heme-human serum albumin as heme sources [175,176]. The inner-membrane heme transporter has not been definitively identified, but the Hip proteins have been implicated [174].

Additionally, *H. influenzae* encodes three receptors, HgpA, HgpB, and HgpC, that can acquire heme from Hp–Hb and Hp bound myoglobin, albeit it at greater concentrations than thought to be physiologically relevant [177,178]. While the contribution of the Hgps seems redundant, HgpB has been demonstrated to be most important for utilization of Hp–Hb and Hp–myoglobin.

There are many outstanding questions regarding *H. influenzae* heme uptake. Many proteins have been attributed to be involved in heme uptake, but their function requires further investigation [179–183]. The regulation of the heme uptake system expression is not well described, except that *hxuCBA* and the *hgp* genes are expressed under *in vitro* iron/heme deplete conditions during experimental infection of the chinchilla ear [184,185]. Lastly, a heme oxygenase of *Haemophilus* has not been described, suggesting that acquired heme is utilized intact and that other iron acquisition pathways, from transferrin and lactoferrin sources, are sufficient for cellular iron needs. However, it is also possible that a heme oxygenase exists and has not yet been identified.

Genetic evidence from clinical isolates suggests that heme uptake is vital to pathogenic strains of *H. influenzae*. Isolates from otitis media infection in children relative to commensal throat isolates exhibit greater rates of *hxuA*, *hxuB*, *hxuC*, and *hgpB* gene prevalence, indicating that heme uptake may be a virulence determinant [186,187]. Several animal models have been used to demonstrate the role of heme uptake during *H. influenzae* infection. In a model of *H. influenzae* bacteremia, infant rats infected with a mutant lacking HbpA completely clear the infection after one week while rats infected with wildtype remain

infected [176]. Likewise, a mutant lacking both HxuC and HgpABC uptake proteins is unable to cause bacteremia in the same rat model [188]. Additionally, the Hgp proteins are required to cause otitis media in a chinchilla model [189]. It is clear that for *H. influenzae* pathogenesis, heme uptake is a critical virulence determinant.

N. meningitidis

N. meningitidis encodes a bipartite heme uptake system consisting of HpuAB and HmbR (Fig. 3). HpuAB is expressed from an iron-repressed operon and consists of the HpuA lipoprotein and HpuB, the TonB-dependent receptor capable of binding Hb, apo-Hp, and Hp–Hb [190,191]. Upon heme transport into the cytoplasm, the HemO heme oxygenase degrades heme to biliverdin and liberates iron. As such, HemO is required for survival using heme, Hb, or Hp–Hb as a sole iron source [192,193]. Heme is extracted from these hemoproteins and is imported intact, as Hb can complement the deficiencies of a heme synthesis mutant in an HpuAB-dependent manner [194]. The inner-membrane transporter has not yet been identified, but a zinc transporter has been implicated [195].

Initial studies of the individual function of HpuA and HpuB failed to describe the role of HpuA in heme acquisition. HpuB is sufficient to bind Hb, but a high-affinity HpuB-Hb complex requires the presence of HpuA, even though HpuA-Hb binding was not detected by a flow cytometry assay [196,197]. Additionally, HpuA is required for growth with Hb as a sole iron source and heme import [198]. However, a recent structural characterization has described a direct, albeit weak, interaction between HpuA and Hb, and a co-crystal structure of Hb and an HpuA homolog from *Kingella denitrificans* has been solved [199]. While these data are not conclusive, they suggest that HpuA and HpuA homologs interact with Hb, and this interaction is required for HpuAB-mediate heme uptake.

HmbR (Hb *r*eceptor) is an additional *N. meningitidis* heme uptake protein that binds host Hb with species specificity, exhibiting a greater utilization of human Hb but is unable to bind the Hp-Hb complex and therefore likely binds free Hb only [200,201]. Like HpuAB, it is subject to phase variation [202]. HmbR, based on spectroscopy and mutational analysis, also coordinates heme with a Tyr residue, which further confirms that diverse heme-binding domains have evolved to utilize tyrosine as the axial ligand [203]. The mechanism of heme extraction by HmbR, the associated inner-membrane heme transporter that partners with HmbR extraction, and structural descriptions of ligand binding are still undescribed for HmbR heme uptake.

In *N. meningitidis,* expression of *hemO* and *hmbR* is regulated by Fur as well as the MisRS TCS [204,205]. MisRS activates the expression of *hemO* and *hmbR* independent of Hb and iron concentration, which suggests an additional layer of regulation for Hb acquisition. However, the activating signal of MisRS has not yet been described.

The genetic diversity of *N. meningitidis* clinical isolates has highlighted the importance of heme uptake to meningococcal virulence. While not all *N. meningitidis* strains express both the HmbR and HpuAB systems, most express at least one. Most pathogenic isolates express at least HmbR, but HpuAB expression is equally associated with disease and carriage isolates, which indicates that HmbR is an indicator of pathogenesis [206,207]. *N.*

meningitidis serotype B isolates associated with disease also exhibit “on” phase variation of HmbR, correlating virulence with the expression of HmbR [208]. Additionally, HmbR is required for virulence in an infant rat model of meningitis [200]. These data implicate heme uptake, particularly HmbR, as an important component of *Neisseria* infection.

Current challenges and opportunities

Study of heme uptake strategies has offered great insight into bacterial pathogenesis and nutrient acquisition. There is still great opportunity for discovery. For most bacterial heme-binding motifs, the transfer from host hemoprotein has not been demonstrated as either passive dissociation or active extraction. The redundancy of heme uptake systems in pathogens like *B. anthracis*, *P. aeruginosa,* and *H. influenzae* is well appreciated, but the role of each system during infection of various niches or commensal environments has yet to be fully elucidated. The relative contribution of host heme to iron acquisition by bacterial pathogens during infection is understudied. It is unclear if pathogens rely on heme for iron in unique spatiotemporal niches and rely on ferrous iron and siderophore acquisition systems in other niches. Opportunity abounds to understand the role of heme-iron utilization across time and tissues during infection. Finally, while global abundance of heme and Hb in the host has been measured, the local availability of heme and hemoproteins during infection has not been described and presents an opportunity to understand the microenvironment of an infectious niche as well as the host response to infection.

In terms of clinical application, heme uptake systems may be attractive therapeutic targets. *S. aureus* Isd proteins have been the target of vaccine development with mixed success and monoclonal antibodies against IsdB have been studied for therapeutic use [209–213]. Considering the importance of heme acquisition to infection, using surface-exposed heme uptake proteins as targets for vaccine and antibodies should continue to be investigated. Additionally, the *Mycobacterium tuberculosis* heme uptake system, which comprises three unique proteins and is sufficient to rescue the growth of a heme auxotroph, has been proposed as a new mycobacteria-specific antimicrobial target to be explored [214–216]

The interactions between host hemoproteins and bacterial hemoprotein binding proteins offer an excellent opportunity to study host–pathogen co-evolution. It has been recently demonstrated that the human and primate iron-binding protein transferrin has undergone positive selection at the interface of binding by bacterial transferrin receptors, suggesting that the co-evolution of humans and pathogens has produced an evolutionary arms race in the context of nutritional immunity [217,218]. In the same vein, the Hb-binding IsdB of *S. aureus* exhibits species specificity and more efficiently utilizes human Hb relative to mouse Hb [86]. In keeping with this, transgenic mice expressing human Hb are more susceptible to *S. aureus* disease [86]. The contribution of bacterial heme acquisition to human evolution presents ample opportunity to further investigate co-evolution and nutritional immunity.

Heme Toxicity and Tolerance

Bacterial pathogens dedicate extensive cellular machinery to the synthesis and acquisition of heme. Paradoxically, excess heme is toxic and thus during infection, invading pathogens must contend with heme toxicity as a component of pathogenesis. While heme toxicity is

well studied in eukaryotes, less is known in bacteria [25,219,220]. A brief description of heme toxicity in bacteria and strategies utilized to combat toxicity follow.

Multi-faceted mechanism of heme toxicity

The reactive nature of heme that makes it such a versatile cofactor also results in toxicity at excess concentrations. While the toxicity of heme toward bacteria has been observed for over 60 years, a complete understanding of the mechanisms of heme toxicity is lacking [26,221]. Free heme is rapidly bactericidal toward various Gram-positive and Gram-negative pathogens in low- to mid-micromolar concentrations [33,222-225]. However, investigation of heme toxicity in a variety of bacterial species has led to a model of heme inducing iron- and non-iron-related damage to the cell.

The accumulation of heme results in excess iron by one of two mechanisms, both of which are likely at play under aerobic conditions. First, a portion of iron is freed by the heme oxygenases. Second, iron itself may be liberated from the porphyrin ring upon reaction with reactive oxygen species (ROS). Irrespective of the source, iron can cycle between ferrous and ferric states *via* Fenton chemistry and the Haber-Weiss reaction (reaction 1), yielding a regenerating supply of ROS.

$$\mathrm{Fe^{2+}} + \mathrm{H_2O_2} \rightarrow \mathrm{Fe^{3+}} + \mathrm{HO\bullet} + \mathrm{OH^-} \quad (1)$$

$$\mathrm{Fe^{3+}} + \mathrm{H_2O_2} \rightarrow \mathrm{Fe^{2+}} + \mathrm{HOO\bullet} + \mathrm{H^+} \quad (2)$$

Iron-mediated production of ROS can damage DNA, lipids, and proteins [226,227]. Further evidence for the contribution of oxidative stress to heme toxicity comes from *S. aureus.* In conditions of excess heme toxicity, membrane proteins are highly oxidized and superoxide is formed by redox cycling of heme-iron through membrane menaquinone [228]. Superoxide production is a separate source of oxidative damage from ferrous iron-mediated ROS and is a major component of heme damage in *S. aureus* [228]. In addition to experimentally validating that heme-mediated ROS is a key to heme toxicity, this work also localized heme toxicity primarily to the membrane. The lipophilic nature of heme suggests that it partitions to the membrane of bacteria, and this has been demonstrated in *S. aureus,* likely resulting in damage to membrane proteins and lipids [84].

Further evidence suggests that iron-mediated ROS production and subsequent membrane damage are an insufficient description of heme toxicity. First, heme is toxic in anaerobic conditions, and second, non-iron protoporphyrins are toxic to bacteria and activate the cellular response to heme toxicity [229–231]. Also, porphyrins cause significant damage to bacterial DNA [232]. Finally, resistance to heme toxicity is in part mediated in *N. meningitidis* by Ght (gene of hydrophobic agent tolerance), suggesting that damage by heme is similar to other hydrophobic molecules and may disrupt the Gram-negative outer membrane [224,233]. The toxicity of heme is likely the result of a combination of

membrane disruption, membrane protein and lipid oxidation, and DNA damage. However, a total understanding of heme-mediated damage is far from complete.

Strategies to overcome heme toxicity

While the direct result of excess heme is unclear, it is evident that bacteria must contend with heme damage and have evolved a variety of strategies to overcome heme toxicity (Fig. 4). These systems consist primarily of efflux and sequestration. Additionally, the heme oxygenase outlined as part of heme acquisition strategies may contribute to the reduction of heme toxicity by cleaving the porphyrin ring and liberating iron for use.

Heme efflux strategies have been primarily characterized in Gram-positive organisms, potentially because efflux across a single-membrane barrier is simpler to achieve than in Gram-negative pathogens. Three systems have been described, HrtAB, PefAB/CD, and MtrCDE. The *S. aureus h*eme-*r*egulated *t*ransporter HrtAB is required for survival in toxic concentrations of heme. *hrtAB* expression is activated by the HssRS *h*eme *s*ensing TCS [222,234,235]. While the ligand of the HssS histidine kinase has remained elusive, excess exogenous or endogenous heme leads to activation, either directly or indirectly [99]. HrtA is an ATPase that drives efflux by HrtB permease of its ligand, likely heme. Orthologues of HrtAB have been described in *B. anthracis* and *Lactococcus lactis* and are required for resistance to heme toxicity in these organisms [236]. When the Hrt efflux pump is inactivated in both S. aureus and *L. lactis*, levels of intracellular heme increase, suggesting that heme is the substrate of HrtAB export [229,237]. In *B. anthracis*, an HssRS orthologue controls the expression of HrtAB and cross-talks with a second TCS that responds to cellular envelope stresses, further implicating membrane damage as a component of heme stress [238]. HrtAB is actively expressed during murine anthrax, suggesting that organisms that replicate in the bloodstream must tolerate heme toxicity [33].

Additional efflux systems exist, suggesting that this strategy is well conserved. *Streptococcus agalactiae* encodes an orthologue of HrtAB, as well as a dual efflux system PefAB and PefRCD [223]. In heme stress, *hrtAB* and *pefAB*/*RCD* are expressed at high levels, and the Pef systems are required for resistance to heme toxicity [223]. The Gram-negative *N. gonorrhoeae* encodes an efflux pump, MtrCDE, for hydrophobic molecules that is required for resistance to heme stress [239].

Heme sequestration and storage is a second theme in strategies to resist heme toxicity. The conserved HemS family has been described in *Yersinia enterocolitica*, *Y. pestis, Shigella dysenteriae* (termed ShuS), *P. aeruginosa* (called PhuS, detailed above), and *E. coli* (ChuS, which also has heme oxygenase activity) [146,160,225,240–244]. While a variety of heme storage, transfer, and degradation properties have been assigned to these proteins, their involvement in resisting heme toxicity is clear. Additionally, non-HemS family proteins have been found to bind heme and play a role in heme homeostasis, including the small outer-membrane Protein E of *H. influenzae* and the Cu,Zn superoxide dismutase of *Haemophilus ducreyi* [245,246].

Current challenges and opportunities

While numerous systems are involved in detoxifying heme, there are many outstanding questions. The efflux systems have been described genetically, but a complete understanding of the ligands exported is still murky. For Gram-positive pathogens, the efflux systems may provide an additional therapeutic target for infection. Inhibition of efflux may offer a treatment option for bloodstream infections by *S. aureus* and *B. anthracis*; presumably the effects of heme toxicity would be deadly to the bacterium if the HrtAB pump were pharmacologically inactivated. This strategy could also pair well with small molecule activation of heme synthesis, which has been developed [99]. In terms of heme sequestration proteins, it has been difficult to fully interpret the contribution of heme sequestration because additional properties like oxygenase (PhuS and ChuS) and DNA binding (ShuS) have been observed. Finally, the role of heme oxygenases in resisting heme stress has not been well studied.

Concluding Remarks

Heme synthesis, uptake, utilization, and toxicity have been an area of intense investigation in bacterial pathogenesis. As outlined throughout, there are many additional questions in this field. Some of the most fundamental aspects of heme homeostasis have not been studied in detail. For most pathogens, regulation of heme synthesis is unclear and the contribution of heme synthesis to infection has not been investigated. For organisms that acquire and synthesize heme, a full model of preference between exogenous and endogenous sources is unknown. Based on limited evidence, exogenous heme is preferred when available, but is there a division between exogenous and endogenous heme in the partitioning to hemoproteins and heme oxygenases? Also, when heme enters the cell or is synthesized, does it exist in the free state or are there heme chaperones analogous to metallochaperones?

As the heme uptake, synthesis, and toxicity processes are well conserved and vital to bacterial pathogenesis, they present an opportunity for therapeutic intervention. As the field gains further insight into these processes, hopefully academia and industry will pursue small molecule interventions and vaccine candidates for the treatment of the bacterial pathogens outlined in this review, many of which are recalcitrant current treatment options.

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Abbreviations

Hb	hemoglobin
Hp	haptoglobin
ALA	δ-aminolevulinic acid
PBG	porphobilinogen

HMB	hydroxymethylbilane
Hx	hemopexin
SCV	small colony variant
PPIX	protoporphyrin IX
NEAT	near-transporter domain
Isd	iron-regulated surface determinants
TCS	two-component system

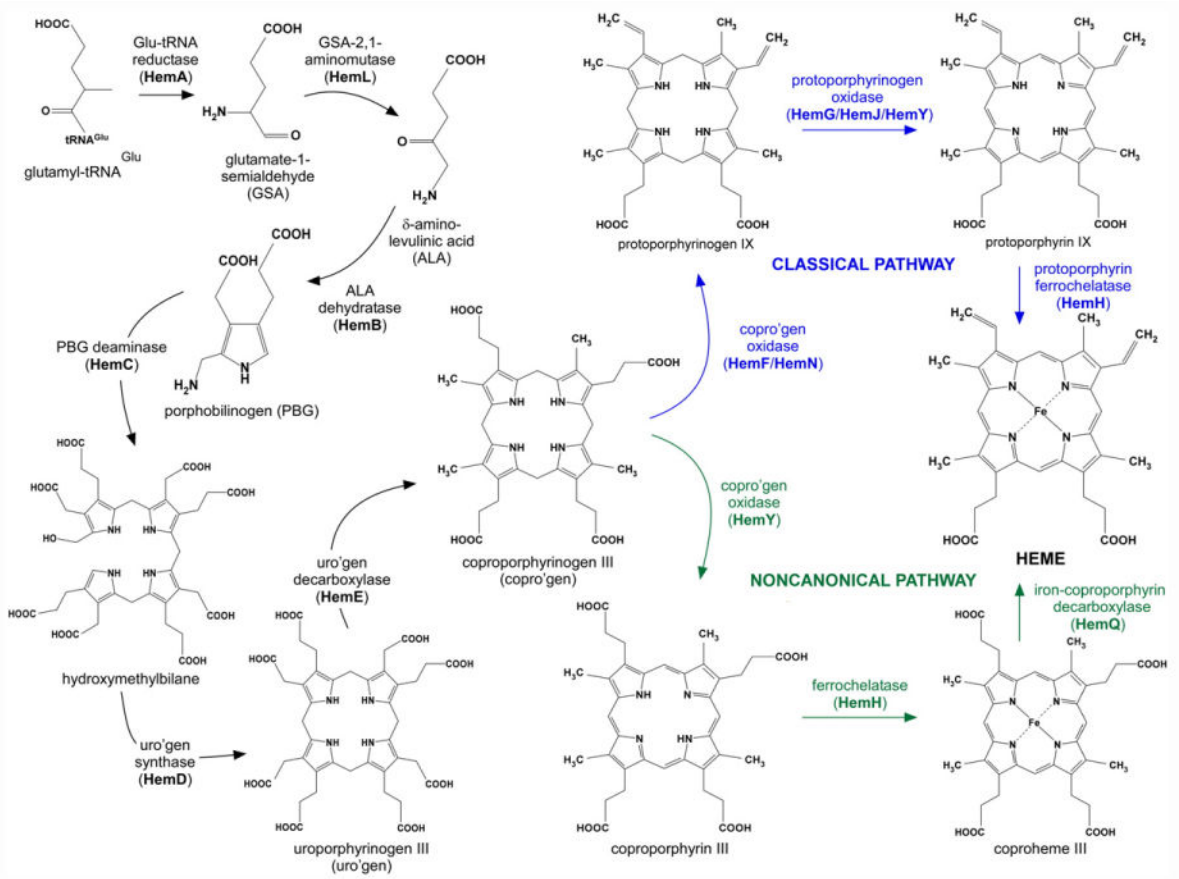

Fig. 1. Bacterial heme biosynthesis

The heme synthesis pathway of most bacteria begins with charged glutamyl-tRNA^{Glu} to form the universal precursor ALA, and coproporphyrinogen III isformed through a series of conserved enzymatic steps. The classical pathway (blue) forms heme through the protoporphyrinogen IX intermediate; most organisms including Gram-negative bacteria and eukaryotes use this pathway. The noncanonical pathway (green), performed by most Gram-positive bacteria, produces heme through the coproporphyrin III intermediate. Shown for each step is the enzyme name followed by the common protein annotation in bold.

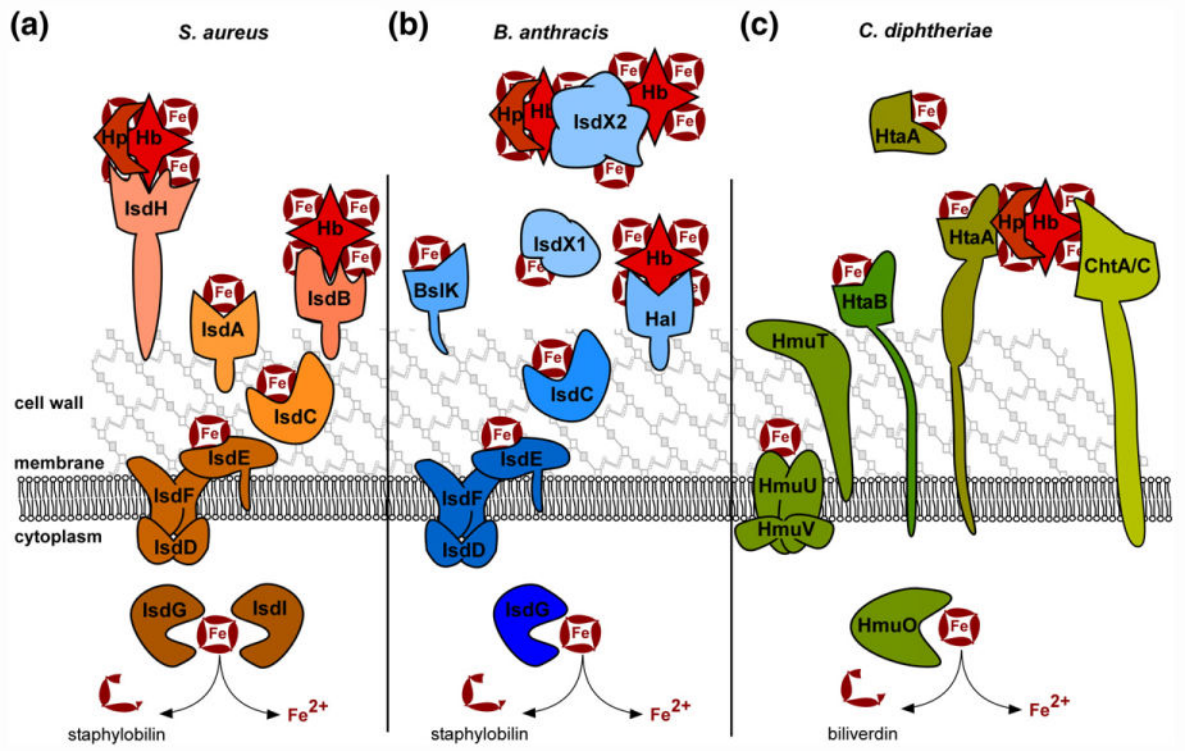

Fig. 2. Gram-positive heme uptake systems

The *i*ron-regulated *s*urface *d*eterminant (Isd) systems for heme acquisition in *S. aureus* and *B. anthracis*, as well as the non-Isd systems of *C. diphtheriae* are diagrammed. Host Hb, Hp-bound Hb, and free heme (Fe-containing ring) can serve as heme sources during infection. (a) In *S. aureus*, IsdH is the primary Hp–Hb receptor and IsdB is the principle Hb receptor. Both are sortase-linked on the surface of the cell wall, bind host hemoproteins with NEAT domains, and extract heme using additional NEAT domains. IsdA can bind free heme or accept heme from IsdB and IsdH. Heme is transferred to IsdC, which is embedded in the cell wall and transits heme to the membrane complex IsdDEF. IsdDEF transports heme to the cytoplasm for utilization intact or for degradation by the heme oxygenases IsdG/I. (b) Similarly, *B. anthracis* uses Isd proteins to acquire heme. IsdX1 and IsdX2 are secreted hemophores that bind Hb, Hp–Hb, or free heme as depicted. IsdX2, which has five NEAT domains, may also serve as a heme storage protein. Additionally, the sortase anchored Hal serves as a Hb receptor on the cell surface and uses its NEAT and leucine-rich repeat domains to acquire heme. BslK is cell wall associated and binds heme *via* its NEAT domain. IsdC transports heme to the IsdDEF membrane importer for utilization or degradation by IsdG. (c) *C. diphtheriae* utilizes a unique set of heme uptake proteins for heme utilization. HtaA is a cell wall spanning lipoprotein that can acquire heme from Hp–Hb in conjunction with ChtA or ChtC. HtaB can bind free heme or accept heme transfer from HtaA and transfers heme to the HmuTUV membrane transporter. A portion of HtaA may also serve as a secreted hemophore. *C. diphtheriae* HmuO heme oxygenase can liberate iron from imported heme.

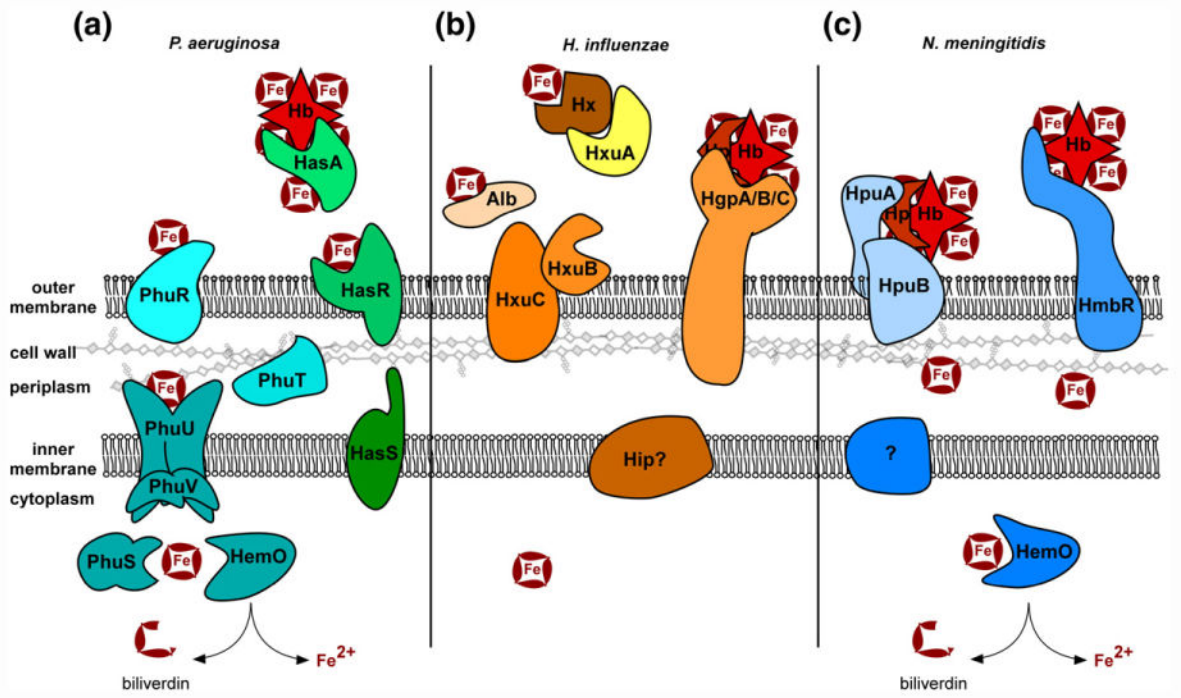

Fig. 3. Gram-negative heme acquisition

The heme uptake systems as described in the text are depicted. (a) *P. aeruginosa* PhuR binds heme at the outer membrane and imports heme into the periplasm in a TonB-dependent manner. Heme is transferred to PhuT, which subsequently transfers heme to the PhuUV inner-membrane transporter for transit into the cytoplasm. There, PhuS binds and stores heme or transfers heme to the heme oxygenase HemO for iron utilization. *P. aeruginosa* also secretes the hemophore HasA which binds Hb or free heme, and transfers heme to the TonB-dependent outer-membrane receptor HasR. The fate of HasR imported heme is not fully understood, but may be trafficked to PhuTUV for import. HasS serves as an inner-membrane sensor and regulates expression of the *has* and *phu* systems through the sigma factor HasI (not shown). (b) *H. influenzae* can utilize a variety of host heme sources. Secreted HxuA specifically binds hemopexin (Hx), and heme from Hx is transferred into the periplasm when HxuA interacts with HxuBC at the outer membrane. Independent of HxuA, HxuC can also import heme from serum albumin (Alb). HgpA, HgpB, and HgpC are highly similar outer-membrane receptors for heme acquisition from Hb complexed with Hp, free Hb, and Hp bound to myoglobin (not shown). The inner-membrane heme transporter has not been clearly defined, but the Hip system has been implicated for heme transit into the cytoplasm. Interestingly, all imported heme may be utilized intact, as no heme oxygenase has been identified yet. (c) The *N. meningitidis* outer-membrane, TonB-dependent complex of HpuAB can acquire heme from Hb and Hp–Hb and bring heme into the periplasm. Additionally, the HmbR outer-membrane receptor specifically extracts heme from Hb for transport. The identity of the inner-membrane heme transporter is unclear at this time, but heme somehow enters the cytoplasm where it can be utilized or degraded by the HemO heme oxygenase.

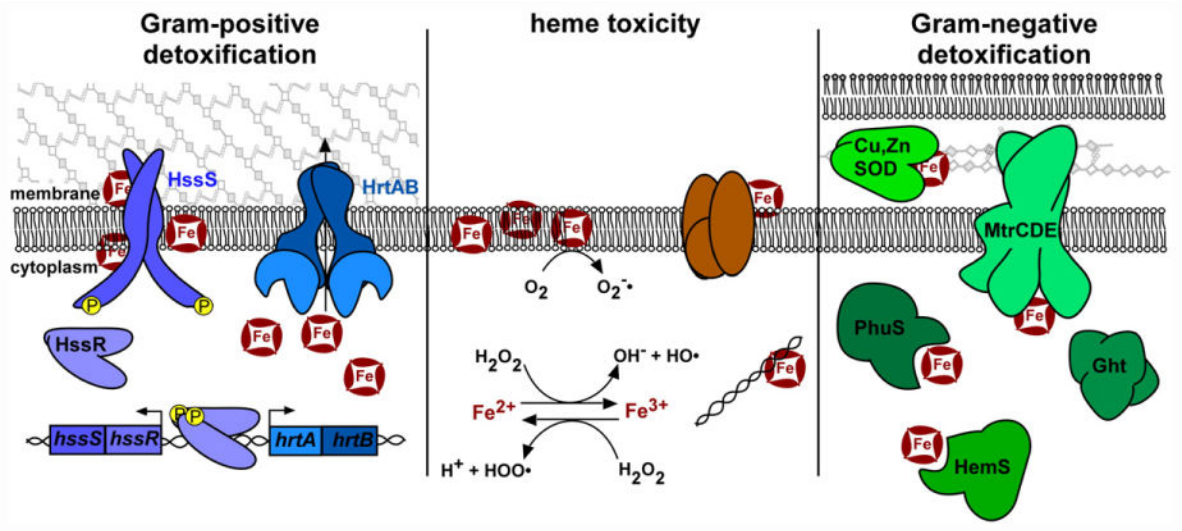

Fig. 4. Strategies to avoid heme toxicity

Heme toxicity (center) is a combination of heme damage to membrane lipids, membrane proteins, and DNA, and oxidative damage. Oxidative damage is mediated by the production of superoxide dismutase (O_2^-•), hydroxyl radical (HO•), and hydroperoxyl radical (HOO•). To reduce heme damage, many Gram-positive organisms (the *S. aureus* system is diagrammed here) encode the HrtAB efflux pump. The HssRS two-component system responds to excess heme and activates the transcription of the *hrtAB* system, thus preventing the accumulation of toxic levels of heme. Alternatively, Gram-negative organisms rely on intracellular heme sequestration proteins (PhuS of *P. aeruginosa*, HemS of *Yersinia*), the periplasmic heme-binding, copper and zinc dependent superoxide dismutase (Cu,Zn SOD, of *H. ducreyi*), and systems that respond to hydrophobic molecules, including heme (MtrCDE efflux and Ght of *Neisseria*).