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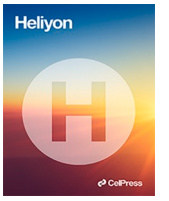

Review article

Heme acquisition and tolerance in Gram-positive model bacteria: An orchestrated balance

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ABSTRACT

As a nutrient, heme is important for various cellular processes of organism. Bacteria can obtain heme via heme biosynthesis or/and uptake of exogenous heme from the host. On the other side, absorption of excess heme is cytotoxic to bacteria. Thus, bacteria have developed systems to relieve heme toxicity and contribute to the maintenance of heme homeostasis. In the past decades, the mechanisms underlying heme acquisition and tolerance have been well studied in Gram-positive model bacteria, such as *Staphylococcus*, *Streptococcus* and other Gram-positive bacteria. Here, we review the elaborate mechanisms by which these bacteria acquire heme and resist heme toxicity. Since both the heme utilization system and the heme tolerance system contribute to bacterial virulence, this review is not only helpful for a comprehensive understanding of the heme homeostasis mechanism in Gram-positive bacteria but also provides a theoretical basis for the development of antimicrobial agents.

1. Introduction

As an essential nutrient for most organisms, heme is involved in diverse cellular processes, such as oxygen storage and transportation, electron transfer, aerobic respiration, and gas sensing [1–5]. In vertebrates, free heme is rare because most of it is bound to hemoglobin (Hb), myoglobin (Mb) and hemopexin (Hpx) [3,6]. Infectious bacteria have developed a high-affinity heme utilization system to transport heme from their host to establish infection. Once the bacterial heme utilization system is destroyed, the virulence of the bacteria will be attenuated [2,7,8]. Regarding Gram-positive bacteria, the heme transport system of *Staphylococcus aureus* was the first identified and is well-studied, which was called the iron-regulated surface determinant (Isd) system [9].

However, excess heme is toxic to bacteria, it causes membrane disruption, membrane protein and lipid oxidation, and DNA damage

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[2,10]. To overcome heme toxicity, bacteria employ multiple heme tolerance systems to detoxify, including efflux, degradation and sequestration. The mechanisms of heme detoxification were well studied in Gram-positive bacteria, such as *S. aureus*, *Streptococcus agalactiae* and *Clostridioides difficile*. When the heme is overloaded, *S. aureus* sense the signal through the two-component signal transduction systems (TCS) HssRS, which leads to heme efflux through the heme efflux system HrtAB [11]. Other Gram-positive bacteria, such as *S. agalactiae* employs porphyrin-regulated efflux system, PefAB and PefRCD, to export heme [12]. *C. difficile* employs the heme-activated operon HatRT and HsmRA to sense and detoxify heme [7,8]. For degradation, IsdG and IsdI heme oxygenases in *S. aureus* degrade heme to free iron, staphylobilins and formaldehyde [13].

In recent years, heme detoxification systems have made more progress in Gram-positive bacteria than heme transport system. Herein, we review the mechanisms of heme utilization and detoxification in the classical model of Gram-positive bacteria, *S. aureus,* and other Gram-positive bacteria.

1.1. Heme acquisition from the host

1.1.1. Heme acquisition mediated by the Isd system of S. aureus

S. aureus is a facultative anaerobe that uses heme during anaerobic nitrate respiration and aerobic respiration [14]. *S. aureus* could lyse erythrocytes to release Hb through secreted hemolysins, and then binds free Hb and extracts heme [15]. Mazmanian et al. (2003) reported that the high-affinity Isd heme acquisition system of *S. aureus* is responsible for Hb binding and the passage of heme into the cytoplasm [9]. The Isd system is composed of nine proteins, which are encoded in five Fur-regulated transcriptional units [16]. IsdA, IsdB, IsdC, and IsdH are cell wall-anchored heme-binding surface receptors; IsdDEF is membrane-associated transport system; and IsdG and IsdI are two cytoplasmic heme oxygenases [17].

Four Isd surface receptors contain an N-terminal secretion signal and a C-terminal sortase signal; IsdA, IsdB, and IsdH are anchored by the cell housekeeping sortase A; and IsdC is attached by StrB [9,17]. All the Isd surface proteins reversibly bind heme through the NEAT (near-iron transporter) domain. NEAT domains consist of approximately 120–125 amino acids and share a β-sandwich fold, and it is primarily responsible for binding Hb and heme [17,18]. IsdA and IsdC contain one NEAT domain each, and binds heme with high affinity. IsdB contains two NEAT domains: domain one (N1) binds Hb and domain two (N2) binds heme [17]. IsdH contains three NEAT domains: domain one and two (N1 and N2) are for Hb and Hb-Hp binding, and domain three (N3) is for heme binding [19,20].

The surface receptors IsdB and IsdH are the first step to extract heme molecules from Hb using a tri-domain unit joined by a helical linker domain. The tri-domain unit in IsdB is formed by domains N1 and N2, and that in IsdH is formed by domains N2 and N3 [21,22]. IsdB binds to the β-subunits of the Hb tetramer, leading to Hb dimerization, and IsdB binds to the α-chain of Hb. Then, heme is extracted by IsdB, and the F-helix in the β-subunits is unfolded [23]. IsdH binds the α-globin F helix of Hb, then the F-helix undergoes conformational change that disrupts heme pocket structure and promotes heme release [24]. The IsdH and Hb complex is dynamic. In this complex, the N2 domain binds Hb, and the inter-domain motions within IsdH enable the N3 domain to transiently distort the Hb's pocket resulting in heme exposure [21,25].

Following capture by IsdH and IsdB, heme is unidirectionally passed to IsdA, and subsequently transfers to IsdC [17]. Heme is transferred between NEAT domains through the formation of handclasp complexes [16,26]. Subsequently, heme is transferred to the heme-specific lipoprotein IsdE, and then the permease IsdF transfers heme across the membrane. IsdD was speculated to associated with IsdEF, its function remains to be identified [9]. *S. aureus* possesses special compartments, functional membrane microdomains [FMMs], for coordinating diverse cellular functions [27]. IsdF is energized by the housekeeping ATPase FhuC, and IsdF interacts with the FMM scaffolding protein flotillin A (FloA), and co-localize on the intact bacterial cells [27]. Isd-dependent heme utilization and proliferation in *S. aureus* requires FMMs and floA [27].

Cytoplasmic heme can be degraded by heme oxygenases IsdG and IsdI to release free iron or be incorporated into protein as a co-factor [2,17]. Mutations in the Isd system components lead to it being highly deficient in virulence, indicating that heme acquisition is important for *S. aureus* infection and is a potential target for the development of novel antibiotics [28,29]. Homologues of the Isd system were identified in *Bacillus anthracis* [30], *Bacillus cereus* [31], *Listeria monocytogenes* [32] and *Staphylococcus lugdunensis* [33].

1.1.2. Heme acquisition system of Corynebacterium diphtheriae

The human pathogen, *C. diphtheriae* causes respiratory disease and secretes the potent diphtheria toxin (DT), using heme and Hb from the host as an essential iron source [34,35]. Drazek et al. (2009) showed that the *hmu* genetic cluster is involved in heme transportation, and its expression is regulated by the diphtheria toxin repressor, DtxR, and iron [36]. The *hmu* genetic cluster includes ATP binding cassette (ABC) heme transporter HmuTUV and two surface-anchored proteins: HtaA and HtaB. HmuTUV is composed of the heme-binding lipoprotein HmuT, the heme transporter permease protein HmuU and the ATP-binding protein HmuV [35]. HmuT is tethered to the cytoplasmic membrane via an N-terminal lipid region [36]. HtaA and HtaB contain N-terminal signal region and C-terminal transmembrane domain, and HtaA is a membrane-anchored protein and also a secreted protein [36]. HtaA binds heme through two conserved regions: CR1 (conserved region 1) and CR2 [37]. The CR domain contains conserved tyrosine and histidine residues, which is important for heme and hemoproteins binding [38]. The *C. diphtheriae* CR domain has no similarity with NEAT domains [34]. HtaB contains one CR domain, and it functions as a mediator of heme transportation [39].

Allen et al. (2011) showed that two genetic loci *chtA-chtB* and *chtC-cirA* also participate in heme and Hb binding and are regulated by DtxR and iron [39]. ChtA and ChtC are surface-anchored proteins, each contains a single N-terminal CR domain, and ChtB has one CR domain [39]. ChtA has 35% similarity with HtaA, which is only limited to the CR domain, ChtB has 63% similarity with HtaB, and ChtC has 47% similarity with ChtA [39]. This means that *C. diphtheriae* employ multiple-function membrane proteins to fulfill heme requirements. Homologues of the Hmu system, ChtA, ChtC and ChtB are distributed predominantly in *Corynebacteria*.

In addition to the heme-binding proteins mentioned above, a unique iron-regulated, Hb- and Hb-Hp binding protein (HbpA) was also identified in *C. diphtheriae* [40]. HbpA is both located in the membrane and secreted, and exists in a large-molecular-weight aggregate that is important for optimal binding to Hb and Hb-Hp [41]. HbpA does not bind heme and contains a CR domain but can bind Hb and Hb-Hp [40]. Structural analysis of HbpA protein revealed that the C-terminal region is pivotal for the use of heme-iron from Hb-Hp and plays a role in anchoring to the membrane [41]. Homologues of HbpA are only distributed in some of C. *diphtheriae* strains [40].

The heme acquisition system of *C. diphtheriae* acquires heme through the following process. At the first step, most Hb and Hb-Hp are bound by secreted or membrane-associated HbpA, and HbpA can promote the binding of Hb-Hp to surface receptors HtaA and ChtA/C [40]. HtaA and ChtA/C bind heme from Hb or Hb-Hp via CR domains and transport heme to HtaB or ChtB [34,41]. Subsequently, heme is transported by HmuTUV across the cell membrane into the cytoplasm and degraded by heme oxygenase HmuO or bound with different apoproteins as an enzymatic co-factor for utilization or storage [34].

1.2. Excess heme is toxic to bacteria

Although heme is essential for the biological function of proteins, it becomes toxic to bacteria when present in excess. For example, when erythrocytes are lysed, infectious bacteria encounter high concentrations of heme. We named this process nutritional intoxication. Excessive free heme causes deleterious effects, such as membrane disordered, oxidative stress damage, lipid oxidation, and DNA damage [2]. Owing to the natural lipophilicity of heme, it tends to gather in the cell membrane. Then, heme and membrane-associated quinone molecules undergo cyclic redox reactions, and generate highly reactive semi-quinones and reduced heme [42]. Both products can react with atmospheric oxygen to produce superoxide radicals, resulting in oxidative stress damage to the cell membrane [42].

Heme metabolites are also toxic to bacteria. Bilirubin is the terminal metabolite in heme catabolism, and can destroy the structure and function of the bacterial cell membrane via direct intercalation, resulting in membrane destabilization [43]. Excess heme results in redundant iron production in two ways. First, heme oxygenases degrade heme and release free iron. Second, iron itself can secede from the middle of the porphyrin ring when oxidation occurs [2,7]. Fenton chemistry reactions and Haber–Weiss reactions are classical chemical reactions that result in the production of ROS components, such as hydroxyl radicals, as the iron cycle moves between the ferrous and ferric states [2]. The hydroxyl and superoxide radicals have strong oxidizing properties, which can lead to DNA damage, membrane disruption, and membrane protein and lipid oxidation, thus resulting in irreversible damage [2,7,10].

Porphyrin is also toxic to bacteria. The reduced porphyrin is a pro-oxidant, whereas the oxidized porphyrin is photosensitive, releasing labile singlet oxygen or hydroxyl radicals under ultraviolet or visible light, which results in different redox reactions [44]. In the early stages of growth in Gram-positive bacteria, porphyrin is in its photo-activated state and can inhibit the growth of bacteria as a result of lipid peroxidation, oxidation of nucleotides in DNA and amino acids in proteins, and protein cross-linking, which leads to bacterial cell damage and death [45].

2. Strategies of Gram-positive bacteria to resist heme toxicity

To overcome heme toxicity, Gram-positive bacteria have evolved and developed various heme detoxification strategies, including efflux, degradation, and sequestration.

2.1. Bacterial heme efflux pump

Bacterial efflux pumps are responsible for the efflux of natural substances produced by the host, such as bile, hormones, and other substances [46,47]. Currently, six families of efflux pumps have been described. These include the primary active transporters from the ABC family, which are dependent on ATP hydrolysis to drive transport [46]. The other five families include the major facilitator superfamily (MFS), resistance-nodulation-cell division (RND) superfamily, small multidrug resistance (SMR) family, multidrug and toxin extrusion (MATE) family, and proteobacterial antimicrobial compound efflux (PACE) family, which are powered by proton motive force (PMF) [46]. In Gram-positive bacteria, the ABC family transporter HrtAB in *S. aureus*, the MFS family transporter HatT in *C. difficile*, the MFS family transporter PefA and the ABC family transporter PefCD in *S. agalactiae* mediate heme efflux [7,12,48,49].

Table 1
The heme efflux pumps and its regulators.

Bacteria	Transporters	Regulators	signaling	References
S. aureus, *B. anthracis*, *B. thuringiensis*, *S. agalactiae*, L. monocytogenes	HrtAB	HssRS	heme	[52–55,86]
C. diphtheriae	HrtAB	ChrSA	heme	[56]
L. lactis	HrtAB	HrtR	heme	[57]
E. faecalis	HrtAB	FhtR	heme	[58]
S. agalactiae	PefAB/PefCD	PefR	heme	[12]
C. difficile	HatT	HatR	heme	[7]

2.2. Heme efflux regulated by the HssRS and HrtAB system of S. aureus

S. aureus can adapt to high concentrations of heme (10 μM) after being pre-exposed to a sub-toxic concentration (2 μM), indicating that *S. aureus* encode heme detoxification system [50]. Actually, it has been shown that *S. aureus* employs the heme-sensing TCS (two-component signal transduction systems), HssRS, and the heme-regulated ABC transporter, HrtAB to reduce heme toxicity [51] (Table 1). HssRS is composed of the response regulator, HssR and the histidine kinase protein, HssS. HrtAB consists of the cytoplasmic HrtA and the integral membrane permease protein HrtB [11,51]. When *S. aureus* is exposed to high concentrations of heme, heme-responsive sensor HssS undergoes auto-phosphorylation at histidine 249 and subsequently transfers its phosphate group to aspartate 52 of HssR. Phosphorylated HssR binds to a direct repeat sequence within the *hrtAB* promoter and induces the expression of HrtAB, which is responsible for the efflux of excess heme to the extracellular environment [11,51]. Consistently, deletion of *hssRS* or *hrtAB* results in high heme sensitivity [48]. HssS-HssR dependent regulation of HrtA and HrtB to alleviate heme pressure is important for *S. aureus* survival and infection.

The homologues of HssRS and HrtAB have been characterized in other Gram-positive bacteria, including *B. anthracis* [52], *Bacillus thuringiensis* [53], *Streptococcus agalactiae* [54], and L. monocytogenes [55], indicating that the heme detoxification strategies of HssRS and HrtAB systems are generally conserved in these bacteria (Table 1). However, although the homologues of HrtAB have been described in *C. diphtheriae* [56], *Lactococcus lactis* [57], and *Enterococcus faecalis* [58], no TCS HssRS homologues identified in these bacteria, suggesting that they have a unique transcriptional regulator for activating HrtAB function [58].

C. diphtheriae employs the TCS consisting of cytoplasmic membrane sensor ChrS and response regulator ChrA to mediate the transcription of *hrtAB* [56] (Fig. 1A) (Table 1). ChrS senses heme and undergoes auto-phosphorylation [59,60]. The signal is transferred to ChrA, and then phosphorylated ChrA binds to the target promoter regions, activating the transcription of *hrtAB* [56] (Fig. 1A). ChrA consists of an N-terminal regulatory domain and a C-terminal DNA-binding region, and the linker region of the N-terminal could create a dimerization interface binding DNA when receiving a heme-sensing signal [61]. Deletion of *chrAB* failed to trigger the expression of *hrtAB* under heme pressure, and mutations in *hrtAB* cause high heme sensitivity and the accumulation of cytoplasmic heme [56,62].

Recently, the crystal structures of the *C. diphtheriae* HrtAB and HrtAB-heme complex was resolved [63]. The entire HrtAB comprises two copies of HrtA-HrtB pair, HrtA contains a nucleotide-binding domain, and HrtB contains a transmembrane domain and an extracytoplasmic domain [63]. The HrtB dimer contains a Glu219 residue for the heme-binding site, which is located on the surface of the outer leaflet of the membrane. The heme-binding site captures heme from the membrane, and the heme is placed in the rearranged transmembrane helix bundle. The HrtA dimerization is driven by ATP-binding, and the heme-binding site is squeezed to extrude the bound heme from the cytoplasmic membrane [63]. The reduction of heme in the cytoplasmic membrane leads to a corresponding decrease in heme in the cytoplasm, as heme is evenly distributed between the cytoplasm and the membrane [64,65].

L. lactis is generally used for industrial fermentation and requires heme to activate aerobic respiration. HrtA and HrtB from *L.* lactis shares 50% and 37% homology with HrtA and HrtB from *S. aureus*, respectively. The heme-regulated transport regulator, HrtR, belonging to TetR family, is responsible for heme sensing and is adjacent to *hrtAB* [57,66] (Fig. 1B) (Table 1). The expression of *hrtA* and *hrtB* is repressed by HrtR through direct binding to the 15-nt palindromic sequence within the *hrtAB* promoter [57]. HrtR binds heme at the histidine residues His72 and His149, resulting in a coil-to-helix transition of the α4 helix in the heme-sensing domain and structural changes in HrtR, further leading to HrtR dissociating from the binding site of the *hrtAB* promoter [57,66]. Therefore, the expression of the HrtAB transporter alleviates heme pressure. Consistently, a hrtRAB mutant of L. lactis exhibited deficient growth when plated in a solid medium containing excess heme [57]. The homologues of HrtR have also been identified in other food and commensal bacteria, such as *Lactobacillus amylolyticus* and *Leuconostoc citreum* [57].

E. faecalis colonizes the human intestinal flora and is a threat to immunocompromised patients leading to severe diseases [67]. Heme is beneficial for *E. faecalis* as it facilitates the activation of respiration and the expression of heme-dependent catalase A (*katA)*

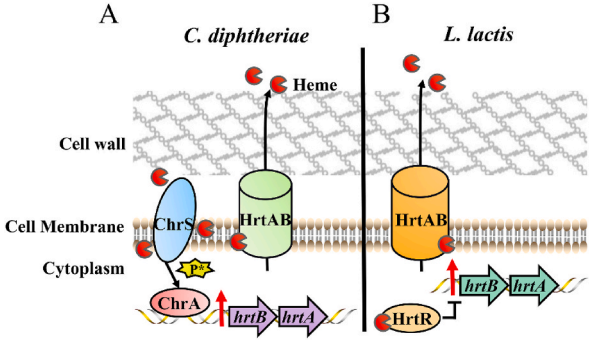

Fig. 1. Heme efflux systems of *C. diphtheriae* and *L. lactis* [56,57]. (A) In *C. diphtheriae*, cytoplasmic membrane ChrS senses heme and auto-phosphorylation occurs. The phosphate group is transferred to ChrA such that phosphorylation ensues. ChrA binds the DNA sequence within the promoter of *hrtAB*, thereby activating the expression of *hrtA* and *hrtB* and resulting in the extrusion of heme via the HrtAB efflux pump. P* represents the phosphate group. (B) In L. *lactis*, the regulator HrtR binds to heme and alleviates the inhibition on the hrtAB promoter, leading to the extrusion of heme by HrtAB.

[58]. HrtA and HrtB from *E. faecalis* share 24% and 45% homology with HrtA and HrtB from *S. aureus*, respectively [58]. Neither *hssRS* nor *hrtR* homologues have been identified in *E. faecalis*. The *E. faecalis* heme transport regulator, FhtR belongs to the TetR family and is located upstream of *hrtAB* [58] (Table 1). FhtR binds the two distinct 14-nt inverted repeat regions within the *hrtAB* promoter and inhibits *hrtAB* expression [58]. FhtR binds internalized heme at a pivotal tyrosine residue Tyr132, and FhtR-heme complexes dissociated from the *hrtAB* operon, resulting in HrtAB-mediated heme exportation [58]. The homologues of FhtR primarily distributed in enterococci, vagococci, and carnobacteri [58].

In summary, considering the contribution of HrtAB to heme toxicity and its widespread conservation in Gram-positive bacteria, targeting HrtAB as a designed drug to inhibit its function would be beneficial for limiting bacterial infections.

2.3. Heme efflux mediated by the Pef system of Streptococcus agalactiae

The opportunistic pathogen *S. agalactiae* infects the human gastrointestinal tract, female genitourinary tracts, and bovine mammary glands, causing septicemia and meningitis [12,54]. *S. agalactiae* cannot synthesize heme and requires exogenous heme for respiration via the activation of the terminal oxidase cytochrome *bd* quinol oxidase (CydAB) [54]. Heme-regulated detoxification systems HrtAB and HssRS have been identified in *S. agalactiae* [54]. Furthermore, it was demonstrated that another porphyrin-regulated efflux system, PefAB and PefRCD, is also important for heme detoxification [12] (Fig. 2) (Table 1). PefAB is composed of the MFS transport protein, PefA and hypothetical protein, PefB. PefRCD is composed of the MarR-superfamily protein PefR and the ABC multidrug transporter PefCD [12]. PefR represses the expression of *pefAB* and *pefRCD* operons by binding to the DNA inverted repeat motifs (IR) within the promoter regions. When heme is in excess, the PefR-heme complex is formed. PefR then dissociates from the promoter regions, and PefAB and PefCD expel excess heme [12] (Fig. 2).

The *pefR* mutant strain displays a reduced respiration rate, due to the over-expression of *pefAB* and *pefCD*, indicating that PefR is important for restricting the expression of Pef efflux pumps. This allows maintenance of intracellular heme homeostasis and ensures that respiration and proliferation function optimally in *S. agalactiae* [12]. Compared with the HrtAB/HssRS system, the Pef system is activated under lower heme conditions (0.1–0.5 μM) [12]. This distinction allows *S. agalactiae* to rapidly adapt to intracellular heme levels under different cellular physiological conditions [12,54]. In summary, *S. agalactiae* relies on the HrtAB/HssRS and PefAB/-PefRCD systems to synergistically maintain heme homeostasis.

The homologues of PefAB are distributed in *Enterococcus faecalis*, Streptococcus uberis, and *Lactobacillus* gasseri, and the homologues of PefRCD are primarily distributed in *Streptococcus* genus, including *Streptococcus dysgalactiae, Streptococcus thermophilus*, and *S. uberis* [12].

2.4. Heme efflux mediated by the HatRT system of Clostridioides difficile

C. difficile is a spore-forming obligate anaerobe that infects the colon and causes severe damage to the intestinal epithelial layer, thereby causing inflammation and bleeding through the release of the bacterial toxins, TcdA and TcdB. The release of toxins leads to high levels of heme from lysed erythrocytes, causing heme toxicity [7]. *C. difficile* does not encode homologues of HrtAB, PefAB, or PefRCD. Compared to untreated cultures of *C. difficile*, the expression of an operon comprising two genes was upregulated by 5- to 11-fold the concentrations of heme-treated cultures (50 μM) in *C. difficile* [7]. These genes were shown to encode a TetR family transcriptional regulator and an MFS family multidrug transporter referred to as heme activated transporter regulator (HatR) and heme activated transporter (HatT), respectively [7] (Fig. 3) (Table 1).

Compared to the wild-type strain, *hatT* and *hatR* mutant strains were more sensitive to heme, and the sensitivity of the *hatT* mutant strain was even more prominent when exposed to excess heme [7]. Despite the constitutive expression of *hatT*, the *hatR* mutant strain was still sensitive to heme, indicating that HatR may function as a heme-binding protein where the histidine residue His99 was identified to be crucial for heme binding [7].

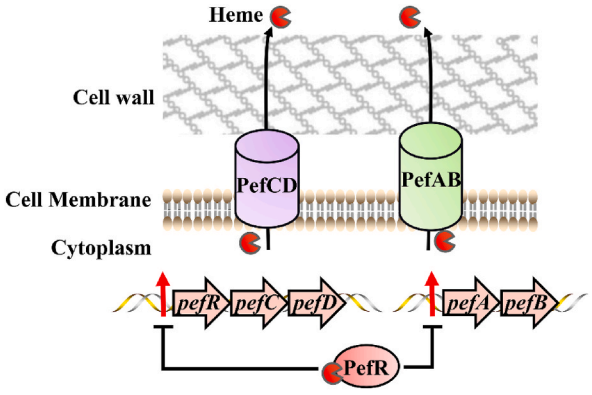

Fig. 2. Pef system of *S. agalactiae* [12]**.** PefR binds heme and thus derepresses the *pefAB* and *pefRCD* operon. Consequently, PefAB and PefCD are able to efflux heme and protect *S. agalactiae* from heme damage.

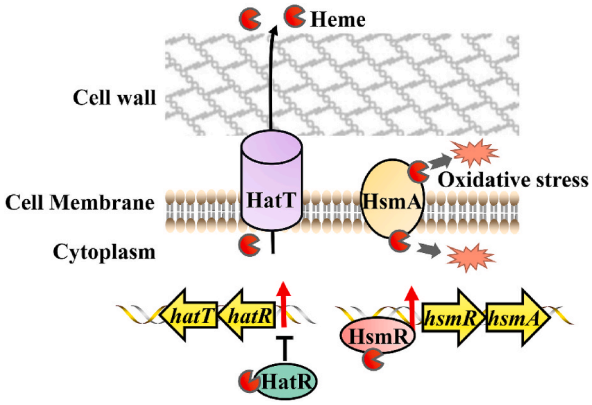

Fig. 3. HatRT and HsmRA systems of *C. difficile* [8]. HatR binds heme and derepresses the *hatRT* operon, which allows HatT to extrude heme. Similarly, HsmR senses heme and activates the expression of the *hsmRA* operon, and then HsmA binds heme and resists oxidative stress.

In general, in the presence of excess heme, HatR binds heme directly to form the HatR-heme complex and dissociates from the *hatRT* operon [7]. Subsequently, *hatT* transcription is activated and excess heme is pumped out [7] (Fig. 3). The HatRT system is essential for protecting *C. difficile* from damage caused by heme toxicity, for growth and for maintaining heme homeostasis.

2.5. Heme degradation and sequestration

2.5.1. Heme degradation mediated by HO-1 and IsdG-like heme oxygenases

Heme oxygenases (HOs) are mainly divided into two families in Gram-positive bacteria: the canonical HO-1 heme oxygenases and the non-canonical IsdG-like heme oxygenases (Fig. 4). The first HO-1 heme oxygenases is HO-like family protein (HmuO) in *C. diphtheriae*, which has significant homology with the human heme oxygenase protein HO-1 (33% identity and 70% similarity) [68]. The HO-1 heme oxygenases are a monomeric helical protein with an α-helical fold that similar to mammal HO-1, and the fold forms a heme-binding pocket containing two ordered water molecules [69,70]. HmuO oxidatively cleaves the α-carbon of heme to produce α-biliverdin, carbon monoxide (CO), and free iron [34,70] (Fig. 4A). The *hmuO* gene is activated by two paralogous TCSs, ChrS-ChrA and HrrS-HrrA in a heme- and Hb-dependent manner and is repressed by DtxR under iron-replete conditions [71,72]. Under heme accumulation conditions, the sensor kinases ChrS and HrrS detect heme and activate the phosphorylation of the response regulators ChrA and HrrA. The phosphorylated response regulators directly bind upstream of the *hmuO* promoter, thereby activating expression [72].

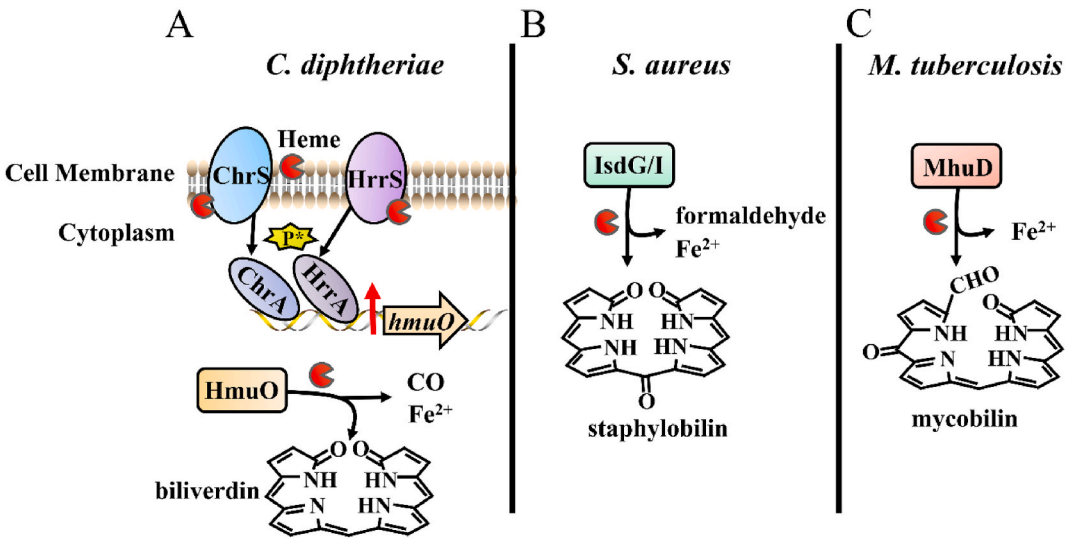

Fig. 4. The heme degradation systems [72,73,79]. (A) In *C. diphtheriae*, the sensors ChrS and HrrS sense heme and transfer signals to ChrA and HrrA. ChrA and HrrA bind the *hmuO* promoter, and the expression of *hmuO* is activated. HmuO degrades heme to Fe2+, CO, and biliverdin. (B) In *S. aureus*, heme oxygenases IsdG and IsdI degrade heme to Fe2+, formaidehyde, and staphylobilin. (C) In *M. tuberculosis*, MhuD degrades heme to Fe2+ and mycobilin.

The IsdG-like heme oxygenases degrade heme without CO production, which is distinct from HO-1 protein, and have no similarity to the HO-like family proteins. The IsdG-like heme oxygenases include IsdG/I in *S. aureus* and MhuD in *Mycobacterium tuberculosis* [13,70,73]. IsdG and IsdI share 64% identity, can degrade heme to release free iron, linear tetrapyrrole staphylobilins (5-oxo-δ-bilirubin and 15-oxo-β-bilirubin), and formaldehyde in the presence of NADPH cytochrome P450 oxidoreductase or ascorbate [74,75] (Fig. 4B). MhuD degrades heme to mycobilin isomers and free iron, and has 45% similarity with IsdG/I [73] (Fig. 4C).

Structural analysis of IsdG-like heme oxygenases revealed that these proteins have a ferredoxin-like fold and a β-barrel at the dimeric interface, and form homodimers of subunits containing only one polar amino acid, which is distinct from HO-like family proteins [76]. After being bound by IsdG-like proteins, heme undergoes significant heme ruffling [77]. Each dimer of IsdG and IsdI contains heme-binding pockets, and the residues responsible for heme binding are conserved between them, including His, Trp and Asn residues [76,77]. MhuD of *M. tuberculosis* can bind one or two molecules of heme at the same active site, whereas IsdG/I just binds a single heme at an active site [73]. Both diheme-MhuD and monoheme-MhuD complexes are enzymatically active and are able to degrade heme [78]. IsdG-like proteins have also been identified in *S. epidermidis* [79], *B. anthracis* [80], and *L. monocytogenes* [81].

2.5.2. Heme sequestration mediated by the HsmRA system

In *C. difficile,* the *hatRT* mutation did not influence colonization or persistence, indicating that *C. difficile* possesses another mechanism to resist heme toxicity [7,8]. Knippel et al. (2020) identified the heme-sensing membrane protein HsmRA, which is regulated by heme and responsible for heme sequestration and oxidative stress resistance [8]. HsmRA is composed of the MarR family transcriptional regulator HsmR, and the membrane protein HsmA. Excessive heme is bound by HsmR through a conserved histidine residue, His50, and then induces *hsmRA* transcription. HsmA reduces free heme concentrations through sequestration; moreover, heme-HsmA complexes could provide protection from oxidative stress [8,82] (Fig. 3). Consistently, *hsmR* or *hsmA* mutant strains are increasingly sensitive to excess heme when compared to the wild-type strain [8]. Homologues of HsmR are widely distributed in *Clostridia*, with a homology of 53%–78% [8]. Homologues of HsmA are widely distributed in *Clostridia*, *Bacillus*, *Lactobacillus*, *Enterococcus*, *Bacteroides,* and *Geobacter*, with a homology of 37%–74%, indicating that HsmA is a conserved strategy for heme sequestration across several bacterial genera [8].

2.5.3. Heme homeostasis mediated by HrrSA of Corynebacteria

The TCS HrrSA system, which is highly conserved among almost all corynebacterial species, including *C. diphtheriae* and *Corynebacterium glutamicum*, plays a crucial role in maintaining the homeostasis of heme when there is an excess of exogenous or endogenous heme [60,83,84]. The similarity between HrrSA in *C. glutamicum* and HrrSA in *C. diphtheriae* is 87% [84].

The regulatory functions of HrrSA encompass a wide range of activities. Under excessive heme conditions, HrrSA inhibits heme synthesis by suppressing the expression of heme synthesis enzymes such as GtrR, UroD, and CpfC [72,83,84]. Additionally, HrrSA activates bacterial respiration by upregulating the expression of genes involved in the respiratory chain, including *ctaE-qcrCAB*, *ctaD*, *ctaF*, and *cydAB* [84]. HrrSA also activates the expression of the gene *katG*, which encodes catalase, to counteract oxidative stress induced by heme [84]. Moreover, HrrSA stimulates the expression of genes related to cell membrane remodeling, such as *murA* and *aftC* [84]. Furthermore, HrrSA can activate the expression of other transcriptional regulators, including *ramA*, *ramB*, and *amtR* [84]. In summary, the HrrSA system coordinates the expression of genes involved in heme biosynthesis, respiration, oxidative stress response, and cell membrane remodeling to maintain heme homeostasis. The regulatory network of the HrrSA system is highly complex, and its conservation among corynebacterial species suggests its potential as a target for drug development.

3. Conclusion and perspective

Gram-positive bacteria employ finely regulated heme uptake and tolerance systems to adapt to varying heme levels in different environments. The mechanisms of heme uptake include the secretion of haemophore, active uptake and transport processes involving various receptors and associated uptake systems. Recently, it was shown that Gram-positive bacterium, Dietzia sp. DQ12-45-1b, releases membrane vesicles (MVs) to participate in extracellular heme capture [85]. Whether haemophore is secreted as MVs requires further investigation. On the other hand, when exposed to excessive heme, tolerance systems play a vital role in protecting bacteria from the toxic effects of heme, including mechanisms such as heme oxygenases, heme-binding proteins, and heme efflux pumps. Among six different efflux systems, only two efflux systems have been found to be involved in heme efflux. Other types of heme efflux systems may be identified in the future. Taken together, these heme acquisition and tolerance systems provide valuable insights into the adaptation and survival strategies of Gram-positive bacteria in diverse environments. Understanding the physiological and pathological implications of these systems may lead to novel therapeutic approaches and the development of drugs targeting heme-related processes in bacterial infections.

Author contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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