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Journal
Current Opinion in Chemical Biology, 19(1)

ISSN
1367-5931

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Publication Date
2014-04-01

DOI
10.1016/j.cbpa.2013.12.014

Peer reviewed

Published in final edited form as:
Curr Opin Chem Biol. 2014 April ; 0: 34–41. doi:10.1016/j.cbpa.2013.12.014.

Heme uptake in bacterial pathogens

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Abstract

Iron is an essential nutrient for the survival of organisms. Bacterial pathogens possess specialized pathways to acquire heme from their human hosts. In this review, we present recent structural and biochemical data that provide mechanistic insights into several bacterial heme uptake pathways, encompassing the sequestration of heme from human hemoproteins to secreted or membrane-associated bacterial proteins, the transport of heme across bacterial membranes, and the degradation of heme within the bacterial cytosol to liberate iron. The pathways for heme transport into the bacterial cytosol are divergent, harboring non-homologous protein sequences, novel structures, varying numbers of proteins, and different mechanisms. Congruously, the breakdown of heme within the bacterial cytosol by sequence-divergent proteins releases iron and distinct degradation products.

Keywords

pathogenic bacteria; heme acquisition; hemophore; heme-degrading; iron; heme transport

Introduction

Pathogenic bacteria have evolved sophisticated pathways to satisfy their iron requirements. In the human host, elemental iron can be acquired via siderophores–secreted bacterial high-affinity iron chelators–from host iron-containing proteins such as transferrin, lactoferrin and ferritin. However, heme represents the major source of iron in humans. Five distinct bacterial heme uptake systems have been identified (Table 1), three of which are illustrated in Figure 1. This review highlights recent advances in structural and mechanistic understanding of pathogenic bacterial heme-iron acquisition, including host heme sequestration, bacterial transmembrane heme transport, and bacterial cytosolic heme degradation.

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Heme external sources

Hemoglobin (Hb) accounts for the majority of heme found in humans. Tetrameric Hb dissociates to methemoglobin (MetHb), an α/β heterodimer containing a ferric heme molecule in each subunit. MetHb is further stabilized by irreversibly binding to haptoglobin (Hp), a serum glycoprotein that prevents heme dissociation from MetHb and hence oxidative damage [1]. Both MetHb and the MetHb-Hp complex represent the most predominant host heme reservoirs for bacterial heme acquisition. Other host heme sources include hemopexin, leghemoglobin, and myoglobin [2]. Bacteria can sequester heme from host hemoproteins via secreted heme-scavenging proteins known as hemophores and/or membrane-bound or cell-wall embedded hemoprotein receptors.

Heme acquired via secreted proteins

HasA-type hemophores

HasA, part of the heme acquisition system (Has), Figure 1A, has been identified in several Gram-negative bacteria including *Serratia marcescens* ($HasA_{SM}$), *Pseudomonas aeruginosa* ($HasA_{PA}$), and *Yersinia pestis* ($HasA_{YP}$). Several studies suggest heme transfer from host hemoproteins to HasA is affinity-driven without the formation of a stable protein-protein complex (reviewed in [2]). Heme-bound structures of $HasA_{SM}$ and $HasA_{PA}$ revealed similar overall α/β folds (Figure 2A) with His32 (the proximal heme-iron ligand) located on Loop 1, and Tyr75 (the distal heme-iron ligand) located on Loop 2 (Figure 2A). A comparison of these binary structures with their apo counterparts and complementary biochemical studies suggest that heme binding is dependent on the movement of Loop 1; binding of Tyr75 triggers the closing of Loop 1 facilitating His32 binding (reviewed in [2]). An extensive spectroscopic-based study recently demonstrated that $HasA_{SM}$ retains its ability to bind heme despite the loss of a single axial ligand (His32Ala or Tyr75Ala mutation), as coordination is most likely compensated by water [3]. Interestingly, Loop 1 adopts a closed conformation for both mutants. In agreement with this observation, the structure of His32Gln $HasA_{YP}$ mutant reveals heme is coordinated by Tyr75 and a sixth solvent molecule ligand, while Loop 1 still adopts a closed conformation [4].

HxuA-type hemophores

Haemophilius influenzae HxuA is a large 100 kDa hemophore which, in concert with HxuB and HxuC, is able to scavenge heme from the host hemopexin [5,6]. A recent study demonstrated that HxuA forms a tight complex with hemopexin, resulting in heme release and subsequent heme accessibility for binding to the heme receptor, HxuC. Interestingly, while it appears to be essential for heme uptake/transfer, HxuA is not a conventional hemophore due to its inability to bind heme [7].

NEAT-type hemophores

The Gram-positive iron surface determinant (Isd) heme uptake pathway (Figure 1B) is typified by Isd proteins harboring one or more near-transporter (NEAT) domains. Despite low sequence identity, NEAT domains have similar immunoglobulin-like folds. *Bacillus anthracis* secretes two hemophores, IsdX1 and IsdX2, which acquire heme from MetHb [8].

IsdX1 contains one NEAT domain whereas IsdX2 has five tandem NEAT domains. The IsdX1 NEAT domain (Figure 2A) and the IsdX2 NEAT5 domain ($IsdX2^{N5}$) comprise two anti-parallel β-sheets that form a β-sandwich with a short 3_{10}-helix within the vicinity of the heme-binding site [9,10]. In both holo-complexes, heme is coordinated by a conserved Tyr, which is in turn stabilized by another conserved Tyr (*i.e.*, Tyr136/Tyr140 and Tyr108/Tyr112 in IsdX1 and $IsdX2^{N5}$, respectively) and by the 3_{10}-helix. To investigate the contribution of the 3_{10}-helix, a comprehensive set of IsdX1 single point mutations were shown to affect heme and/or Hb binding, implicating its role in IsdX1 function [9]. Mutants with both heme and Hb binding defects were also unable to rescue a hemophore-deficient *B. anthracis* strain when grown in Hb-supplemented media [9]. Importantly, Gln29 within the 3_{10}-helix from $IsdX2^{N5}$ is essential for heme extraction from MetHb [10]. Accordingly, Gln29 is conserved in IsdX2 NEAT domains capable of scavenging heme from MetHb (*i.e.*, NEAT1 and NEAT5); substitution of Gln29 with Thr to mimic IsdX2 NEAT domains unable to extract heme from MetHb (*i.e.*, NEAT2, NEAT3, and NEAT4) renders $IsdX2^{N5}$ inactive in MetHb heme extraction [10].

Rv0203

The recently discovered *Mycobacterium tuberculosis* (Mtb) heme uptake pathway (Figure 1C) features a secreted mycobacterial-specific heme-binding protein, Rv0203, involved in sequestering heme from Hb [11]. The crystal structure of tetrameric Rv0203 reveals a novel fold (Figure 2A). Furthermore, despite having no sequence or structural similarities, Rv0203 possesses a similar heme-binding motif (Tyr59, His63, and His89) to that of HasA [11]. Further characterization suggests that Rv0203 is unable to scavenge heme from Hb via an affinity-driven mechanism due to its low heme affinity as compared to Hb [12].

Heme acquisition and transport across the membrane

Gram-negative bacterial systems

The energy for heme transport across the outer membrane in Gram-negative bacteria is coupled to the bacterial proton motive force [2], utilizing an inner membrane complex, TonB/ExbB/ExbD, in which TonB's periplasmic domain interacts with a weakly conserved TonB box from a TonB-dependent outer membrane transporter (TBDT), Figure 1A. Heme is eventually shuttled across the inner membrane by an ATP-binding cassette (ABC) transporter [2].

Outer membrane transport

Spanning the outer membrane, TBDTs share structural homology consisting of a 22-stranded anti-parallel β-barrel that is occluded by an N-terminal plug, containing the TonB box. TBDTs are able to acquire heme directly from Hb, as observed in *Haemophilus ducreyi* HgbA. It was previously shown that *H. ducreyi* HgbA extracellular loops 5 and 7 were essential for Hb interaction [13]. Recently, a comprehensive mutational study on two conserved motifs demonstrated that the Loop 5 GYEAYNRQWWA motif is important in binding Hb while only the Phe692 from the Loop 7 FRAP motif is essential for heme uptake [14]. In contrast, the conserved Arg and/or Pro from *Porphyromonas gingivalis* TBDT

(HmuR) FRAP motif were shown to be required for heme acquisition, suggesting that this discrepancy may be attributed to subtle mechanistic differences [15].

P. gingivalis possesses an additional outer membrane lipoprotein, HmuY, able to sequester and deliver heme from host hemoproteins to HmuR. The structure of HmuY harbors a novel β-fold with bis-His coordinated heme [16]. Additionally, *P. gingivalis* has serine proteases (R-gingipain and K-gingipain proteases), which have recently been shown to cooperatively assist HmuY in the acquisition of heme through the degradation of host Hb [17].

TBDTs can also acquire heme via a cognate hemophore, as in *S. marcescens* HasR. *S. marcescens* HasB is a TonB-like protein that interacts with HasR. The recently solved NMR structure of HasB periplasmic domain ($HasB_{CTD}$) revealed three α-helices sitting on top of a three-stranded anti-parallel β-sheet [18]. $HasB_{CTD}$ is structurally related to TolA, a membrane protein involved in colicin (a bacterial toxin) uptake. Additionally, a docked model of HasR's TonB box peptide to $HasB_{CTD}$, using several NMR-derived restraints, shows that the peptide aligns lengthwise, parallel to $HasB_{CTD}$'s β3, to form an intermolecular four-stranded β-sheet [18]. The promise of the plug domain structure of HasR [19] could ultimately provide pertinent molecular details of HasR and HasB interaction interface and residues critical for heme transfer into the periplasm.

Inner membrane transport

In *Y. pestis*, the periplasmic protein, HmuT, delivers heme to HmuUV, which constitutes the ABC transporter where HmuU and HmuV correspond to the transmembrane and nucleotide binding domains, respectively. Woo *et al*. solved the structure of heterodimeric HmuUV, Figure 2B [20], revealing a periplasmic-facing heme translocation funnel located at the two-fold interface of membrane-associated HmuU as well as several residues (i.e., Gly164 and Arg176) conserved among ABC transporters. The authors further developed a novel assay to assess heme transport into proteoliposomes and showed that these residues play important roles in HmuT binding, ATP hydrolysis, and heme transport.

Gram-positive bacterial systems

Gram-positive bacteria have an envelope composed of a thick peptidoglycan layer embedded with proteins, carbohydrates, and teichoic acids. Within some species, there are also capsule polysaccarides and crystalline protein S-layers [2]. The *S. aureus* heme uptake system has been extensively studied, Figure 1B [21]. Four heme-binding proteins (IsdA, IsdB, IsdC and IsdH) are anchored to the cell wall, all of which contain NEAT domains–described in the hemophore section. IsdB (two tandem NEAT domains) and IsdH (three tandem NEAT domains) physically interact and capture heme from host Hb, which is transferred to the cytoplasmic membrane via a heme transfer cascade to IsdC via IsdA. Heme is then transported across the membrane by a membrane lipoprotein (IsdE) and an ABC transporter (IsdDF).

In the last two years, there have been several papers that shed light on the mechanism of *S. aureus* IsdH (and closely related IsdB, Figure 2B) heme acquisition from host Hb. IsdH-NEAT1 ($IsdH^{N1}$) and IsdH-NEAT2 ($IsdH^{N2}$) bind Hb and/or Hp, whereas IsdH-NEAT3

(IsdH^{N3}) binds heme [22,23]; IsdB-NEAT1 (IsdB^{N1}) binds Hb and IsdB-NEAT2 (IsdB^{N2}) binds heme [24,25]. The structure of IsdH^{N1} in complex with MetHb demonstrates that IsdH^{N1} binds to the α-Hb subunit in the vicinity of the NEAT domain heme-binding site, although too far from the α-Hb heme-binding site (<20 Å) to scavenge its heme [26]. IsdH^{N2} also binds to the α-Hb subunit, most likely at the same site as IsdH^{N1}. In contrast, IsdH^{N3} does not bind to the α-Hb subunit and no interaction was also observed for IsdH^{N1}, IsdH^{N2} or IsdH^{N3} to the β-Hb subunit. Furthermore, Hb mutational analysis at the IsdH binding interface to IsdH^{N1} limited the capacity of *S. aureus* to utilize Hb as an iron source. Recently, the NMR structure of the linker region between IsdH^{N2} and IsdH^{N3} was determined to be a 3-helix bundle (Figure 2B) required to position the two NEAT domains (IsdH^{N2,N3}) in a dumbbell-shaped structure [27]. In studies pioneered by Martin Stillman, elucidation of heme transfer pathways was achieved via electrospray ionization mass spectrometry (ESI-MS) [28-30]. Spirig *et al.* elegantly showed by ESI-MS that this linker region was required for the IsdH$^{N2\text{-linker-}N3}$ fragment to acquire heme from Hb but also promotes dissociation of tetrameric Hb into its dimeric form by possibly inducing steric strain upon Hb, thereby promoting heme release, Figure 2B [27].

Interestingly, the *S. aureus* Isd system has no hemophores in contrast with that of *B. anthracis*, which has two hemophores, IsdX1 and IsdX2 [7] (see hemophore section). The *B. anthracis* Isd system also has a membrane-associated heme-acquisition leucine rich repeat protein (Hal) [31] that contains a NEAT domain capable of binding and acquiring heme from Hb. Furthermore, genes contained in the *S. lugunensis* Isd operon suggest a larger repertoire of proteins involved in heme acquisition as compared to both *S. aureus* and *B. anthracis* [32].

The discovery that Mtb can utilize heme as an iron source is recent [11,33], and thus not all protein players involved in heme uptake are known; however, two inner membrane proteins, MmpL3 and MmpL11, have been implicated in heme acquisition [11]. Both proteins have similar predicted secondary structures, comprising eleven transmembrane helices, one intracellular domain, and two extracellular domains. Recently, demonstration that Rv0203 may transfer heme to one of the extracellular domains of both MmpL3 and MmpL11 at a rate faster than passive heme dissociation from Rv0203 suggests a protein-protein interaction-driven mechanism [34].

Mechanisms of heme degradation

Conventional cytosolic heme-degrading proteins, heme oxygenases (HOs), catalyze the oxidation of heme to biliverdin, ferrous iron, and CO, in a seven-electron process requiring three equivalents of molecular oxygen (Figure 3). NADPH supplies electrons necessary for heme degradation via NADPH-cytochrome P450 reductase [35]. Bacterial HOs have been previously reviewed [36], although the recent report of *Corynebacterium diphtheriae* HmuO describes a possible mechanism by which the active site is rearranged upon heme binding, also alluding to a possible iron release mechanism upon cleavage of the tetrapyrrole ring [37]. The active site of *C. diphtheriae* HmuO narrows, facilitated by the proximal helix that is partially unwound in its apo form but forms a continuous helix upon heme binding (Figure 3). This results in His20 acting as the proximal heme-iron ligand and movement of the distal

helix to close the heme-binding pocket. Upon heme binding, a network of water molecules form around the distal side of the pocket positioned for catalytic activity. The structure of biliverdin-HmuO provides insight into iron release (Figure 3); His20 is flipped out of the pocket and no longer coordinates iron, while biliverdin is stabilized by hydrogen bonds to water molecules allowing for iron release [37]. *P. aeruginosa* heme degradation is driven by the interaction of the cytoplasmic heme transport protein PhuS, forming a 1:1 complex with iron-regulated HemO for heme degradation [38,39]. The interesting discovery regarding PhuS-HemO heme-degradation is coupled regulation of heme uptake: when heme levels are low, equilibrium is shifted toward apo-PhuS and heme-bound HemO, whereas when heme levels are high, equilibrium shifts toward holo-PhuS; thus no further transfer of heme to HemO occurs [39].

Another group of bacterial heme-degrading enzymes include *S. aureus* IsdI and IsdG [40] and the homologous Mtb MhuD [41]. *S. aureus* IsdG and IsdI as well as Mtb MhuD demonstrate significant heme distortion (ruffling) proposed to be important for heme degradation, Figure 3. Both proteins produce unique heme degradation products without the production of CO; *S. aureus* produces staphylobilins [42] and formaldehyde [43] while Mtb produces only mycobilins [44]. Interestingly, MhuD can accommodate two heme molecules in its heme pocket (rendering the enzyme inactive) as opposed to only one observed in HOs and IsdG/I enzymes (Figure 3). The importance of MhuD-diheme conformation is currently unknown; however, the discovery that CO (an important Mtb signaling molecule) is not a by-product has implications regarding Mtb pathogenesis. A recent report has identified the likely electron donor important for Isd-mediated heme degradation in *S. aureus*. Iron utilization oxidoreductase, IruO, was identified as necessary for NADPH-dependent heme degradation by IsdG/IsdI [45]. IruO homologs have been found in other Gram-positive bacteria, suggesting that heme degradation using Isd-type proteins could utilize homologous IruO reductases for heme acquisition.

Concluding remarks

Since the discovery of bacterial heme uptake pathways, great progress has been made in identifying key players involved in heme uptake, determining structures of proteins involved in the various pathways, and unraveling the heme transfer mechanism from the extracellular environment to the bacterial cell. In this review, we survey a number of studies that propel our knowledge of bacterial heme uptake and utilization. Of particular relevance, the first structure of the inner membrane ABC transporter for HmuUV, whose report was greatly complemented by the development of a novel assay to investigate heme uptake for membrane proteins [20]. Additional studies on Isd-type proteins, such as IsdH, further explicate the importance of linker regions in the capture of heme from Hb [27]. Once the bacterial cell has internalized heme, heme is degraded to release iron in concert with a newly identified reductase, IruO [45]; however, Isd-type heme degraders do not produce CO like conventional HOs [42, 43, 44].

The battle for iron is rampant between pathogenic bacteria and the human host. Free iron or heme is toxic to the cell [1,4]; therefore both the host and bacteria tightly regulate iron and heme release. The tug-of-war for iron sources is of great research interest as bacterial access

to host iron sources exacerbates infection, creating difficulties for successful clearance. The infected host sequesters iron sources while pathogenic bacteria circumvent sequestration by secreting cytolytic toxins that damage host cells, thereby releasing host heme [40,46]. In an attempt to exploit the bacterial desire for host heme, recent developments in porphyrin-based therapeutics have made important discoveries. The potential of gallium-based anti-infectives has been reviewed, noting that metalloporphyrin-based derivatives can target bacterial heme-uptake pathways [47,48]. For example, these non-iron metalloporphyrin analogs have high affinities to HmuY hemophore preventing heme association [49], accentuating the anti-microbial potential of porphyrin derivatives. As porphyrin-based analogs surface as possible anti-microbials, the potential impact of identification and in-depth characterization of bacterial heme uptake pathways is commanding the spotlight.

Acknowledgments

Authors of this review were supported by the National Institutes of Health, R01-AI081161 (C.W.G) and F31-GM101945 (H.C.). We thank Parker M. Johnson and Alex Chao for critical reading of this article, and Adam P. Sienicki for graphical design assistance.

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Highlights

- HmuUV is the first structure of a heme uptake inner membrane ABC transporter
- A novel assay was developed to assess *in vitro* heme uptake for transmembrane proteins
- IsdH structured linker region between NEAT domains is required to release heme from hemoglobin
- Unlike canonical heme oxygenases, Isd-type heme degraders do not produce carbon monoxide
- IruO represents the first electron donor for Isd-like heme-degraders

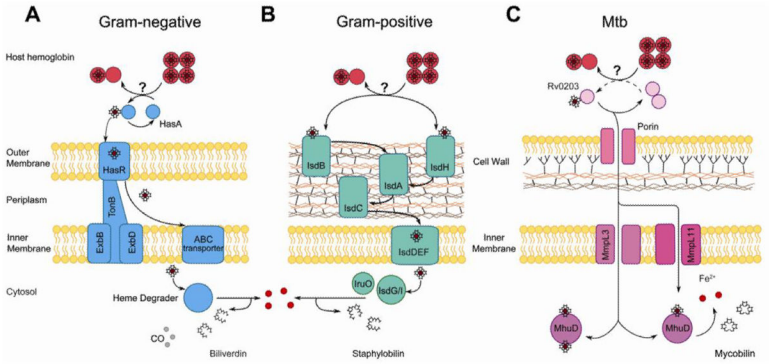

Figure 1.

Schematic overview of heme transport in (**A**) Gram-negative bacteria, (**B**) Gram-positive bacteria, and (**C**) Mtb. Hemoglobin is depicted as a tetramer which can dissociate into dimeric methemoglobin; the precise oligomeric state of hemoglobin from which heme is sequestered *in vivo* is not well established and is represented by a question mark (?). Heme extraction from host hemoglobin in the Mtb is depicted with dashed lines to reflect an unknown mechanism, as there is no evidence to suggest Rv0203 is able to directly scavenge heme from hemoglobin.

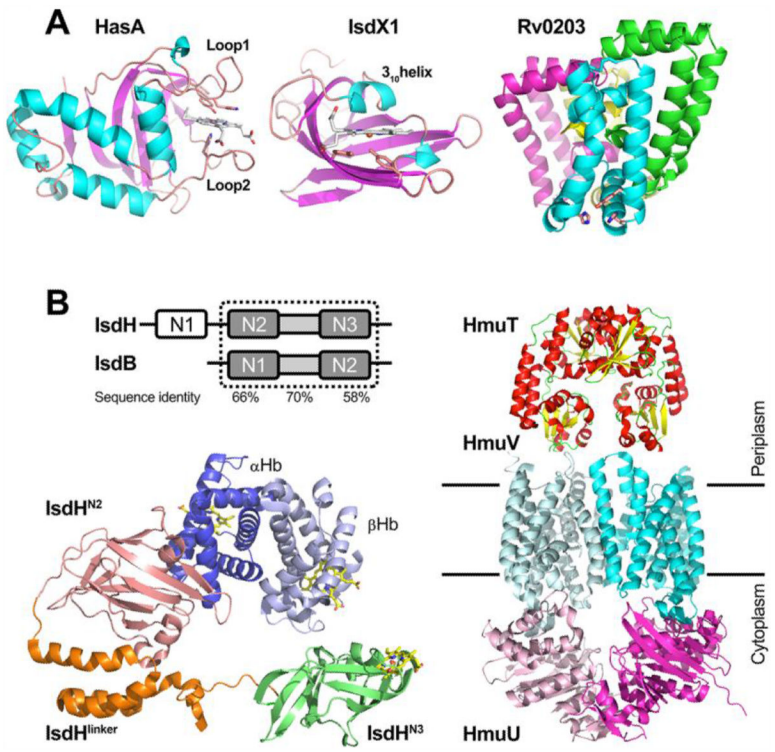

Figure 2.

Structures of proteins involved in heme uptake. (**A**) Hemophores from left to right; *S. marcescens* HasA, *B. anthracis* IsdX1 NEAT domain and Mtb Rv0203. (**B**) Homology between *S. aureus* Hb receptors IsdH and IsdB. A structural model based on the structure of $IsdH^{N1}$ and Hb complex, a ribbon representation of $IsdH^{N2}$ in complex with MetHb with $IsdH^{linker}$ and $IsdH^{N3}$ modeled into the schematic. Heme molecules are shown in yellow stick model (left panel). Ribbon representation of HmuUV, and HmuT (right panel).

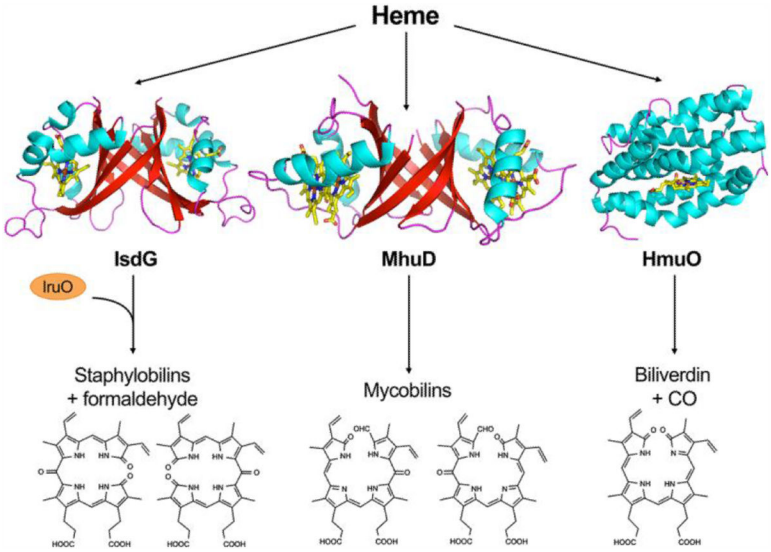

Figure 3.

Ribbon diagrams of bacterial heme degraders (*S. aureus* IsdG, inactive Mtb diheme-MhuD, and *C. diphtheriae* HmuO) with heme in yellow stick model, and chemical structures of their products.

Table 1

Key players involved in various heme uptake pathways, where Mtb is *Mycobacterium tuberculosis*.

	Heme source	Hemophore	Receptors	Membrane proteins	Degraders
Gram-positive	Hb, Hp/Hp	IsdX1, IsdX2	IsdB, IsdH	IsdA, IsdC, IsdDEF	IsdI, IsdG
	Hb	HasA	HasR	HasB, TonB	BphO, HemO
Gram-negative	Hb		HmuY, HmuR	HmuTUV	HmuS
	Hp	HxuA		HxuC, HxuB (secretes HxuA)	-
Mtb	Hb	Rv0203		MmpL3, MmpL11	MhuD