



Published in final edited form as:

Arch Biochem Biophys. 2009 January 1; 481(1): 1–15. doi:10.1016/j.abb.2008.10.013.

Bacterial heme-transport proteins and their heme-coordination modes

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Abstract

Efficient iron acquisition is critical for an invading microbe's survival and virulence. Most of the iron in mammals is incorporated into heme, which can be plundered by certain bacterial pathogens as a nutritional iron source. Utilization of exogenous heme by bacteria involves the binding of heme or hemoproteins to the cell surface receptors, followed by the transport of heme into cells. Once taken into the cytosol, heme is presented to heme oxygenases where the tetrapyrrole ring is cleaved in order to release the iron. Some Gram-negative bacteria also secrete extracellular heme-binding proteins called hemophores, which function to sequester heme from the environment. The heme transport genes are often genetically linked as gene clusters under *Fur* (*Ferric uptake repressor*) regulation. This review discusses the gene clusters and proteins involved in bacterial heme acquisition, transport and processing processes, with special focus on the heme coordination, protein structures and mechanisms underlying heme transport.

Keywords

Heme; transport; structure; bacteria; iron; pathogen

Heme, iron and bacteria

Iron is an essential element for almost all organisms, including bacteria. As a catalytic center for redox reactions in many enzymes, iron facilitates numerous cellular processes such as electron transport, peroxide reduction and nucleotide biosynthesis [1–4]. Upon entry into the mammalian host, bacterial pathogens have to acquire iron from tissues to survive [5]. Almost all bacterial pathogens require iron to be infectious [6]. However, free iron is scarcely available inside animal hosts (free iron concentration at $\sim 10^{-18}$ M within the human host) [7,8], albeit it is the most abundant metal on the Earth [9]. Most of the iron in the human body is sequestered by high affinity iron-binding proteins, such as transferrin (Tf), lactoferrin (Lf) and the iron storage protein ferritin, or by incorporation into a protoporphyrin ring as heme in hemoproteins [5,10,11].

Bacteria have developed sophisticated mechanisms for the uptake of iron from the environment. A common mechanism, seen in many bacteria, is to secrete siderophores to chelate iron from their surroundings [3]. A second mechanism, found only in pathogenic

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bacteria, is to steal iron from the host by capturing iron from Tf, Lf or ferritin via specific outer membrane receptors [3,4,10,12,13]. A third mechanism, also identified mostly in bacterial pathogens [14–16] is to take up iron either from free heme or from heme-containing proteins, such as hemoglobin or hemopexin.

Heme refers to the ferrous iron (Fe^{2+}) bound to tetrapyrrole, a macrocyclic porphyrin; whereas the oxidized form (Fe^{3+}) is known as hemin. However, both are commonly called “heme”, so we will apply this convention throughout this paper. As a prosthetic group in many proteins and enzymes, heme plays significant roles in many fundamental biological processes [17], including O_2 storage and transport (e.g. myoglobin and hemoglobin), respiration and electron transfer (e.g. cytochromes), activation of O-O bond (e.g. P450 enzymes and peroxidase) [18], signal transduction and gas sensing (e.g. CooA, nitric oxide synthase (NOS) and soluble guanylate cyclase (sGC)) [19], and control of gene expression and microRNA processing [20]. Heme can also serve as a source of both iron and porphyrin for the microbes. Recently, it has been discovered that pathogenic bacteria *Staphylococcus aureus* significantly prefers heme iron over transferrin iron during the early stage of infection [21].

Heme is a lipophilic molecule with low molecular weight (616.48 Da), so it can easily intercalate into membranes and thus impair lipid bilayers and organelles, such as mitochondria and nuclei, and destabilize the cytoskeleton [22]. Furthermore, because heme catalyzes the formation of reactive oxygen species, accumulation of heme in cells will result in cellular damage, oxidative stress and tissue injury [23,24]. Therefore, free heme is also poorly tolerated by cells. When heme-containing molecules are degraded, released heme will be rapidly bound by molecules such as hemopexin ($K_d \sim 10^{-12}$ M) and serum albumin ($K_d \sim 10^{-8}$ M) [25]. In the presence of the scavenging proteins, the concentration of free heme within the animal host is maintained at very low levels [26].

Heme sources

The total body intracellular iron pool (~ 4 g) consists of heme which bound to hemoglobin (2.67 g), myoglobin (0.2 g), some heme enzymes (0.08 g), insoluble ferric irons that were stored in ferritin (1 g), and a poorly defined labile iron compartment (0.07 g) [27]. Hemoglobin and myoglobin are the most abundant heme sources in the body. Free hemoglobin, like free heme, is not tolerated by the body [28], and therefore upon being liberated by intravascular hemolysis from erythrocytes, hemoglobin dimers are bound with high affinity ($K_d \sim 10^{-12}$ M) by haptoglobin, a serum heterotetrameric glycoprotein [29,30]. Then the haptoglobin-hemoglobin complexes are subsequently delivered to the reticulo-endothelial system by CD163 receptor-mediated endocytosis [31]. Although not all bacterial pathogens are able to use haptoglobin-bound hemoglobin as an iron source, it has been reported that *Neisseria* spp., *Hemophilus influenzae* and *Porphyromonas gingivalis* are capable of utilizing iron through specific haptoglobin-hemoglobin receptors [32–35].

Upon heme is released from hemoproteins such as hemoglobin, as occurs in the erythrocyte lysis, the free heme will be sequestered immediately by hemopexin, which is a 60-kDa plasma glycoprotein with a high heme affinity ($K_d \sim 10^{-13}$ M), in an equimolar complex [30,36,37]. Hemopexin is a major heme transportation vehicle in the plasma, functioning to prevent heme-mediated oxidative stress and heme-bound iron loss. The heme-hemopexin complex is routed to the liver, where apo-hemopexin is recycled after heme is discharged intracellularly.

Human serum albumin (HSA), the most abundant plasma protein (~ 640 μM), which has a lower affinity ($K_d \sim 10^{-8}$ M) for heme compared to hemopexin [38], may also contribute to heme transport [39,40]. HSA consists of three homologous domains (I, II and III). Each of which is made up of two separate subdomains (designated A and B) that are connected by a random coil. The crystal structure shows that heme binds to a hydrophobic, narrow D-shaped

cavity in subdomain IB and the propionate side chains of heme are coordinated with a triad of basic residues at the entrance of the pocket. The central iron atom of heme is coordinated by Tyr161 [25,41].

It is interesting to note that the high and low density lipoproteins (HDL and LDL, respectively), which are the most oxidatively intolerant components in plasma, also bind to heme with high affinity ($K_d \sim 10^{-11}$ M to 10^{-10} M), and the binding is kinetically faster than serum albumin and hemopexin [42], although the role of the transient heme binding to HDL and LDL is not clear yet.

The heme-containing proteins, such as hemoglobin, myoglobin, haptoglobin–hemoglobin complex, HSA, HDL, LDL, or peroxidases, catalases, cytochrome P450s and cytochromes, provide multiple potential iron sources to the infectious bacteria seeking this essential nutrient.

An overview of heme uptake systems in Gram-negative bacteria

Three major heme acquisition systems have been identified in Gram-negative bacteria. The well-characterized system involves direct binding of heme or heme proteins (e.g. hemopexin, hemoglobin, hemoglobin-haptoglobin) to specific outer membrane receptors on the bacterial surface (named “direct heme uptake system” here). The heme, is then transported into the cell via ATP-binding cassette (ABC) transporters. The best studied systems in this category consist of the *hemR-hemSTUV* system in *Yersinia enterocolitica* [43], the *hmuRSTUV* system in *Yersinia pestis* [44], *shuASTUV* system of *Shigella dysenteriae* [45,46], and the *phuRSTUVW* system in *Pseudomonas aeruginosa* [47–49]. The second heme uptake system is comprised of an outer membrane receptor, an ABC transporter and extracellular hemophores. Best known in the *hasADEB* system in *Serratia marcescens* [16], hemophores function to take up free heme or extract heme from hemoproteins in the external medium and shuttle it to the cell surface, where it interacts with a TonB-dependent hemophore specific outer membrane receptor [50,51]. A few researchers classified the bipartite hemoglobin receptor HpuAB in *Neisseria meningitidis* as a third heme acquisition system [52]. HpuAB is composed of a TonB-dependent outer membrane receptor HpuB and an accessory outer membrane lipoprotein HpuA [34,53], which is analogous to the bipartite transferrin receptor Tbp1 and Tbp2 [54]. It has been demonstrated that the *N. meningitidis* HpuAB bipartite receptor is capable of transporting intact porphyrin from hemoglobin and haptoglobin-hemoglobin complexes [55]. It is of interest to note that heme can passively diffuse across model lipid bilayers, although the outer membrane permeability in Gram-negative bacteria is less than 600 Da [56].

Hemophore-mediated heme uptake systems

HasA hemophore

Two types of hemophore systems have been reported so far. One is HasA (heme acquisition system), which has been identified in *Serratia marcescens* (Figure 1) [57–59], *Pseudomonas aeruginosa* [60,61], *Pseudomonas fluorescens* [60,62], *Yersinia pestis* [63], and *Yersinia enterocolitica* [43,64]. The other type of hemophore is HxuA (heme/hemopexin utilization) which has been described only in *Hemophilus influenzae* [65,66], a species that lacks the heme biosynthetic pathway and requires exogenous heme for aerobic growth.

The HasA from *S. marcescens* (HasA_{SM}) was the first identified hemophore. It is a 19 kDa monomer that binds one *b*-type heme per molecule with high affinity ($K_d \sim 10^{-11}$ M) [67]. The crystal structure of HasA_{SM} has been determined at 1.9 Å resolution [58] (Figure 2a). The HasA_{SM} shows an original α/β fold in which seven β -strands form an antiparallel β -sheet on one face of the protein, and the four α -helices are positioned on the other face. The heme is held between two loops and is located at the interface between the α - and β -fold of the molecule

(Figure 2b). In contrast to the bis-histidinyl ligation based on EPR studies [68], heme iron is ligated by His32 and Tyr75 [58]. The possible heme binding and release mechanism has been proposed to be involved in a tight hydrogen bond between the N δ of His 83 and the phenolate group of Tyr75. If Tyr75 is deprotonated, heme ligation is favored. In contrast, heme can be released by weakening the Fe-Tyr75 bond through H-bonding of the Tyr75 phenolate group with His83 N δ [58]. His83, which has been confirmed by mutagenesis study to play a critical role in the stability of axial coordination, was suggested to be an alternative iron ligand in the absence of Tyr75 [67,69]. Recently, it has also been proposed to be involved in heme delivery to the receptor [70].

Sequence alignment (Figure 3) reveals that HasAs share about 30–50% identity. The high variable regions are the two heme binding loops H1 (ruler: 30–44) and H2 (ruler: 85–97), and the C-terminal stretch. The relative high flexibility of the heme binding pocket formed by H1 and H2 is suggested to be important for heme recognition and/or for the recognition by the receptor HasR [59]. The last four residues in the C-terminus containing the secretion information is very conserved. However, one of the heme ligation residues, His32, is not conserved and is replaced by either Gln or Asp. His83 and Tyr75 are conserved among all the species. Taken together, the heme ligation environment among HasAs is similar, but may have some slight differences.

The receptors of HasA hemophore

Like other outer membrane receptors, the hemophore receptor, HasR, was proposed to be dependent on HasB (a TonB-like protein) for energy [71–73]. Based on sequence and functional similarities with other known TonB-dependent receptors, a HasR structure was predicted to be composed of a 99-residue amino-terminal extension, followed by a plug domain and a β -barrel domain with lumen closed by the plug [74]. HasR is highly homologous to other heme and hemoprotein receptors, in particular the conserved FRAP/NPL sequences in the β -barrel and two conserved histidines (His189 in the plug and His603 in the barrel) [75]. HasR also functions as a heme receptor, taking up free heme ($K_d \sim 10^{-6}$ M) and hemoglobin-bound heme, albeit less efficiently than taking up hemophore-bound heme ($K_d \sim 10^{-9}$ M) [26,69].

HasA has a markedly higher affinity ($K_d \sim 10^{-11}$ M) for heme than its receptor HasR ($K_d \sim 10^{-6}$ M) [76], which raises the question of how heme could transfer from a high-affinity protein to a lower-affinity protein. The interactions between HasA, hemoglobin and HasR have been characterized [68,77]. It has been biochemically demonstrated that heme transfer *in vitro* from HasA to HasR is energy- and TonB-independent and is only driven by protein-protein interactions [78]. The binding of HasA and HasR is mediated by two independent hemophore-binding regions, β strands 51–60 and 95–105 [79]. Based on the three-dimensional HasR model and mutagenesis studies, three loops L6, L8, and L9 of HasR are proposed to contribute either directly or indirectly to one or several HasA binding sites. By investigating the HasA with direct-detected ^{13}C NMR, Caillet-Saguy proposed that upon binding to HasR, the high-spin/low-spin equilibrium shifts toward the high-spin species. The affinity of HasA for heme in the HasA-HasR complex would become lower than that of HasR, thus allowing heme transfer to HasR [70].

HxuA hemophore

The gene cluster *hxuCBA* in *Hemophilus influenzae* type b (Hib), a species that lacks the heme biosynthetic pathway and requires exogenous heme for aerobic growth, has been shown to be necessary for utilization of hemopexin-bound heme [65,80]. Sequence analysis of the *hxuA* gene and N-terminal amino acid analysis of the HxuA protein revealed that the mature protein was 99 kDa with a leader peptide removed from a 101 kDa precursor [81]. HxuA was first identified to bind heme-hemopexin [65]. The HxuA-heme-hemopexin complex is presumably

bound by the bacterial cell and the heme is internalized; the outer-membrane HxuA receptor has not been identified albeit it may be HxuC [66,82]. The exact mechanism by which Hib acquires heme from the heme-hemopexin complex is yet unknown.

The 60-kDa HxuB protein was proposed to be involved in the release of the soluble HxuA molecule from the Hib cell. HxuA and HxuB were believed to be involved only in the heme-hemopexin utilization process [82]. While the 78-kDa outer membrane protein HxuC is required for Hib to acquire the very low concentrations of free heme [80], heme-albumin complex [82], or hemoglobin in the absence of the Hgps [83]. All the three genes in *hxuABC* are essential for Hib to grow on free heme or heme-hemopexin medium. The Hxu system of Hib differs from the Has system of *S. marcescens* in that HxuA actually extends the range of the substrates rather than simply increasing its efficiency [66].

Direct heme uptake systems in Gram-negative bacteria

The direct heme uptake systems in Gram-negative bacteria are similar and are encoded by a Fur regulated gene cluster (Figure 4 shows the gene cluster from *P. aeruginosa* [84]). This system involves a single outer membrane heme receptor (e.g., PhuR) (Figure 5A), a periplasmic heme transport protein (e.g., PhuT) (Figure 5D), inner membrane proteins (e.g., PhuUVW) (see Figure 5C, D and E) typical for an ABC transport system, and a cytoplasmic protein (PhuS, possible for heme storage/chaperone) (See Figure 11, the crystal structure of a PhuS analogue, HemS). Based on the current understanding, a possible heme acquisition and transport pathway is illustrated in Figure 6. Bacterial pathogens secrete hemolysin to lyse red blood cells and release hemoglobin (80–800 nM) into the serum. Hemoglobin is rapidly complexed by the excess quantities of serum haptoglobin (5–20 μM) and delivered to the liver for removal. Heme, after being released from the hemoglobin (mechanism is unclear) is rapidly sequestered by hemopexin (12 μM , with a high affinity to heme, $K_d \sim 10^{-13}$ M), or by excess quantities of serum albumin (640 μM , $K_d \sim 10^{-8}$ M). The bacteria heme receptor (PhuR) can recognize heme, hemoglobin, haptoglobin-hemoglobin, hemopexin, and myoglobin, assimilating the heme and passing it *via* a TonB dependent process to the periplasmic heme transporter (PhuT), which is proposed to shuttle the heme between the outer and inner membrane [49]. Heme is then passed to inner membrane ATP-dependent permeases (PhuUVW) which transfer the heme to a cytoplasmic heme-binding protein (PhuS) [47,85]. Once internalized into the cytosol, heme is rapidly transferred to heme oxygenase (HO) which opens the porphyrin ring producing biliverdin, carbon monoxide and iron [85,86].

Heme transport across the outer membrane

In Gram-negative bacteria, specific outer membrane receptors sequester heme from free heme or host hemoproteins and bring it into the periplasmic space via a TonB-dependent process. The receptors identified so far include heme receptors, hemopexin receptors, hemoglobin (Hb) and hemoglobin-haptoglobin (Hb-Hp) receptors. Over thirty outer membrane heme receptors have been reported from a wide variety of Gram-negative bacteria [87]. The receptors range from 70 kDa to 120 kDa in size, and vary in their binding diversity, from interacting with a large number of ligands to only one or a few. Heme receptors have been subdivided into three categories: the first group recognizes heme; the second group binds host hemoproteins; and the third group interacts with hemophores [87].

The heme receptor HemR from *Y. enterocolitica* has been most investigated [75,87]. Mutational analysis has postulated that heme-binding involves two conserved histidine residues, His128 and His461 which are located between two conserved motifs, the FRAP and NPNL boxes, identified by comparison of the HemR sequence with other heme receptors [75]. HemR also recognizes other hemoproteins such as, myoglobin, hemoglobin, hemopexin, haptoglobin-hemoglobin and heme-albumin complexes. Similar to HemR, outer membrane

heme receptor BhuR in *Bordetella pertussis* also recognizes heme, hemoglobin, haptoglobin-hemoglobin and the heme-albumin complex. Mutation of the *bhuR* gene makes *B. pertussis* incapable of utilizing any of these compounds, suggesting that BhuR is essential for the heme uptake system [52]. However, in contrast to HemR, the BhuR receptor from *B. pertussis* and *B. bronchiseptica* as well as other heme receptors PfhR (*P. fluorescens*) and PhuR (*P. aeruginosa*) are predicted to lack the conserved histidine residue in the FRAP and NPNL motifs [52,88], suggesting that the heme binding mechanism between different heme receptors may be somewhat different.

Some hemophore receptors may also function as heme receptors, such as HasR in *S. marcescens*, not only binding hemophore-bound heme, but also taking up free and hemoglobin-bound heme [26].

Hemopexin receptors are not ubiquitously expressed [89]. It has been reported that hemophore HxuA in Hib, is not only released into the culture supernatant, but also presented on the bacterial cell surface as an outer membrane hemopexin receptor, which is regulated by the availability of iron in the growth medium [66,80,90]. In response to iron depleted conditions, *H. influenzae* 706705 and DL42 do not express the 99-kDa hemopexin receptor [65]. Wong et al. also suggested that some *H. influenzae* strains could possess at least two hemopexin receptors and the expression of which is determined by the prevailing growth medium [90].

Two Hb receptors have been described in *Neisseria meningitidis*, HmbR and HpuB. HmbR is an 89.5 kDa single-component TonB-dependent outer membrane receptor. It has been identified that the *N. meningitidis* *hmbR* mutant was unable to use Hb but could still use heme as the sole iron source [91]. Further evidence demonstrated that HmbR is not involved in the acquisition of iron from Hb-Hp or heme [55]. Studies also suggested that the two-component receptor HpuAB in *N. meningitidis*, composed of HpuB (a TonB-dependent transport protein) and the lipoprotein HpuA, was required for the utilization of either Hb or Hb-Hp complex [34,53,55].

Three Hb/Hb-Hp receptors, HgpA, HgpB and HgpC, have been identified from *H. influenzae* HI689. Expression of any one of these three proteins is adequate to support growth with Hb-Hp as the heme source [32,92,93]. Simpson and co-workers characterized a TonB-dependent outer membrane receptor HmuR (Heme utilization receptor) in *Porphyromonas gingivalis* [88]. HmuR, in concert with another outer membrane putative heme-binding lipoprotein HmuY [94], is involved in the utilization of both heme and Hb in *P. gingivalis*. HmuR shares extensive sequence homology with TonB-dependent outer membrane heme/Hb receptors of other Gram-negative bacteria, such as HemR of *Y. enterocolitica* and ShuA of *S. dysenteriae* [88]. The N-terminal regions of the *hmuR* gene are 100% identical with the previously reported *hemR* gene, but their C-terminal regions are strikingly different [95]. The role of the *hmuR* gene in heme uptake has been well studied [88,96,97]. It has been proposed that HmuR may bind heme through histidine residues [56]. A three-dimensional homology model of HmuR suggested a β -barrel structure with two distinct domains. The first domain is a β -barrel with long loops on the extracellular side and short turns on the periplasmic side. The second domain is a globular amino-terminal domain that folds into the barrel pore [98]. This barrel-like structure has been suggested to be necessary for the heme transport.

Recently, a 73 kDa outer membrane receptor ShuA of *S. dysenteriae* has been characterized [46]. It has been proposed that oxidized hemoglobin is the most likely physiological substrate for ShuA. Mutagenesis studies demonstrate that His-86 and His420 are essential for substrate recognition, heme coordination and transfer.

The TonB-ExbB-ExbD complex

Heme transport across the outer membrane is an energy-requiring process. The energy for transport of heme into periplasmic space in most Gram-negative organisms is provided by cytoplasmic membrane (CM) protein TonB in association with two helper proteins, ExbB and ExbD (Figure 7), which form a complex in a molar ratio of 1:7:2, respectively [99–102]. This is an active process driven by the proton motive force generated from the CM. TonB-homologues are widespread in bacteria where they may have similar functions in heme uptake systems [103]. TonB is composed of three domains [104]: the N-terminal domain which is anchored to the CM, interacts with ExbB and ExbD to form an energy transducing complex; the C-terminal domain directly contacts receptors in the outer membrane; and the intermediate domain which is a proline-rich region, consists of alternating Pro-Glu and Pro-Lys repeats. ExbB possesses three transmembrane segments with the bulk of the protein in the cytoplasm, while ExbD has only one transmembrane segment and most of the protein is located in the periplasm [105].

Figure 7 schematically illustrates the protein complex involved in energy transduction in Gram-negative bacteria. Since no energy source is available in the outer membrane, TonB-dependent outer membrane receptors must utilize the energy provided by the proton motive force through the TonB-ExbB-ExbD complex. The TonB N-terminal domain contains a histidine residue, which probably binds proton together with ExbB and ExbD, which induces the TonB structural change into an energized form. Then the energized TonB will interact with the outer membrane receptor via TonB box, which is in the N-terminal domain of the receptor and shares amino acid homology in several regions [106–109]. After energy transduction, ExbB and ExbD will retrieve TonB from the outer membrane.

A *tonB* mutation prevented *S. marcescens* growth in iron-restricted medium, but not free heme or heme-hemoglobin, indicating a TonB-like protein may be involved in the hemophore-dependent heme acquisition system. Paquelin and co-workers [103] characterized this TonB-like protein (named HasB) and found that those two proteins have partially redundant function with respect to heme uptake. In addition, a second *tonB* gene (*tonB2*) in *P. aeruginosa* was proposed to be linked to the *exbB* and *exbD* genes, but *tonB2exbBD* is not required for heme acquisition [110]. Two sets of *tonB*, *exbB*, *exbD* genes have been reported in *V. cholerae* [111–113]. They have at least partially overlapped functions in iron or heme utilization. The mechanism of redundant TonB proteins in the bacteria is not yet clear.

Heme transport in the periplasmic space

Once across the outer membrane, heme is internalized into periplasm and is then bound by a heme-specific periplasmic transport protein (HTP), which mediates heme transfer to the cytoplasm through ATP-binding cassette (ABC) transporter.

A few periplasmic HTPs have been identified at genetic level, such as HemT (*Y. enterocolitica*), HmuT (*C. diphtheriae*), ShuT (*Shigella dysenteriae*), PhuT (*P. aeruginosa*), HutB (*Vibrio cholerae*), and ChuT (*E. Coli*), which share 30 to 90% identical amino acid residues [85]. HTPs have been proposed to bind heme in the periplasm and deliver it to the membrane-bound ABC transporter [49,114]. It has been considered that heme is bound in a cleft between the two subdomains of HTP, which will induce a conformational change in HTP and this allows the holo-HTP to interact with the transmembrane domain of ABC transporter. As a result, heme will face to the channel of ABC transporter and be delivered across the inner membrane.

HbpA, a 51-kDa lipoprotein from Hib purified in a heme-agarose affinity purification protocol, was initially proposed to possess heme transport or heme binding functions when expressed

in *E. coli* [115,116], but the role of HbpA in heme utilization was not identified until recently by an insertional mutation of hbpA [117]. However, the protein sequence of HbpA is not homologous to any known HTPs, but instead shares 53% sequence identity with the *E. coli* periplasmic dipeptide transport protein DppA [116]. Interestingly, two well known periplasmic dipeptide-binding protein DppA and MppA, the L-alanyl-gamma-D-glutamyl-meso-diaminopimelate binding protein, with a low heme-binding affinity (K_d : $\sim 10^{-5}$ - 5×10^{-5} M) have been recently demonstrated to function in heme transport in the periplasmic space of *E. coli* under the condition of the absence of a heme-specific HTP [118].

Two of the better characterized periplasmic heme transport proteins are ShuT from *Shigella dysenteriae* [45] and PhuT from *Pseudomonas aeruginosa* [49]. ShuT was expressed as a monomeric recombinant protein of 28.5 kDa, binding one *b*-type heme per monomer and bearing no significant homology with other known heme proteins [45]. Resonance Raman, MCD, and UV-visible spectra of the hemin-loaded ShuT indicate a five-coordinate high spin heme with a Tyr proximal ligand, which is supported by site-directed mutagenesis studies [45]. Recombinant PhuT from *P. aeruginosa* was purified as a 33 kDa His-tagged protein with $\sim 50\%$ heme-bound in the native form [49]. Similar to that in ShuT, the heme is noncovalently attached as a five-coordinate, high-spin, ferric heme and can be transferred to apo-myoglobin in vitro. The heme in PhuT can be reduced by dithionite but not by either DTT or ascorbate. A Tyr-to-heme charge transfer band was identified in the oxidized form of holo-PhuT, suggesting a Tyr-heme coordination. This Tyr was identified to be Tyr71 in PhuT by site-directed mutagenesis [49]. Apo-PhuT binds heme or protoporphyrin IX at 1:1 ratio with high affinity ($K_d \sim 1.2$ nM and 14 nM, respectively).

A BLASTP search revealed ca. 52 putative bacterial heme-specific HTPs, which can be grouped into 6 classes (Figure 8), most of which are associated with pathogenic bacteria. Multiple sequence alignment analysis (Supporting info.) reveals that these putative periplasmic heme transporters share low sequence homology and have no conserved common binding motif for heme-ligation. However, a tyrosine residue (Tyr71 in PhuT) is highly conserved in all but one of the 52 HTPs, implying that Tyr is a possible axial heme ligand in these HTPs.

Recently, the crystal structures of PhuT and ShuT have been solved in apo- and heme-bound forms (Figure 9) [48]. They share common structural features with 2 topologically similar globular domains and a long, rigid α -helix as an interdomain linker, typical of a Class III periplasmic binding protein, such as BtuF, a vitamin B12 transporting protein [119,120] and PhuD, an *E. coli* periplasmic protein that binds hydroxamate-type siderophores [121]. In terms of ligands, heme, B12 and peptide siderophores, PhuT/ShuT are more closely resemble BtuF, which shows the modest 1Å shrinking of the B12 binding pocket upon ligand binding. It has been proposed that heme binding to PhuT/ShuT may only induce a minor conformational change of the heme binding pocket, including a reorientation of the Tyr heme ligand, which may play a role in the heme binding/release process.

In PhuT, the heme prosthetic group is embedded in a narrow cleft between the 2 globular domains. As we expected, the heme is pentacoordinate with Tyr71 from the C terminal end of the β -3 strand in the N terminal domain serving as the proximal heme ligand [49]. The heme propionate side chains are pointing outward to the surface of the protein, and each is bent to the opposite side of the heme plane, respectively (Figure 10). One propionate side chain is directly hydrogen bonded to Arg228 which is stacked against the distal surface of heme. In the close vicinity of the other propionate side chain, Arg73, on the proximal side of heme, is hydrogen bonded to the ligand Tyr71. However, in ShuT, Lys69, which is the homolog to the PhuT Arg73, is far from the heme ligation residue Tyr67. In order to form a H-bond between Lys69 and Tyr67 to mimic the Tyr71-Arg73 interaction in PhuT, there must be a reorientation

of the Lys69 side chain. Therefore, PhuT and ShuT may have similar conformational changes upon binding/releasing heme, but they may have slightly different mechanism.

Heme transport across the cytoplasmic membrane

In Gram-negative bacteria, once heme is transported into the periplasmic space, it will be translocated across the cytoplasmic membrane by specific ABC transporters. The ABC transporters mediate active translocation of a variety of molecules across cell membranes in both Gram-positive and Gram-negative pathogens [114,122]. When associated with heme-specific HTP, it forms a complete heme-uptake permease. The first heme permease was identified from *Y. enterocolitica*, composed of one periplasmic heme-binding protein (HemT), one transmembrane protein (HemU), and one ATPase protein (HemV) [85].

The inner membrane proteins in *P. aeruginosa* which are proposed to be involved in heme transport include PhuU, PhuV and PhuW. No biochemical studies on these proteins have been reported yet, but the homology models of PhuU, PhuV have been shown in Figure 5. Function of PhuW is not known yet, but recently, an analogue protein ChaN, which is an iron-regulated lipoprotein from *Campylobacter jejuni*, has been characterized [123]. The crystal structure of ChaN (Figure 5) reveals that it consists of a large parallel β -sheet with flanking α -helices and a smaller domain with α -helices. Interestingly, two cofacial heme groups (~ 3.5 Å apart with an inter-iron distance of 4.4 Å) bind in a pocket formed by a dimer of ChaN monomers. Tyr148 residue was found to be involved in coordination with heme iron (2.47 Å) [123]. The EPR spectrum at acidic pH is consistent with a five-coordinate ferriheme center with a proximal Tyr ligand, while the spectrum at alkaline pH is more complex.

Interestingly, the PhuUV analogues in *S. dysenteriae*, ShuUV, have recently been cloned and incorporated into artificial liposomes [122]. By encapsulation of ShuT into the lumen of the liposomes, the transport of heme from heme-bound ShuT to apo-ShuS (the cytoplasmic heme-binding protein) was observed. The heme translocation process is found to be coupled with ATP hydrolysis and probably via direct complex formation between ShuT and ShuU [122]. Site-directed mutagenesis studies revealed that 2 histidine residues on ShuU are critical in the translocation of heme across the membrane [122].

Heme transport, degradation and iron releasing in cytosol

Once internalized into the cytoplasm, heme is rapidly catabolized by heme oxygenase (HOs), which opens the heme porphyrin ring. HOs, first identified in mammals, are primarily involved in protecting cells against the toxic effect of heme, whereas bacteria utilize HOs to access heme as a nutrient iron source [124]. HOs are ubiquitous in nature, and function to catalyze the oxidation of heme into biliverdin, carbon monoxide, and free iron [125,126]. However, before heme is delivered to a HO, it may be sequestered by a PhuS analogue, because the free heme is toxic to cells.

HemS (Figure 11), a 40 kDa protein from *Y. enterocolitica*, is a PhuS analogue. It is thought to be responsible for oxidative cleavage of the heme, protecting the cellular environment from the heme toxicity effects [64]. However, no biochemistry study on the function of this protein has been reported yet. More recently, Schneider et al. reported the crystal structures of apo- and heme-bound HemS, thus providing penetrating evidence into its mechanism of heme binding and release [127]. HemS associates with heme through a pocket, which is formed between a large β -sheet dome and a three-helix subdomain. The His196 coordinates the iron in the heme-HemS complex, which is also stabilized by a distal Arg102 residue. A “heme-induced fit” binding mechanism based on the striking conformational change was revealed by comparison of apo- and holo-formed HemS crystal structures, indicating that HemS is an intracellular heme transporter rather than a heme oxygenase.

In *Shigella dysenteriae*, the cytoplasmic protein, ShuS, which shares 64% sequence identity with HemS and binds one heme per monomer ($K_d \sim 13 \mu\text{M}$), has been shown to function as both a heme storage protein and a DNA binding protein for protection against heme-induced oxidative damage, but does not exhibit any ability to break down the heme [128]. A *shuS* mutant study identified that ShuS improves heme utilization efficiency at low heme concentration and protects cells from growth inhibition by heme at high heme concentration [129]. However, carbon monoxide detection confirmed that the cytoplasmic analogue ChuS from *E. coli* O157:H7 is a HO, which shares 98% identity with the ShuS sequence [126]. DLS (Dynamic Light Scattering) analysis demonstrated that ChuS and ChuS-heme both form high molecular weight aggregates after incubation for 4 days in Tris buffer at 4°C. Suits et al. suggested that this aggregation could be the reason why ShuS is absent of HO activity [126], while it is also possible that the different phenotype is due to the different genetic backgrounds. The crystal structure of ChuS displays a previously uncharacterized fold and is unique when compared with known mammalian and bacterial HOs. In addition, *E. coli* ChuS is capable of using ascorbic acid or cytochrome P450 reductase-NADPH as an electron donor for heme degradation.

HmuS in *Y. pestis*, was originally proposed to be a heme oxygenase [64]. However, inactivation of *Y. pestis hmuS* showed that these mutants were still able to use hemin and all hemoproteins as an iron source [130,131]. Therefore, another hypothesis is that HmuS is probably involved in heme storage and/or chaperoning. Since then, heme oxygenases have been found in several pathogenic bacteria including *N. meningitides*, *C. diphtheriae* and *P. aeruginosa*.

The cytoplasmic heme-binding protein PhuS from *P. aeruginosa* has been characterized recently [47,132]. PhuS binds one heme per monomer with alternate His ligands at positions 209 and 212. A second distinct six-coordinated low-spin heme site is present in dimeric PhuS, but the coordination residues have not been identified [47,132]. In contrast to the previous reports, Block and co-workers [132] provided compelling evidence that PhuS is not a HO but a heme chaperone responsible for passing heme to a *pa*-HO, which is driven by a specific protein-protein interaction. It has been proposed that the specific and unidirectional heme transfer from PhuS to *pa*-HO was triggered by a spin-state crossover, according to the experiments conducted in the presence and absence of cyanide [133]. Due to the fact that the transfer rate from heme-loaded PhuS ($K_d \sim 0.2 \mu\text{M}$) to *pa*-HO is 30-fold faster than that to apomyoglobin ($k_H = 10^{-8} \mu\text{M}$), Bhakta et al. also suggested that heme transfer is independent of heme binding affinity.

Most heme proteins participating in O₂ activation have imidazolate of histidine or thiolate of cysteine as proximal ligands and a few have a neutral imidazole as the proximal ligand [134]. In contrast to periplasmic heme transport protein PhuT and ShuT, HO binds substrate heme at the specific position of its pocket to form a heme-HO complex with histidine as a heme-iron ligand, as proposed in *pa*-HO [135], *nm*-HO from *N. meningitides* [136], and *cd*-HO from *C. diphtheriae* [137].

The mechanism of heme oxidation by the HO has been proposed as follows [14,134]: the reaction starts with the formation of the ferric heme-HO complex with histidine as an iron ligand. Ferric iron is then reduced to a ferrous iron by the electron donated from NADPH-cytochrome P450 reductase. Next, oxygen binds to the complex forming a metastable oxy-form. The iron-bound oxygen is converted to a hydroperoxide intermediate ($\text{Fe}^{3+}\text{-OOH}$), by receiving another electron from the reductase and a proton from the distal pocket water. Lastly, the terminal oxygen of $\text{Fe}^{3+}\text{-OOH}$ attacks the α -*meso*-carbon of the porphyrin ring to form ferric α -*meso*-hydroxyheme, which is converted to the ferric verdoheme in the presence of oxygen. The mechanism of the verdoheme degradation to ferric biliverdin is not well understood.

Heme uptake in Gram-positive bacteria

Unlike that of the Gram-negative bacteria, the envelope of Gram-positive bacteria have a thick cell wall composed of the murein sacculus and the attached polysaccharides, teichoic acids, and cell wall proteins [138]. Studies of bacterial heme uptake systems have been mainly focusing on Gram-negative microbes, thus relatively less is known about the Gram-positive bacteria heme uptake pathway. Progress has recently been made in exploring the heme acquisition in certain Gram-positive bacteria. In *Corynebacterium diphtheriae*, the outer membrane receptors as well as the Ton system are missing; a HemT-like lipoprotein, HmuT, serves to function as a heme receptor [122,139]. However, the ABC transporters are required for heme uptake not only in *C. diphtheriae* [122], but also in *Streptococcus pyogenes*, or group A streptococcus (GAS) [140–142], and *S. aureus* [21]. Cell surface-binding proteins (named Shr and Shp) and the ABC transporter lipoprotein (named HtsA) in human pathogen GAS have been identified recently [143,144]. The Shr of *S. pyogenes* can bind heme and transfer it to the streptococcal heme-binding protein Shp [144]. Shp may then relay its heme to apo-HtsA to across the bacterial envelope. The HtsA (SiaA) from *S. pyogenes* has been characterized recently [145]. Resonance Raman (rR), magnetic circular dichroism (MCD), and nuclear magnetic resonance (NMR) spectroscopic studies suggest that the heme in SiaA is six-coordinate and low-spin, with methionine and histidine as axial ligands.

The Gram-positive bacterium *S. aureus* has developed a heme-acquisition system encoded by an *isd* (iron-regulated surface determinants) gene cluster (Figure 12) to acquire heme-bound iron during infection, via the secretion of hemolysins (which rupture the red blood cells to release hemoglobin) [146]. The process begins with binding of a hemoglobin or a haptoglobin-hemoglobin complex onto the bacterial surface receptors IsdB and IsdH (Figure 13). Heme is then removed from hemoglobin by IsdA and IsdB, transferred to the cell wall protein IsdC, and then passed through the membrane with the aid from translocation factors (IsdD, IsdE, and IsdF). A recent *in vitro* study [147] demonstrated that heme can be directly transferred from IsdA to IsdC via protein-protein interaction and that the process is rapid and affinity-driven.

The crystal structure of the heme-IsdC complex was solved (Figure 14) [148]. IsdC has an architecture with a distinct binding pocket with the ligand located next to the hydrophobic core of the β -sandwich. The heme-iron is coordinated with a tyrosine surrounded by several non-polar side chains which cluster into a tightly packed proximal side. Meanwhile, the distal side is relatively exposed to a short helical peptide segment which acts as a lip and clasps onto almost half of the porphyrin plane. This structural feature was proposed to play a role in the mechanism of heme binding and release through conformational changes.

The crystal structure of the soluble portion of the IsdE lipoprotein in complex with heme (Figure 14) was reported recently [149]. The structure reveals a bi-lobed topology formed by an N- and C-terminal domain bridged by a single α -helix. The heme is bound in the groove of the domain interface, which is six-coordinated in a novel fashion for heme transporters by Met78 and His229. Both heme propionate side chains are secured by H-bonds to the IsdE main chain and side chain.

Once inside the cytoplasm, IsdG and IsdI, the cytoplasmic heme-iron binding proteins, may be involved in removing iron from heme. Heme may also be complexed with some unknown membrane-associated proteins as an enzyme cofactor. Another possibility is that excess heme or heme metabolites are pumped by HrtAB out of the cytoplasm to protect the bacteria from its toxicity [146].

IsdA and IsdB as well as IsdC are covalently anchored by sortases to the bacterial peptidoglycan. The LPXTG anchor structure is proposed to allow *staphylococci* to use IsdA and IsdB as receptors, whereas the NPQTN structure in IsdC may function in the transportation

of heme across the cell wall. Magnetic circular dichroism (MCD) analysis of the recombinant IsdA from *S. aureus* suggests that it binds to a five-coordinate, high-spin ferric heme molecule, proximally coordinated by a tyrosyl residue [150]. Reduction of the IsdA heme yields a five-coordinate, high-spin ferrous heme with a neutral axial ligand, most likely the histidine [150]. The *isd* locus is also present in several other Gram-positive bacteria, such as *Listeria monocytogenes*, *Bacillus halodurans*, and *Bacillus anthracis*, suggesting that similar mechanisms of iron acquisition may exist [151].

Regulations on heme uptake

Iron is an essential nutrient for bacteria; however, overloaded iron could be harmful due to its toxic properties. Therefore, expression of iron/heme uptake proteins during different periods of time of infection is regulated in response to iron level in the environment. In many bacterial pathogens, such as *V. cholerae* [111], *Y. pestis* [130], *Y. enterocolitica* [64], and *P. aeruginosa* [84], iron/heme uptake regulation is negatively controlled by the global determinant ferric-uptake regulator protein (Fur) [152]. Fur, a homodimer consisting of 17-kDa subunits [153], are divided into two domains: N-terminal domain, which functions in DNA binding and the C-terminal domain, which is rich in His residues and is responsible for divalent iron binding and dimerization [153,154]. As shown in Figure 15, under iron-rich conditions Fur binds to the divalent iron, which is capable of binding target DNA sequences designated as Fur box, a 19-bp consensus sequence, GATAATGATAATCATTATC. Two Fur dimers are suggested to bind at each Fur box on opposite faces of the DNA helix [155]. Hence, transcription of iron-dependent outer membrane receptor genes will be inhibited. On the other hand, when iron is scarcely available, divalent iron will be released from Fur and the RNA polymerase will gain access to the promoter region, and the genes will be transcribed.

Another regulatory mechanism involves specific extracytoplasmic function (ECF) sigma factors. Figure 1 shows the *has* operon (*hasRADEB*) in *S. marcescens*. It codes for the receptor (HasR), the hemophore (HasA), two inner membrane hemophore secretion proteins (HasD and HasE) [26], and a TonB homolog (HasB), which is involved in heme uptake [103]. Two genes immediately upstream of the *has* operon encode HasI, an ECF sigma factor, and HasS, an anti-sigma factor. When enough heme is available in the media, the anti-sigma activity of HasS is turned off, which is positively regulated by HasI through a signaling cascade triggered by the heme-loaded HasA binding to HasR. When heme is in shortage, apo-HasA is displaced from HasR. As a result, HasS will be re-activated and the transcription of the *has* operon will be turned off.

To date, most of the heme transport systems are negatively regulated by the Fur repressor. Recently, positive regulation of *bhu* gene expression in the Gram-negative respiratory pathogen *Bordetella pertussis* and *Bordetella bronchiseptica* has been reported [156,157]. The heme uptake regulator gene *hurIR* is immediately upstream of the heme uptake *bhuRSTUV* gene cluster and is predicted to encode ECF sigma factor, HurI, and cytoplasmic membrane regulator, HurR. In response to iron starvation conditions, Fur represses the *hurI* promoter activity. When enough iron is available in the media, Fur will derepress *hurI* allowing transcription of HurI and HurR and low levels of the *bhu* gene. However, HurI will remain inactive through its association with HurR until the outer membrane receptor, BhuR, binds heme. A receptor occupancy signal will be transduced through HurR, and HurI will be released and then it will associate with RNA polymerase to direct high levels of transcription of heme uptake genes. Fur repression will resume when intracellular iron stores are replenished.

Concluding remarks

Over the past few decades, it has been well established that heme is a major source of iron for bacteria. Many bacterial pathogens, including both Gram-negative and Gram-positive ones are armed with sophisticated mechanisms to steal heme from the host. Understanding their heme transport pathways is not only of scientific interest, but may also provide a basis for potential novel inhibitors/antibiotics design by targeting the heme transport processes. Structural and mechanistic studies of the proteins involved in the heme trafficking processes have been providing important insight to our understanding of this process at the molecular level. However, it is still challenging to understand the structures of the membrane proteins involved. Comparing with the heme-binding process, the mechanism of heme-releasing process is less understood. Although thermodynamics may play a significant role in certain cases [144,147], it appears that heme transport processes are not simply driven by a sequential increase in heme-affinity from one protein to another in the heme-transport cascade. Specific protein-protein interactions with or without the coupled ATP hydrolysis, changes in heme-coordination and spin states may be critical to certain steps in the heme translocation processes. There is no doubt that further structural and mechanistic studies will be necessary to unravel the details of the heme acquisition processes in bacteria. This information will be important to guide the design of potential antimicrobial agents that target the heme-transport pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was partially supported by the National Institutes of Health and University of Massachusetts Dartmouth. This publication/project was made possible by grant 1 R21 AT002743-02 from the National Center for Complementary and Alternative Medicine (NCCAM). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NCCAM, or the National Institutes of Health. We thank Drs. T. Su, W. Dills, J. D. Smith and Ms Erin Sullivan (UMass Dartmouth, MA, USA) for helpful discussions.

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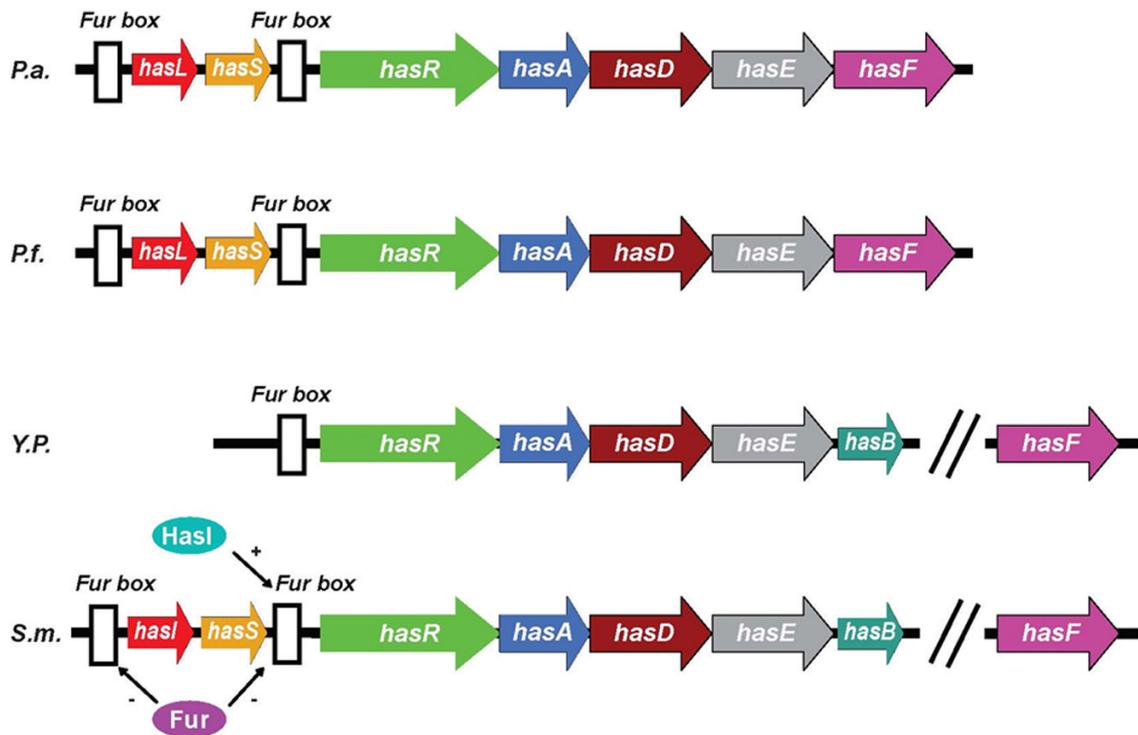


Figure 1.

Genetic organization of the *has* systems in different bacteria. *P.a.*, *Pseudomonas aeruginosa*; *P.f.*, *Pseudomonas fluorescens*; *Y.p.*, *Yersinia pestis*; *S.m.*, *Serratia marcescens*. *HasI* and *hasS* encode sigma and antisigma factors, respectively. The white boxes indicate consensus Fur boxes. The *has* operon is directly controlled by Fur (Ferric uptake regulator) [26] and positively regulated by sigma and antisigma factors [158]. *HasA* is secreted by an ABC transporter consisting of three envelope proteins: *HasD*, *HasE* and *HasF*. *HasD*, an inner membrane ATPase (the ABC protein) provides the energy for the substrate export; *HasE* is another inner membrane component and also a membrane fusion protein; *HasF*, an outer membrane component, is homologous to TolC, which creates a channel in ~ 30Å diameter through the periplasmic space and the outer membrane through which the secreted protein is most likely translocated [159]. Like most proteins secreted by an ABC exporter, hemophores have an α -helical C-terminal secretion signal which can be accessed by SecB, a cytoplasmic chaperone required for hemophore secretion [160]. The secretion signal is recognized by the ABC protein *HasD*, through modulating its ATPase activity, a *HasA-HasDEF* multi-protein complex will be formed [161]. Once translocated into the extracellular medium, *HasA* will bind heme and return it to the *HasR* receptor. Apo-*HasA* will be released into the extracellular medium through interaction with TonB-dependent outer membrane receptor.

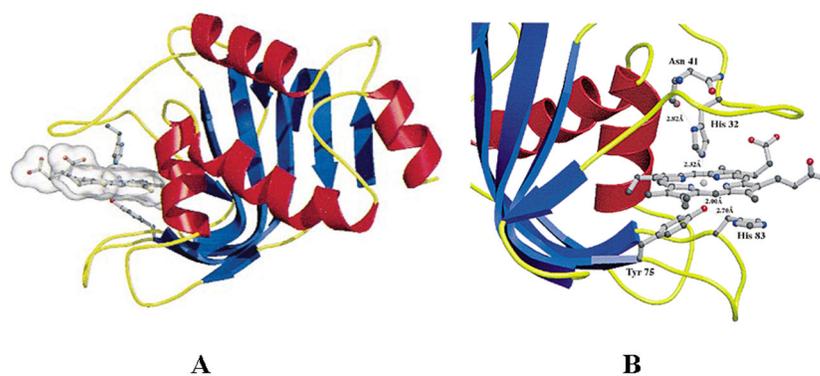


Figure 2. Crystal structures of holo-Has_{ASM} (PDB ID: 1b2v). A: Ribbon diagram with helices colored in red and strands in blue. Heme and the ligands of heme are shown in ball-and-stick representation. B: View of the residues in the heme binding site. Adapted from [58].

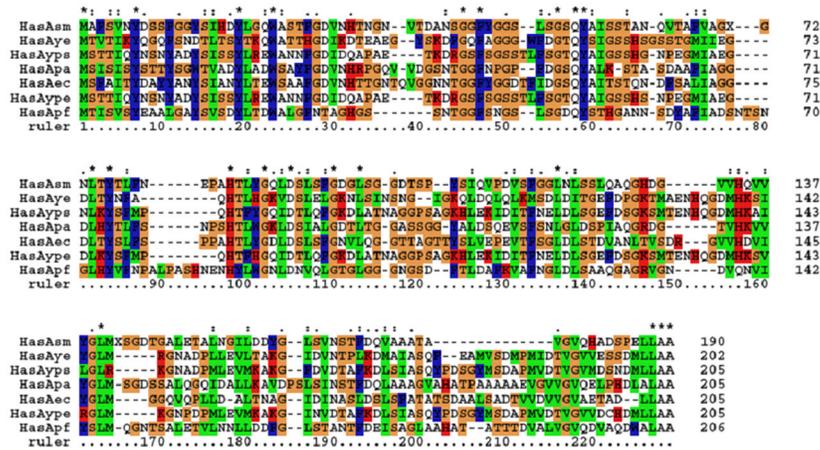


Figure 3. Multiple sequence alignment of HasAs from *Serratia marcescens*, *Yersinia enterocolitica* strain 8081, *Yersinia pseudotuberculosis*, *Pseudomonas aeruginosa* PAO1, *Erwinia carotovora* subsp. *Atroseptica*, *Yersinia pestis* and *Pseudomonas fluorescens*.

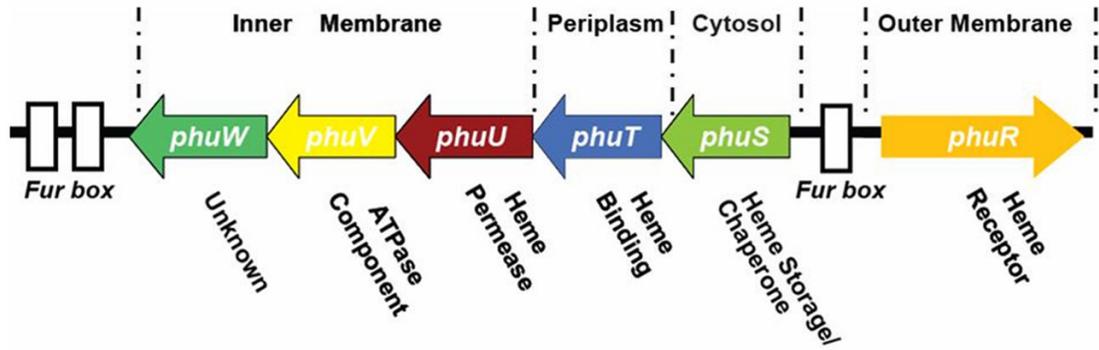


Figure 4. Map of the *P. aeruginosa* heme uptake locus containing the *phuR* gene and the *phuSTUVW* operon. Three *Fur* binding elements are shown as white boxes (adapted from [49]).

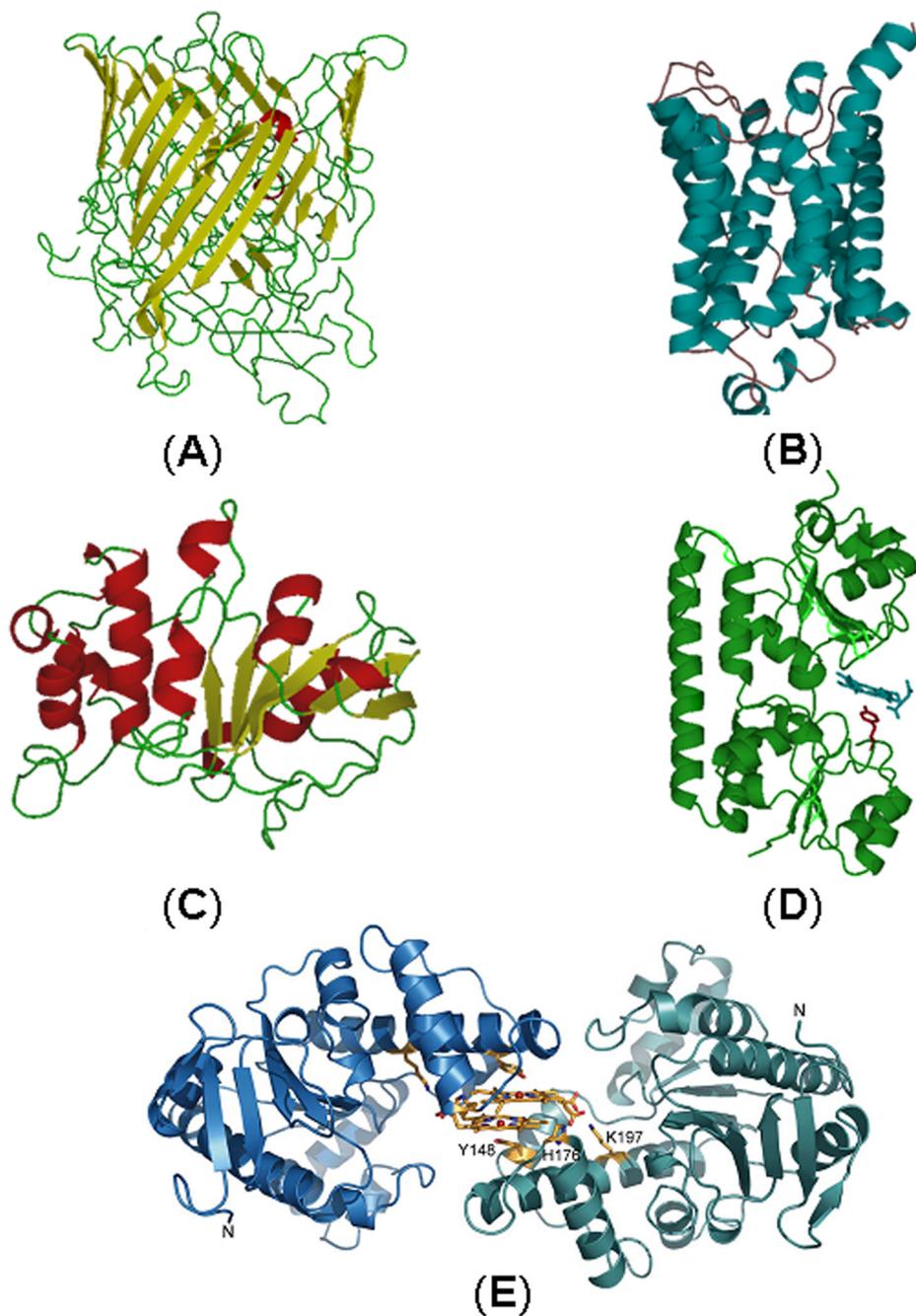


Figure 5. Homology models and structures of heme transport proteins of *P. aeruginosa* in the *phu* locus: PhuR (A); PhuU (B); PhuV (C); Crystal structure of PhuT (D) [48] and Crystal structure of a PhuW analogue, ChaN (adapted from [123]) (E).

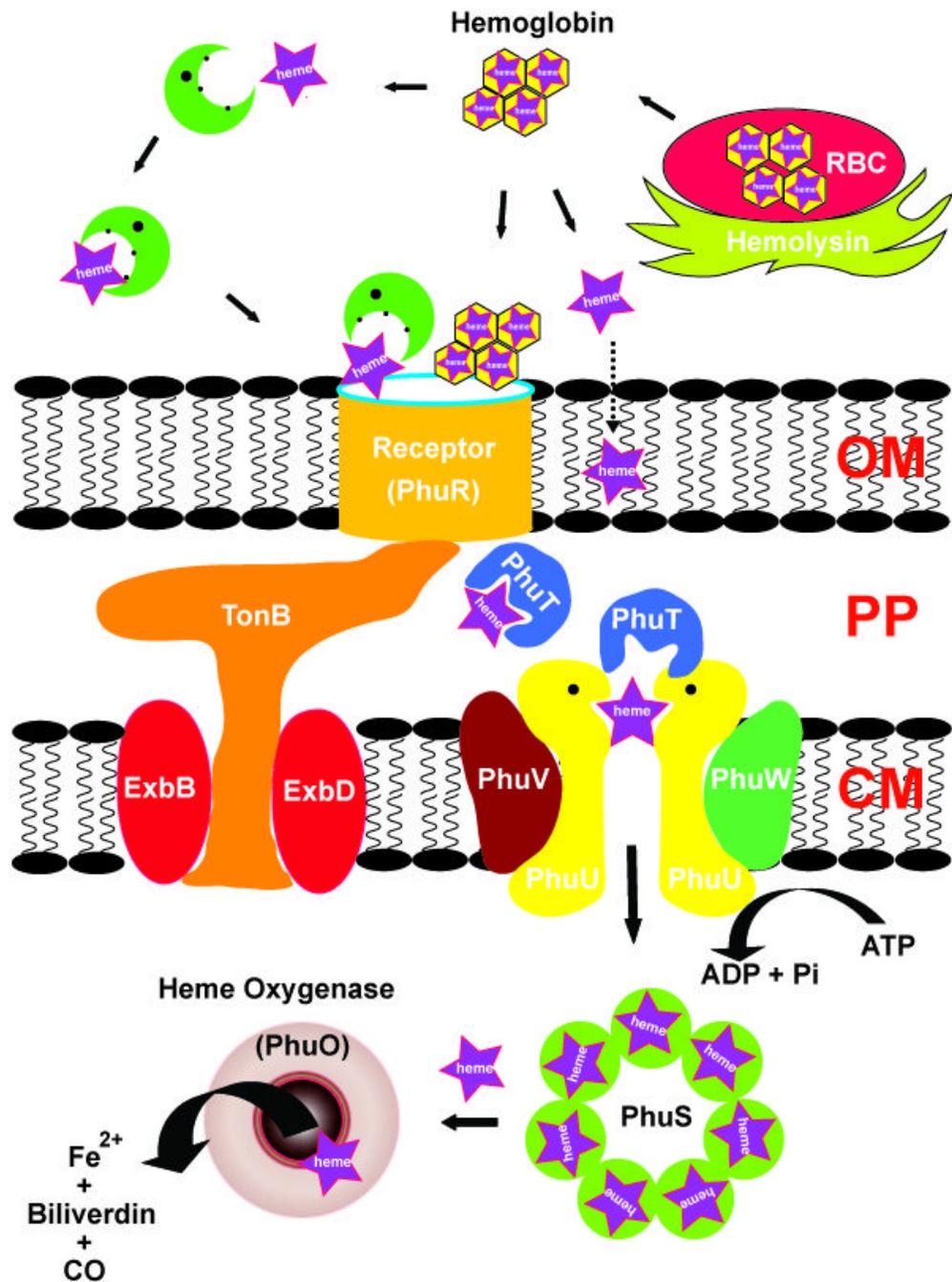


Figure 6. Cartoon showing the organization and the key elements of heme (purple star) acquisition systems in Gram negative bacteria *P. aeruginosa* (adpted from [49]).

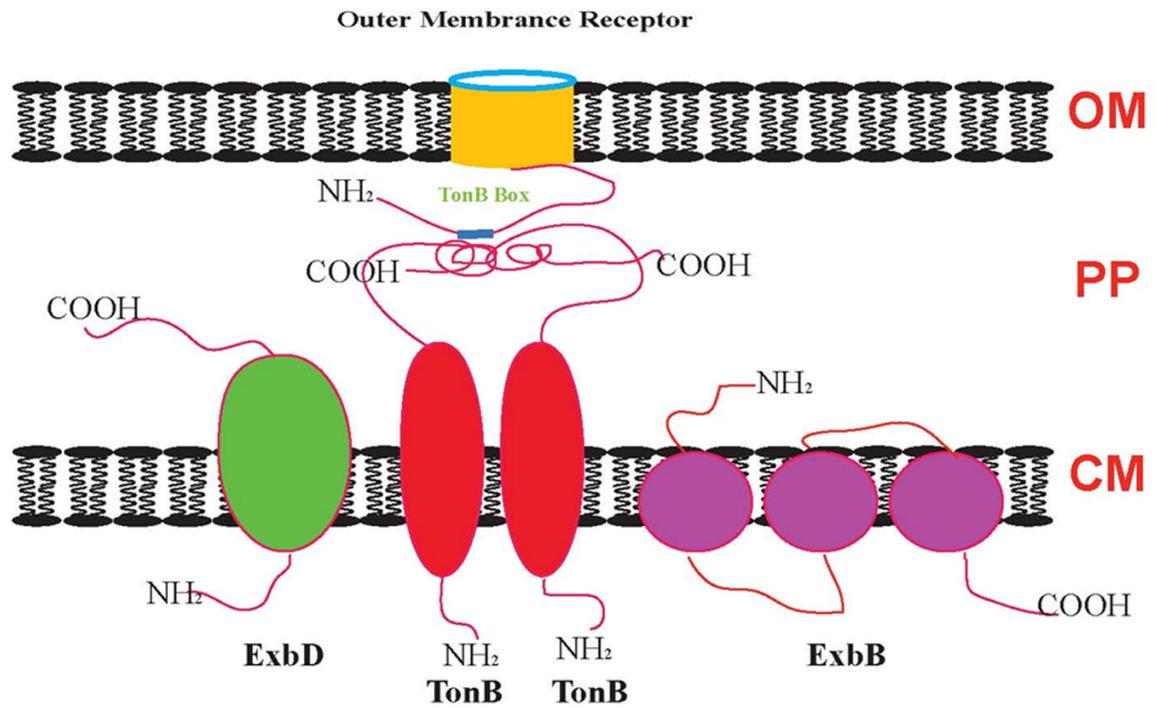


Figure 7. Schematic illustration of the protein complex involved in energy transduction from the cytoplasmic membrane to the outer membrane in Gram-negative bacteria. OM: outer membrane; PP: periplasmic space; CM: cytoplasmic membrane (modified from [97]).

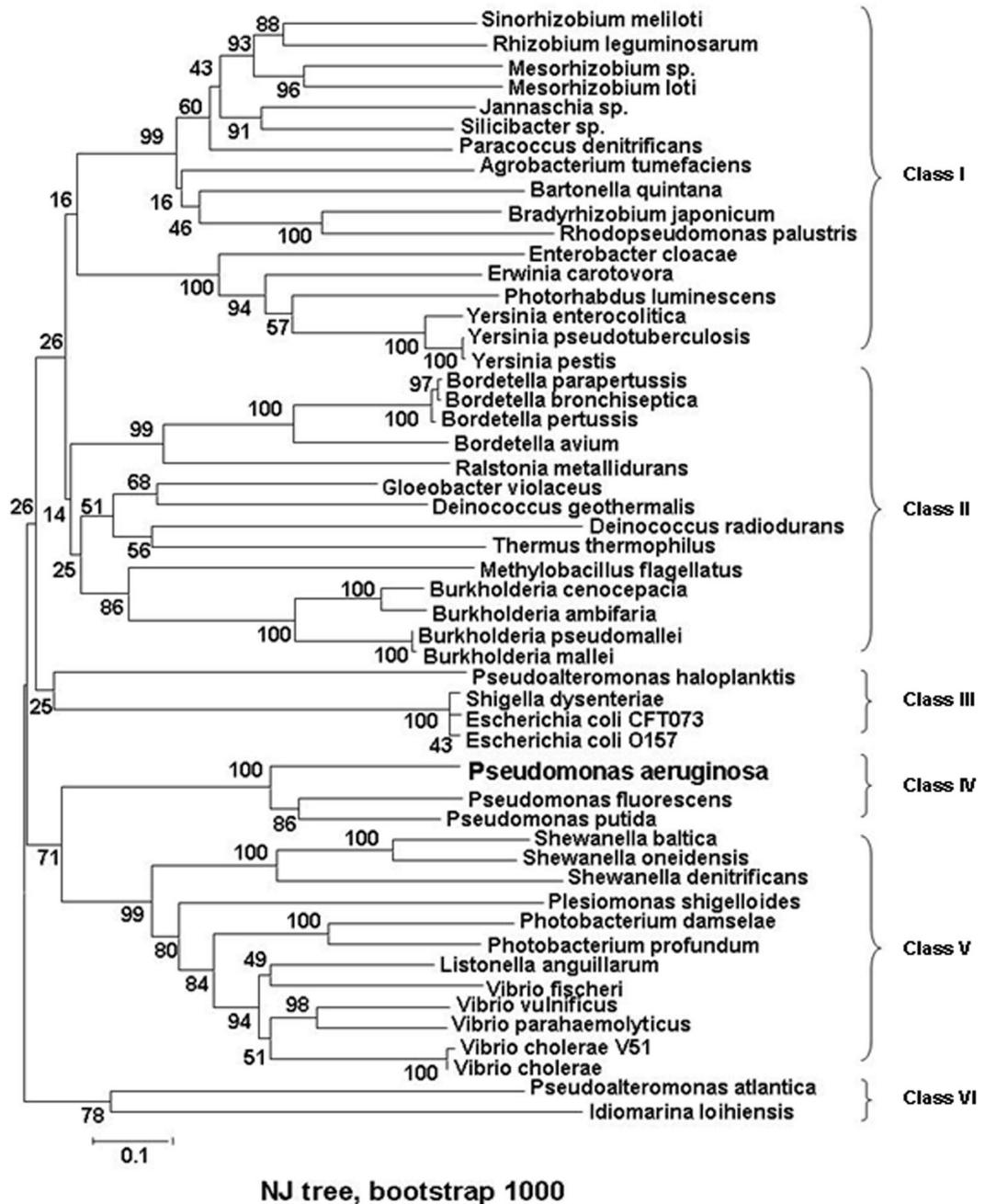


Figure 8.

Unrooted NJ tree of HTP sequences of 52 taxa was conducted using *MEGA* version 3.1. Eight clusters are indicated and are grouped into 6 classes. *Pseudomonas aeruginosa* (*P.ae*), AAC13287; *Pseudomonas fluorescens* Pf-5, YP_262338; *Pseudomonas putida* KT2440, NP_746798; *Bordetella avium*, AAM28270; *Burkholderia cenocepacia* AU 1054, ZP_00457560; *Burkholderia pseudomallei* 668 (*B.ps*), ZP_00488203; *Burkholderia ambifaria* AMMD, ZP_00689266; *Escherichia coli* CFT073, NP_756175; *Burkholderia mallei* SAVP1, ZP_00449888; *Shigella dysenteriae* (*S.dy*), AAC27815; *Escherichia coli* O157:H7 EDL933, NP_290082; *Bordetella pertussis* Tohama I, NP_879218; *Vibrio vulnificus* CMCP6, NP_763480; *Bordetella parapertussis* 12822, NP_886318; *Bordetella*

bronchiseptica RB50, NP_891189; *Ralstonia metallidurans* CH34 (*R.me*), ZP_00596240; *Gloeobacter violaceus* PCC 7421, BAC88521; *Vibrio parahaemolyticus* RIMD 2210633, NP_799933; *Shewanella baltica* OS155, ZP_00581516; *Vibrio cholerae* V51, ZP_00751095; *Shewanella oneidensis* MR-1, NP_719214; *Paracoccus denitrificans* PD1222 (*P.de*), ZP_00628787; *Photobacterium damsela* subsp. *Piscicida*, CAE46553; *Listonella anguillarum* serovar O1, CAF25487; *Photobacterium profundum* SS9, YP_130306; *Erwinia carotovora* subsp. *Atroseptica* SCRI1043, CAG74748; *Pseudoalteromonas haloplanktis* TAC125, YP_341564; *Chloroflexus aurantiacus* J-10-fl, ZP_00768862; *Sinorhizobium meliloti*, CAC47008; *Vibrio fischeri* ES114 (*V. fi*), YP_204605; *Shewanella denitrificans* OS217, EAN70978; *Shewanella putrefaciens* CN-32, EAO95810; *Vibrio cholerae*, AAB94547; *Jannaschia* sp. CCS1, ZP_00557202; *Deinococcus geothermalis* DSM 11300, ZP_00395505; *Rhizobium leguminosarum*, CAC34393; *Plesiomonas shigelloides*, AAK38770; *Methylobacillus flagellatus* KT, ZP_00566306; *Deinococcus radiodurans* R1, NP_051557; *Yersinia enterocolitica*, CAA54866; *Yersinia pseudotuberculosis* IP 32953, YP_068884; *Bradyrhizobium japonicum*, CAC38745; *Enterobacter cloacae* (*E. cl*), CAD61864; *Mesorhizobium* sp. BNC1, EAN05942; *Silicibacter* sp. TM1040, ZP_00621030; *Yersinia pestis* biovar *Medievalis* str. 91001, AAS60707; *Agrobacterium tumefaciens* str. C58, NP_533132; *Pseudoalteromonas atlantica* T6c, ZP_00775180; *Thermus thermophilus* HB8, YP_145459; *Photorhabdus luminescens* subsp. *Laumondii* TTO1, NP_929869; *Rhodopseudomonas palustris* CGA009, NP_947465; *Mesorhizobium loti* MAFF303099, NP_102804; *Idiomarina loihiensis* L2TR (*I.lo*), YP_154505; *Bartonella quintana* str. Toulouse, CAF25913. The values at each node are bootstrap value on 1000 replications.

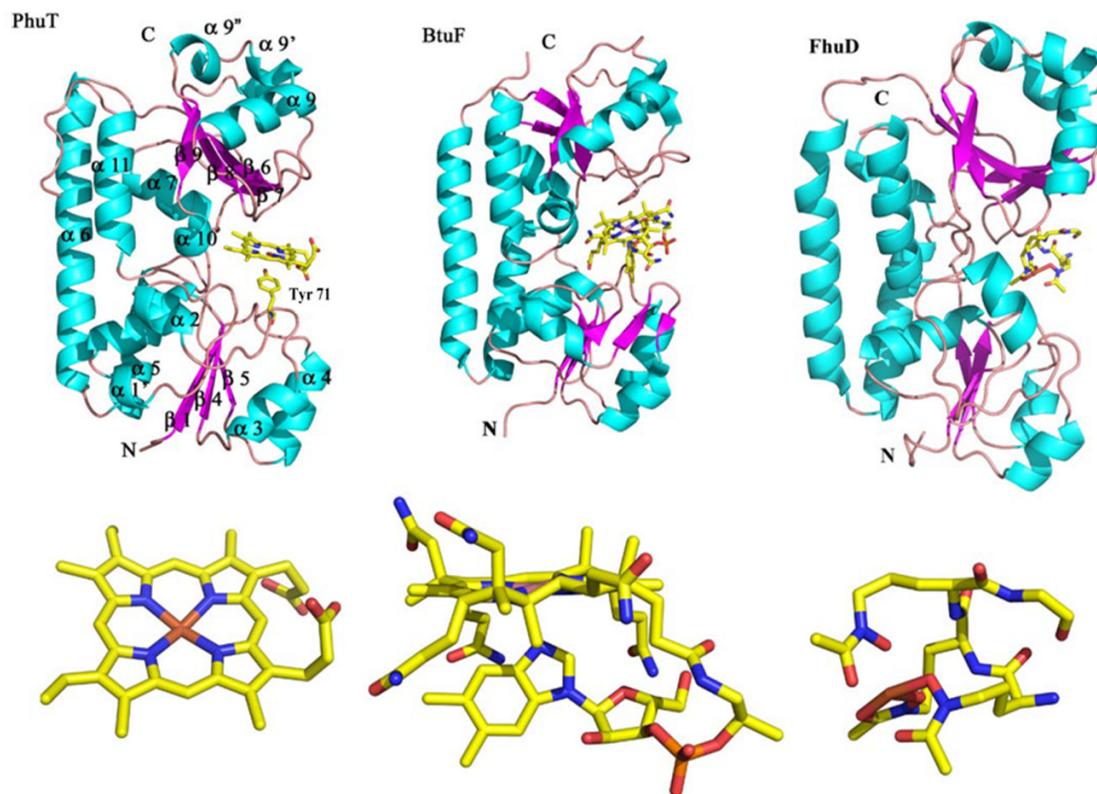


Figure 9.

Structure of PhuT compared with BtuF and FhuD. The major elements of the secondary structure according to the BtuF structure are shown on the PhuT structure. The ligands for each protein are also shown. The PDB accession code for BtuF and FhuD are, 1N2Z and 1K7S, respectively (modified from [48]). Figures were made with PyMOL [162].

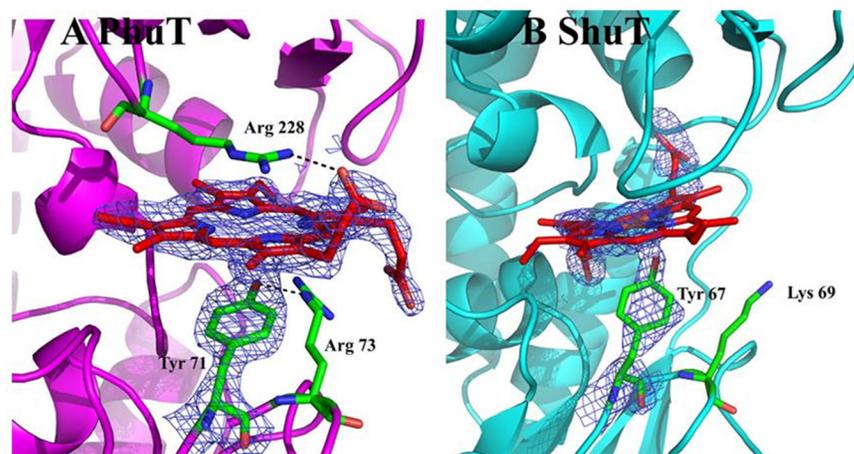


Figure 10. Heme binding pockets in PhuT and ShuT. The maps shown are $2F_o - F_c$ maps contoured at 1.0σ . **(A)** In PhuT, Arg228 H-bonds with one heme propionate while Arg73, in the proximal pocket, is H-bonding with the Tyr71 ligand. **(B)** The partially bound heme in the ShuT structure has Lys69 nearby. In order to form a H-bond with the Tyr ligand, an unfavorable bending of the side chain would be required (adapted from [48]).

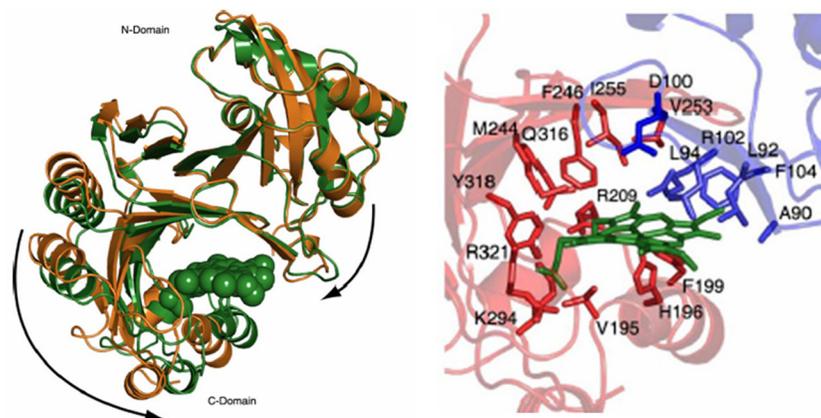


Figure 11.

(A) Crystal structure reveals HemS conformation switches between apo, open state and the heme-bound closed state. The holo-form is shown in green, and the apo-structure is shown in gold. (B) Heme-binding pocket in the heme-HemS complex: His196 coordinates to the heme iron; Arg-102 extends over the porphyrin plane to interact with the heme propionates by electrostatic interactions (adapted from [127]).

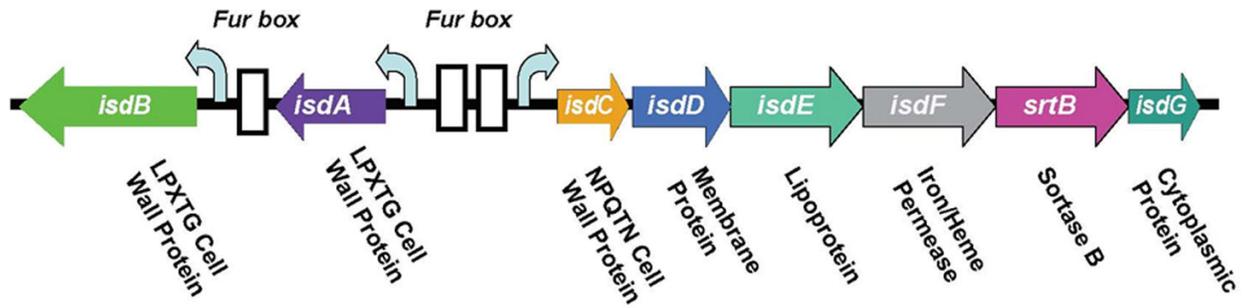


Figure 12. Organization of the *isd* locus of *S. aureus* encodes surface proteins, lipoprotein, membrane transporter, and cytoplasmic proteins (modified from [146]).

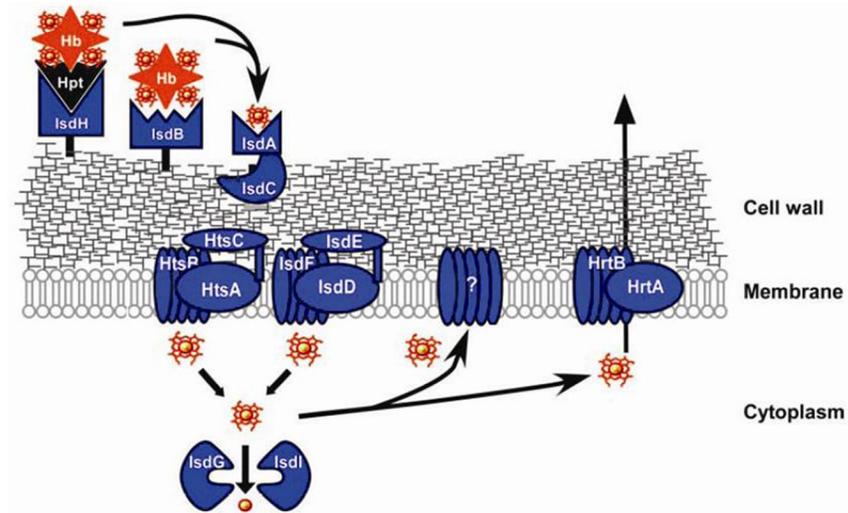


Figure 13. Gram-positive bacteria *S. aureus* heme-acquisition system (adapted from [163]).

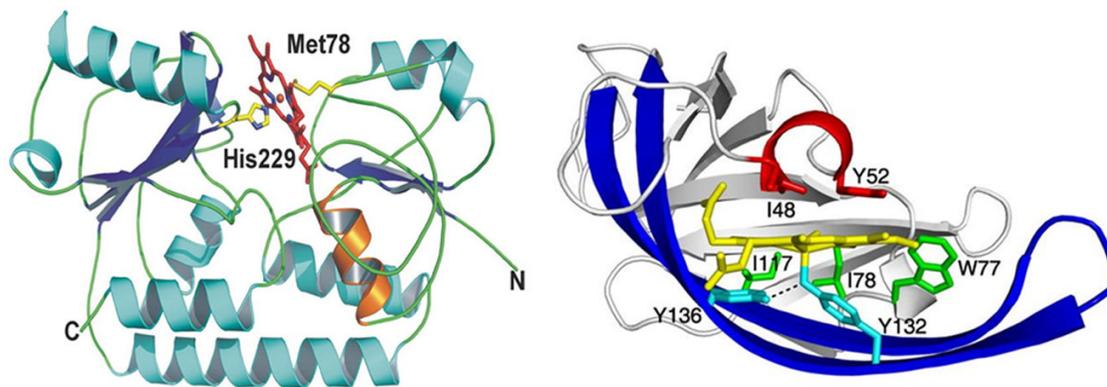


Figure 14.

The overall structure of the IsdE-heme (A) and heme-IsdC complex (B). (A) Schematic representation of bi-lobed IsdE. Secondary structural elements of the protein are represented by strands, loops, and helices colored in blue, green, and cyan, respectively. Propionate stabilizing of helix-1 is represented in orange, and protein termini are labeled with N or C. Heme is shown as sticks within the binding pocket. Heme carbon and oxygen are shown as red, and nitrogen and iron are shown as blue and orange, respectively (adapted from [149]). (B) Schematic representation of IsdC highlights the lip region, shown in red, with the distal Ile48 and Tyr52 over the heme. The prominent β -hairpin structure is in dark blue, with Tyr136 hydrogen-bonded to the proximal Tyr132, both in cyan. Lastly, Trp77, Ile78, and Ile117, in green, pack along the equatorial plane of the porphyrin, effectively interlocking the heme into its binding pocket (adapted form [148]).

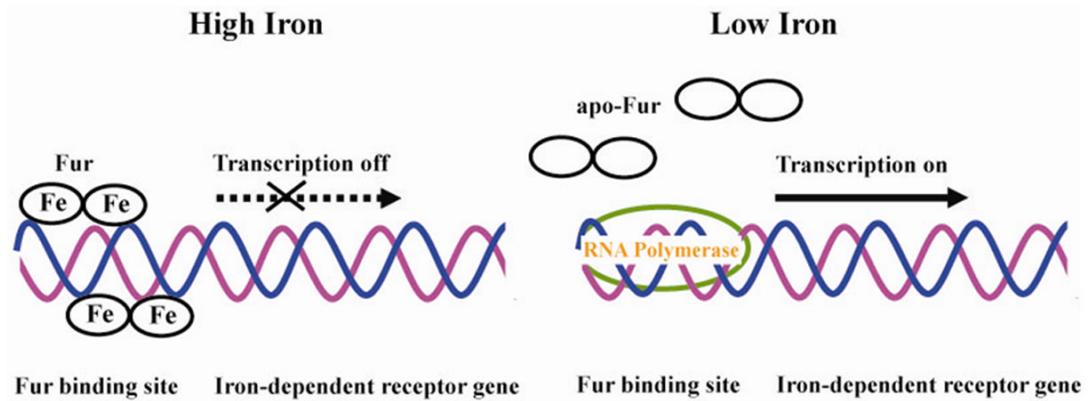


Figure 15.

Schematic representation of Fur regulated gene expression. At high iron concentration, both iron-loaded Fur dimers bind Fur box on opposite faces of DNA. Transcription of the iron-dependent receptor gene will be turned off. When iron concentration decrease, iron is released from Fur and apo-Fur is displaced from Fur box. RNA polymerase binds at the promoter region initiating the transcription.