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Extracellular Heme Uptake and the Challenges of Bacterial Cell Membranes

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Abstract

In bacteria, the fine balance of maintaining adequate iron levels while preventing the deleterious effects of excess iron has led to the evolution of sophisticated cellular mechanisms to obtain, store, and regulate iron. Iron uptake provides a significant challenge given its limited bioavailability and need to be transported across the bacterial cell wall and membranes. Pathogenic bacteria have circumvented the iron-availability issue by utilizing the hosts' heme-containing proteins as a source of iron. Once internalized, iron is liberated from the porphyrin enzymatically for cellular processes within the bacterial cell. Heme, a lipophilic and toxic molecule, poses a significant challenge in terms of transport given its chemical reactivity. As such, pathogenic bacteria have evolved sophisticated membrane transporters to coordinate, sequester, and transport heme. Recent advances in the biochemical and structural characterization of the membrane-bound heme transport proteins are discussed in the context of ligand coordination, protein–protein interaction, and heme transfer.

1. INTRODUCTION

Aerobic respiration has led to natural selection of elements essential for the diversity of chemical reactions required for life in an oxygen environment. Iron due to its versatility in ligand coordination and tunable redox state is an essential element for the survival of most organisms. It facilitates many cellular processes ranging from dioxygen transport, energy transducing pathways, and nitrogen and hydrogen fixation (Aisen, Enns, & Wessling-Resnick, 2001; Papanikolaou & Pantopoulos, 2005). Iron despite being the fourth most abundant metal in the earth's crust is not readily available at physiological pH as it forms insoluble iron (II/III) oxides, hematite (Fe₂O₃) and magnetite (Fe₃O₄). Alternatively, when completely reduced, Fe(II) is soluble and promotes the generation of toxic-free radicals through Fenton chemistry. These reactive oxygen species (ROS) can have deleterious effects on the cell damaging lipids, proteins, and nucleic acids. For bacterial pathogens, iron is most often the limiting factor for growth and a key virulence determinant (Anzaldi & Skaar, 2010; Braun, 2005; Brown & Holden, 2002; Bullen, Rogers, Spalding, & Ward, 2006; Cornelissen & Sparling, 1994; Payne, 1993). Bacterial pathogens face even greater hardship obtaining iron due to the host innate immune response (Doherty, 2007; Ganz, 2009; Nairz, Schroll, Sonnweber, & Weiss, 2010). This response is orchestrated by the combined action of the key regulators of iron homeostasis, hepcidin and ferroportin (De Domenico, Ward, & Kaplan, 2011; Ganz, 2011). Indeed, hepcidin was first isolated as an antimicrobial peptide produced in the liver and macrophages that directly sequestered iron (Verga Falzacappa & Muckenthaler, 2005). Thus, hepcidin has a dual function as an iron chelator and regulator of

iron homeostasis by inducing hypoferremia or more specifically a low-serum iron-binding capacity and increased levels of ferritin within the tissues. In addition, there are other frontline defense proteins that reduce the levels of iron including lipocalin-2, which can directly bind bacterial siderophores such as enterobactin, and lactoferrin (Lf) that sequester any free iron (Bachman, Miller, & Weiser, 2009; Borregaard & Cowland, 2006; Clifton, Corrent, & Strong, 2009). Lipocalin-2 [also known as siderocalin or neutrophil gelatinase-associated protein (NGAL)] is secreted by neutrophils, macrophages, and other cell types and is upregulated as a component of the acute-phase response to infection. Lf, a glycoprotein similar to transferrin (Tf), is found in high concentrations in neutrophils and mucous secretions. Lf possesses a wide range of antimicrobial and immunomodulatory properties in addition to the ability to bind iron (Gonzalez-Chavez, Arevalo-Gallegos, & Rascon-Cruz, 2009; Jenssen & Hancock, 2009).

As a means of circumventing the innate immune response, bacterial pathogens have evolved several redundant mechanisms to obtain iron including the utilization of heme. With 75% of total body iron being complexed in heme, this presents a prime reservoir of iron for invading pathogens (Otto, Sparrius, Wors, de Graaf, & MacLaren, 1994). Recently, it has been shown in *Staphylococcus aureus* and *Bordetella pertussis* that heme is the preferred source of iron during specific stages of infection (Brickman, Vanderpool, & Armstrong, 2006; Skaar, Humayun, Bae, DeBord, & Schneewind, 2004). As would be expected for a biologically active iron chelate, heme, if not adequately sequestered, can generate ROS, and due to its hydrophobic properties, heme can readily intercalate into membrane lipids disrupting organelle and cell structure (Balla et al., 1993; Belcher, Beckman, Balla, Balla, & Vercellotti, 2010; Halliwell & Gutteridge, 1984). As such heme biosynthesis and degradation are tightly regulated and coupled to the cellular heme protein levels. Therefore, capture of heme from cellular heme proteins, most notably hemoglobin (Hb), presents a major challenge to bacterial pathogens. Additionally, heme must then traverse the thick peptidoglycan cell wall of gram-positive organisms and outer membrane (OM) and cytoplasmic membrane (CM) of gram-negative bacteria. The heme uptake systems comprise a concerted network of highly evolved proteins that (a) sequester heme with high affinity in a redox inert state and (b) utilize both the free energy of protein-protein interaction and the energy generating systems of the cell membrane to translocate heme into the cytoplasm.

2. HEME CAPTURE AND TRANSPORT ACROSS THE CELL WALL AND MEMBRANES

2.1. Active Transport Across the Gram-Negative OM

Gram-negative pathogens must actively transport heme through the OM and shuttle it across the periplasmic space (Fig. 1). The OM an asymmetric lipid bilayer protects the organism from the external environment including from the effects of extracellular toxins. The inner layer of the OM is composed of phospholipids and the outer layer of lipopolysaccharide that provides the bacteria with a negatively charged polar surface preventing the influx of lipophilic compounds. Therefore, within the membrane, there are numerous transporters for the active influx and efflux of molecules such as heme. All heme OM transporters have a similar closed β -barrel architecture consisting of 22 antiparallel β -strands and a globular domain most often referred to as the plug, cork, or hatch that occludes the pore of the β -barrel. The β -strands are connected by short turns in the periplasm and by long, hydrophilic loops at the cell surface that play a critical role in protein-protein interaction and heme binding (Fig. 2A). The TonB-dependent OM receptors bear significant sequence and structural homology to the more well-characterized iron-siderophore receptors (Braun & Endriss, 2007; Wandersman & Delepelaire, 2004; Wilks & Burkhard, 2007). In addition to high structural similarities, multiple sequence alignment of all the known heme OM

receptors identified two absolutely conserved histidine residues located in a conserved FRAP/NPNL domains that are essential for receptor function (Bracken, Baer, Abdur-Rashid, Helms, & Stojiljkovic, 1999). The OM heme receptors of gram-negative organisms fall into two classes, those that scavenge heme through the secretion of a hemophore (Cescau et al., 2007; Wandersman & Delepelaire, 2004; Wandersman & Stojiljkovic, 2000) and those that recognize specific heme protein complexes such as Hb or Hb-haptoglobin (Burkhard & Wilks, 2007; Leduc et al., 2008; Rohde & Dyer, 2004; Simpson, Olczak, & Genco, 2000). As is the case with iron-siderophore uptake, there is a level of redundancy whereby bacteria encode several distinct heme uptake systems. In *Pseudomonas aeruginosa*, two heme uptake operons have been characterized, the *has* (heme acquisition system) which encodes a hemophore (HasA) and the *phu* (*Pseudomonas* heme utilization) system that utilizes the hosts' heme proteins directly (Ochsner, Johnson, & Vasil, 2000).

The analogous hemophore system in *Serratia marcescens* has been extensively studied and much of our mechanistic understanding of these heme transporters derives from this system. The OM receptor, HasR, of *S. marcescens* can acquire free heme at micromolar concentrations (Letoffe, Ghigo, & Wandersman, 1994). However, the efficiency of heme uptake is greatly enhanced in the presence of the hemophore, HasA, with heme or Hb (Ghigo, Letoffe, & Wandersman, 1997). HasA binds heme with a very high affinity ($K_a = 5.3 \times 10^1$ /M) (Letoffe et al., 1994). However, there is no apparent affinity for HasA with Hb nor does it seem to increase the rate of heme dissociation from Hb, and it is proposed that heme is captured by HasA as it spontaneously dissociates from Hb (Yukl et al., 2010). HasA coordinates heme axially through His-32 and Tyr-75, with His-83 forming a hydrogen bond to Tyr-75, strengthening the Tyr-iron bond contributing to this high affinity for heme (Deniau et al., 2003; Letoffe et al., 2001). The H83A mutation resulted in a 256-fold decrease in affinity for heme, indicating its importance for stabilizing heme in the binding pocket (Letoffe et al., 1994). The function of holo-HasA is to deliver heme to the OM receptor HasR and it has been shown to form an extremely tight complex with the HasR receptor (Izadi-Pruneyre et al., 2006; Letoffe, Nato, Goldberg, & Wandersman, 1999). HasR, however, has a much lower affinity for heme ($K_a = 5 \times 10^6$ /M) (Izadi-Pruneyre et al., 2006). Surprisingly, heme transfer from HasA to HasR occurs spontaneously and independently of TonB; this otherwise thermodynamically unfavorable transfer of heme from a very high-affinity site to a lower affinity site is driven by the highly exothermic and exergonic protein-protein interaction (Izadi-Pruneyre et al., 2006). Once heme is transferred to HasR, heme is coordinated by two conserved histidine residues, His-189 and His-603. Molecular modeling of the HasR receptor identified His-603 as being located on the extracellular loop L7 and His-189 resides on the extracellular face of the N-terminal "plug" (Barjon, Wecker, Izadi-Pruneyre, & Delepelaire, 2007). These observations have been corroborated by UV-vis and resonance Raman (rR) spectroscopic studies identifying a ferric 6-coordinate low spin (6CLS) (Izadi-Pruneyre et al., 2006).

The mechanism of heme transfer from the high-affinity hemophore to the OM receptor was further revealed on crystallization of the HasA/holo-HasR complex. Sequence alignment of HasR with the closely related siderophore TonB-dependent OM receptors identified exceptionally large extracellular loops L2, L6, and L9 of HasR. Mutagenesis studies revealed that two of these loops, L6 and L9, along with L8 were critical for binding HasA (Barjon et al., 2007). The recently solved crystal structure of the HasA:HasR:heme complex further shows that loops L6, L9, and L8 all form contacts with HasA and bury a large surface area of the protein (Krieg et al., 2009). In the structure, heme is located in the channel of HasR, coordinated by His-189 and His-603, which is a 9.2 Å translation from the binding pocket of HasA, along with rotation of the porphyrin ring and exposing the propionates to solvent (Fig. 2B). Additionally, the loop bearing the coordinating histidine in HasA (His-32) is displaced from the binding pocket by loops L7 and L8 of HasR, thereby

weakening the affinity of heme for the hemophore. Interestingly, superposition of heme in the binding pocket of HasA in the HasA:HasR complex reveals that Ile-671 on extracellular loop L8 of HasR would involve a steric clash with the heme (Krieg et al., 2009). This observation led to a hypothesis for heme release from the high-affinity HasA site to the lower affinity HasR site through protein–protein-induced conformational change. The protein–protein-induced conformational change weakens the His-32 heme ligand of HasA followed by steric displacement of heme by Ile-671 triggering heme release to HasR. Furthermore, His-83 in HasA which hydrogen bonds to the axial Tyr-75 ligand is rotated away from the active site in the HasA/HasR structure which would impede heme sliding back into the HasA binding pocket (Krieg et al., 2009). The mechanisms were further supported on mutation of HasRIle-671 to Gly where the ability to extract heme from HasA was abolished while retaining its ability to bind free heme. Furthermore, the crystal structures of holo-HasA/HasR-I671G revealed that in contrast to the wild-type heme remained bound to HasA. Upon release of heme from the hemophore and capture by the receptor, the energy provided through TonB then supposedly facilitates translocation of heme into the periplasm and returning the receptor to the ground state.

The second class of OM receptors do not rely on recognition of heme-loaded hemophores but instead interact with and directly capture heme from host heme proteins. OM receptors of this type have been identified in many gram-negative pathogens such as *Yersinia* sp. (Rossi et al., 2001; Stojiljkovic and Hantke, 1992, 1994), *Neisseria* sp. (Stojiljkovic, Larson, Hwa, Anic, & So, 1996; Stojiljkovic et al., 1995), *Vibrio* sp. (Henderson & Payne, 1994), and *P. aeruginosa* (Ochsner et al., 2000). These receptors have high sequence identity (25–65%) and structural similarity to the hemo-phore-dependent receptors, and their related iron–siderophore OM receptors (17–23%), suggestive of a common mechanism of heme transport (Braun & Endriss, 2007; Wandersman & Delepelaire, 2004; Wandersman & Stojiljkovic, 2000; Wilks & Burkhard, 2007).

The non-hemophore receptors are typified by ShuA from *Shigella dysenteriae* which interacts and strips heme directly from heme proteins such as Hb. Similar to the bis–His coordination in HasR, ShuA has a conserved His-86 on the N-terminal plug and His-420 on extracellular loop L7. The recent crystal structure of apo-ShuA confirms His-86 and His-420 location on the N-terminal plug and extracellular loop, respectively. However, His-86 and His-420 are 9.86 Å apart, suggesting that a significant conformational change must take place upon heme binding (Cobessi, Meksem, & Brillet, 2010). A mechanism based on the accumulated biochemical data and the HasA/HasR structure has His-420 coordinating to the heme in the first ligation step with hydrophobic residues on the L7 loop interacting with the porphyrin and displacing the heme which is coordinated to the N-terminal plug His which then forms a bis–histidine ligation through the loop His sequestering heme within ShuA and preventing back transfer of the heme. In this way, the heme is coordinated in a redox-inactive state excluded from the extracellular environment by closure of the loops over the barrel. Kinetic analysis of heme transfer from Hb revealed that transfer from oxidized form of the Hb dimer (metHb₂) or tetramer (metHb₄) is unidirectional and on the order of 10⁴ faster than the rate of free heme association and 10³ faster than heme transfer from oxyhemoglobin, indicating that the oxidized form of Hb is most likely the physiological substrate for ShuA (Burkhard & Wilks, 2007). Biochemical characterization of the wild type, His-86 Ala and His-420 Ala mutants, revealed that both residues were required for heme acquisition from methemoglobin (Burkhard & Wilks, 2007). Kinetic studies indicated that heme could be released from Hb to the ShuA H86A or ShuA H420A receptor but in the absence of the second His residue the heme is transferred back to methemoglobin. In contrast, the H86A/H420A ShuA mutant had no residual heme bound to the receptor and was also unable to extract heme from methemoglobin. Thus, both His residues are essential for the unidirectional transfer of heme from methemoglobin to the OM receptor, ShuA.

The mechanism of translocation through the OM β -barrel receptors has been the subject of intense study with conflicting mechanisms having been proposed. As no energy sources are accessible in the periplasm (e.g. ATP, protein gradients), the OM heme receptors must harness energy generated by the proton-motive force of the CM to transport ligands into the periplasm. Heme transport is driven by the CM electrochemical gradient that is then transduced across the periplasm to the receptor through the TonB–ExbB–ExbD complex. The TonB protein interacts with the OM receptor through a consensus sequence on the receptor known as the “TonB box” (Braun & Endriss, 2007). The first step in heme transport across the OM most likely involves a conformational change on heme binding that exposes the TonB box and promotes an interaction between the receptor and TonB (Khursigara, De Crescenzo, Pawelek, & Coulton, 2004, 2005). Crystal structures of the closely related ferrichrome (FhuA) and cobalamin (BtuB) OM receptors show that, upon binding of TonB, an interprotein β -sheet is formed at the TonB box (Pawelek et al., 2006; Shultis, Purdy, Banchs, & Wiener, 2006). The formation of this β -sheet positions the TonB α -helix 1a in proximity to the plug where extensive electrostatic interactions drive conformational changes within the plug (Pawelek et al., 2006; Shultis et al., 2006). Structural and computational experiments to date suggest a shearing or mechanical pulling mechanism, where hydration of the central β -sheet of the cork domain disrupts interactions with the inner face of the β -barrel. The perpendicular force applied to the β -sheet on interaction with TonB results in local unfolding of the cork creating a channel for passage of the ligand (Chimento, Kadner, & Wiener, 2005; Faraldo-Gomez, Smith, & Sansom, 2003; Gumbart, Wiener, & Tajkhorshid, 2007; Pawelek et al., 2006; Shultis et al., 2006). Heme translocated through the OM receptor is captured by the periplasmic heme-binding protein that then acts as a soluble receptor for a heme-dependent ABC (ATP-binding cassette) transporter in the CM (Koster, 2005; Krewulak & Vogel, 2008). The mechanism of heme release from the OM receptors to the periplasmic binding proteins (PBPs) is not known. However, studies have shown that the TonB protein acts as a scaffold for recruitment of the PBP placing it in the vicinity to readily access the ligand and transport it to the ABC transporter (Carter et al., 2006). Presumably, the heme affinity for the OM receptor must decrease upon interaction with the PBP with the energetic cost of heme transfer being met by the free energy from complexation.

2.2. ATP-Dependent Transport Across the Cytoplasmic Membrane

The soluble periplasmic and lipid-anchored substrate-binding proteins deliver heme to a specific ABC transporter in gram-negative and gram-positive pathogens, respectively. The gram-negative bacterial PBPs are critical in shuttling substrates across the periplasm from the OM to the CM. PBPs are divided into three classes based on the number of interdomain connections (Borths, Locher, Lee, & Rees, 2002; Dwyer & Hellinga, 2004). All three classes of PBPs are comprised of N- and C-terminal globular domains that on closure form ligand-binding cleft between the domains. The Class I and II PBPs have two or three interdomain connections, respectively. Class I proteins, including the maltose-binding protein, and Class II proteins, such as ribonucleotide reductase, undergo large structural changes which are required for substrate binding and release (Quirocho & Ledvina, 1996). Class III proteins have one interdomain connection between the globular domains and undergo relatively small conformational changes (Borths et al., 2002; Clarke, Braun, Winkelmann, Tari, & Vogel, 2002; Karpowich, Huang, Smith, & Hunt, 2003). Proteins included in Class III are BtuF (Borths et al., 2002), a vitamin B₁₂-binding protein, and FhuD (Clarke et al., 2002), a protein that binds hydroxamic acid siderophores. The structurally determined heme-binding proteins ShuT and PhuT have very similar architecture to BtuF where the N- and C-domains consist of a five-stranded β -sheet flanked by helices (Fig. 3A). Heme is ligated through a Tyr located on the N-terminal domain at the interface of the heme-binding cleft (Fig. 3B). The Tyr ligand points away from the binding pocket in the apoprotein and on heme

coordination reorients toward the pocket but there is little overall change in the structure. A similar reorganization of the proximal ligand is observed in the ferric-siderophore FhuD protein and has been proposed to play a role in guiding the ligand into the pocket (Zhu, Alexeev, Hunter, Campopiano, & Sadler, 2003). In the *S. dysenteriae* system, ShuT accepts heme from ShuA and forms a highly stable complex with Tyr-94 (Eakanunkul et al., 2005; Ho et al., 2007). Binding affinities for the PBP ShuT have not been measured directly due to residually bound heme present following purification of the protein; however, rate constants of heme extraction by apo-hemoglobin ($K_d = 10^{14}$) indicate a high affinity of ShuT for heme (Eakanunkul et al., 2005). The apparent rate constant of heme exchange from holo-ShuT to apo-myoglobin is 4×10^{-5} M (Eakanunkul et al., 2005). This rate decreases 10^4 -fold when Tyr-94 is mutated to Ala (Eakanunkul et al., 2005). Although mechanistic details of ShuT release from ShuA are not yet known, it is expected that conformational changes resulting from heme coordination to ShuT play a role in release of the PBP to the periplasm where it can interact with the ABC transporter to release heme for transport across the CM. While the proximal face of the heme-binding cleft of ShuT and PhuT are similar, the distal side has significant differences. The largest of these differences is a hairpin loop after the β -6 strand that is pointing up in PhuT and down in ShuT. Due to the positioning of the loop in the ShuT cleft, it has the potential to make van der Waals contacts with the heme. A second hairpin loop in PhuT protrudes into the heme cleft providing an Arg that is able to interact with one of the heme propionates. It has been proposed, based on computational studies, that such hydrogen bonding networks play an important role in heme binding and release, suggesting that sequence diversity in the active site can be tolerated as long as a suitable hydrogen bond network is retained (Liu, Su, et al., 2008). The lipoprotein relay network of gram-positive organisms will be discussed in the following section.

ABC transporters constitute one of the largest protein superfamilies and are primarily responsible for the ATP-dependent transport of substrate molecules across cellular membranes (Higgins, 1992, 2001). The ABC transporters are composed of two transmembrane domains (TMDs), which form the translocation channel, and two cytoplasmic nucleotide-binding domains (NBDs) that bind and hydrolyze ATP. The TMD vary in sequence and chain length allowing for specificity of substrate, while the ATP-binding domains are highly conserved with the Walker A and Walker B ATP-binding motifs, a glutamine residue on the Q-loop, a highly conserved histidine residue in the Switch region, and the LSGGQ “signature motif” which is unique to NBD proteins (Holland & Blight, 1999; Hopfner et al., 2000; Schneider & Hunke, 1998; Walker, Saraste, & Gay, 1982).

Transport of heme across the CM by the ABC transporters is proposed to be similar to that of the previously characterized Vitamin B₁₂ transport system BtuCD. The ABC transporter comprises two transmembrane BtuC subunits and two corresponding ATP-binding BtuD subunits (Hvorup et al., 2007). A general mechanism for ligand translocation in the type II ABC transporters is proposed to be via an ATP-switching model (Fig. 4) (Higgins & Linton, 2004). In such a model, the ligand-bound PBP binds to the TMD of the ABC transporter inducing a conformational change to the outside facing state. This lowers the activation energy for ATP binding to the NBD. ATP binding induces the closed dimer formation of the NBD inducing the inward facing conformation of the TMD for active transport to the cytoplasm. ATP hydrolysis drives release of the ligand to the cytoplasm, following which the NBD dimer is destabilized resetting the transporter. Recent molecular dynamic simulations based on the various structures of the BtuCD complex support the transition from the outward to inward facing state being dependent on conformational changes in the NBDs (Weng, Fan, & Wang, 2012).

In the case of the heme–ShuUV transporter, release of heme from ShuT to ShuU presumably correlates with an opening of the lobes of ShuT and ShuU upon binding. This hypothesis is supported by crystallographic data of the vitamin B₁₂ transporter, BtuCD, complexed with BtuF (Hvorup et al., 2007). Based on current findings with the ShuUV system, this step in ligand release occurs in the absence of ATP and is consistent with a model in which conformational changes reduce the heme affinity of ShuT, releasing heme to the lower affinity site within the transporter (Fig. 4). A sequence alignment of all predicted heme ABC transporters reveals two conserved His residues in ShuU His-252 and His-262 that are also present in the vitamin B₁₂ transporter, BtuC. Crystal structures of BtuCD reveal the His residues to be located in the translocation channel (Hvorup et al., 2007; Locher, Lee, & Rees, 2002). Heme translocation studies show that both His residues are required for transfer of heme through the ABC transporter, ShuUV; however, the detailed molecular mechanism of transport has not yet been determined (Burkhard & Wilks, 2008).

Unlike the well-characterized vitamin B₁₂ ABC transporters release of heme to the cytoplasm requires interaction of the ShuUV transporter with the cytoplasmic heme-binding protein, ShuS (Burkhard & Wilks, 2008). The ShuUV transporter when reconstituted in liposomes requires the cytoplasmic heme-binding protein ShuS to be present in the external media for active translocation of heme from the lumen (Burkhard & Wilks, 2008). In the absence of the cytoplasmic heme-binding protein, no ATP hydrolysis or heme translocation occurs. This was the first direct evidence that the physiological function of the cytoplasmic heme-binding proteins is to capture exogenously acquired heme following translocation through the CM. Earlier studies suggested a homolog of the *S. dysenteriae* ShuS protein from *Y. enterocolitica* (HemS) encoded a hemeoxygenase (HO) required for iron release (Stojiljkovic & Hantke, 1994). However, this annotation was based on the observation that deletion of the gene caused heme toxicity. The ChuS protein of *Escherichia coli* 0157:H7 has been reported to be an HO based on in vitro assays reporting degradation of heme in the presence of a reducing agent (Suits, Jaffer, & Jia, 2006; Suits et al., 2005). However, it has not been determined if the catalytic turnover is greater than one or that the product of the reaction is indeed biliverdin. The enzymatic activity remains questionable given the nonenzymatic “coupled oxidation” of heme described almost half a century ago (O’Carra & Colleran, 1969,1970). Furthermore, it has recently been shown that the role of the cytoplasmic heme-binding proteins is to sequester and traffic heme within the cell (Lansky et al., 2006). In vivo and in vitro studies suggest that once the cytoplasmic heme-binding protein has obtained heme from the ABC transporter, it shuttles the heme to an iron-regulated hemeoxygenase (HemO; previously annotated as both *pa-HO* and *PigA*) for degradation (Bhakta & Wilks, 2006; Block et al., 2007; Kaur, Lansky, & Wilks, 2009; Lansky et al., 2006).

2.3. Transport Across the Gram-Positive Cell Wall

The extensive peptidoglycan cell wall in gram-positive organisms is required for structural rigidity and protection from osmotic lysis; however, these properties pose a significant hurdle to ligand transport. Moreover, the redox reactivity of heme requires sequential delivery via specific protein–protein interactions to prevent oxidation of membrane lipids. The transport of heme through the peptidoglycan cell wall and across the membrane involves several proteins from surface-exposed lipoproteins that are anchored in the cell wall to the ABC transporters in the cell membrane (Grigg, Ukpabi, Gaudin, & Murphy, 2010; Maresso & Schneewind, 2006) (Fig. 4). The most well-characterized gram-positive heme transport system is that of *S. aureus* (Maresso & Schneewind, 2006; Mazmanian et al., 2003) although others have recently been identified in *Streptococcus pyogenes* (Bates et al., 2003; Ekins & Niven, 2003; Hanks, Liu, McClure, & Lei, 2005; Liu & Lei, 2005; Sook et al., 2008; Zhu, Liu, & Lei, 2008), *Listeria monocytogenes* (Jin et al., 2006), and

Corynebacterium diphtheria (Drazek, Hammack, & Schmitt, 2000). A similar system has been identified in *Mycobacterium tuberculosis* and encodes a secreted protein and two membrane proteins required for heme transport across the cell wall (Tullius et al., 2011).

The *S. aureus* iron-regulated heme uptake gene cluster (*isd*) encodes a sortase (*srtB*) that is required for sorting and anchoring the surface-exposed heme transport proteins to the cell wall (Mazmanian, Ton-That, Su, & Schneewind, 2002) (Fig. 4). Heme acquisition by *S. aureus* involves the cell wall-anchored IsdA, IsdB, IsdC, and IsdH (HarA) and IsdD, IsdE, and IsdF that comprise the transmembrane ABC transporter (Mazmanian et al., 2003; Skaar & Schneewind, 2004). In addition to the transport proteins, the *isd* gene cluster encodes the heme-degrading IsdG and IsdI proteins. The surface proteins IsdH and IsdB directly extract heme from Hb and Hb-haptoglobin complexes (Dryla, Gelbmann, von Gabain, & Nagy, 2003; Mazmanian et al., 2003; Pilpa et al., 2009; Torres, Pishchany, Humayun, Schneewind, & Skaar, 2006). Several studies have shown sequential transfer of heme from the surface proteins IsdH and IsdB through IsdA to IsdC which then delivers heme to the membrane proximal IsdE (Fig. 4) (Liu, Tanaka, et al., 2008; Muryoi et al., 2008). Holo-IsdA cannot deliver heme to apo-IsdE and requires facilitation by apo-IsdC (Zhu, Xie, et al., 2008). An alternative study suggests that IsdA and IsdB physically interact and colocalize to a distinct site within the cell wall coordinating the transfer of heme from Hb at the cell surface to the cell wall proteins (Pishchany, Dickey, & Skaar, 2009). In the final uptake step, IsdE, the cell membrane lipoprotein component of the permease transporter (IsdDEF) transfers heme to the membrane-bound transporter IsdD/F.

All cell wall-anchored Isd proteins contain one to three copies of the near iron transport (NEAT) domains (Fig. 5A) (Andrade, Ciccarelli, Perez-Iratxeta, & Bork, 2002; Grigg et al., 2010). The domains acquired their names as a result of their vicinity in the genome to putative iron chelate ABC transporters. The Isd surface protein has a secretion signal peptide for targeting, a sortase anchoring signal for attachment to the cell wall, and at least one NEAT domain. The multiple NEAT domain proteins are separated by sequences (15–20 residues) that are often highly charged and predicted to be disordered (Grigg et al., 2010). In proteins with multiple NEAT domains, such as IsdH, not all domains bind heme but are required to increase the binding affinity to the target protein through increased protein–protein interaction. A number of NEAT domains have been structurally characterized including IsdA N1 (holo and apo), IsdC N1 (holo and apo), IsdH N1 (apo), IsdH N3 (holo and apo) (Grigg, Vermeiren, Heinrichs, & Murphy, 2007; Pilpa et al., 2006; Sharp, Schneider, Cockayne, & Paoli, 2007; Villareal, Pilpa, Robson, Fadeev, & Clubb, 2008; Watanabe et al., 2008), and IsdB N2 (Gaudin, Grigg, Arrieta, & Murphy, 2011). All the NEAT domains have the same overall eight-stranded β -sandwich fold architecture with an α -helix connecting the β 1- and β 2-strands that in the heme-coordinating NEAT domains is stabilized on ligand binding (Fig. 6B) (Pilpa et al., 2006; Villareal et al., 2008). Structural and spectroscopic studies of the holo-NEAT domains reveal an absolutely conserved Tyr ligand located in a hydrophobic pocket on the β 8-strand (Fig. 6A) (Grigg et al., 2007; Mack, Vermeiren, Heinrichs, & Stillman, 2004; Muryoi et al., 2008; Sharp et al., 2007; Tiedemann, Muryoi, Heinrichs, & Stillman, 2008; Vermeiren, Pluym, Mack, Heinrichs, & Stillman, 2006; Watanabe et al., 2008). A second Tyr, located four residues downstream, hydrogen bonds with the coordinating Tyr and forms a π -bond interaction with the heme pyrrole ring. A conserved Ser and several hydrophilic residues on the distal side of the pocket form H-bonds with at least one heme propionate providing a high-affinity site.

Despite a similar overall architecture, minor differences among the Isd proteins are observed in the heme-binding pocket with the major variation in the composition of loops and chain termini. These variations play a role in the unique function of each protein. For example, the nonheme-binding IsdHN1 (Sharp et al., 2007) does not contain Tyr-52, Tyr-132, His-134,

and Tyr-136 residues that are involved in heme binding (Pilpa et al., 2006; Sharp et al., 2007). Additionally, the N1 domain of IsdH has a series of disordered aromatic residues (Tyr-125, Tyr-126, His-127, Phe-128, Phe-129) in loop 2 between the β 1b- and β 2-strands (Pilpa et al., 2006). These residues are conserved in all Hb-binding NEAT domains. It had been proposed that the function of IsdH is to tether Hb to the surface of the cell wall to enable heme transfer through IsdA to IsdC (Sharp et al., 2007). Recent spectroscopic and structural studies of the IsdH N1 domain in complex with Hb revealed a highly specific protein–protein interaction involving significant contributions from the conserved aromatic loops 2 and 4 with the hemoglobin α -subunit (α Hb) (Krishna Kumar et al., 2011). Conservation of loops 2 and 4 in IsdH N2 and IsdB N1 would suggest that they also have similar affinity for binding to α Hb. Furthermore, heme dissociation from Hb on interaction with the IsdH/B N1 domains alone is three to four times slower than for the intact receptors, suggesting that interaction of the Hb-binding domains enhances interaction with the heme acceptor NEAT domains N3 of IsdH and N2 of IsdB domains promoting release of heme from Hb. The structure of the IsdB N2 heme-binding domain was recently solved, revealing a unique heme ligand set contributed by Tyr-440 and Met-362 (Gaudin et al., 2011). Interestingly, the crystal structure revealed a mixture of pentacoordinate and hexacoordinate states with Met-362 partially occupying the sixth ligand position. Based on spectroscopic studies of the Met-362 Leu variant, the increased heme off rates, combined with the decrease in transfer rate, suggest that Met-362 is crucial for heme transfer to IsdA.

The relative heme affinities of the Isd proteins have been estimated from the apparent rate of heme transfer from the proteins to Mb, where IsdC > IsdE > IsdB $\approx$ IsdA (Zhu, Xie, et al., 2008). Kinetic studies of the Isd system in combination with circular dichroism and mass spectral data provided *in vitro* evidence that supports a model (Mazmanian et al., 2003) in which heme transport across the envelope of gram-positive bacteria is based on localization of the proteins in the envelope (Fig. 5B) (Liu, Tanaka, et al., 2008; Muryoi et al., 2008; Zhu, Xie, et al., 2008). Heme is transferred from methHb to IsdB at a rate of 0.31 per second (Zhu, Xie, et al., 2008). This is 80 and 2200 times greater than the rate for dissociation from the β and α subunits of human methHb, respectively. This transfer is a single exponential process unlike that of simple heme dissociation from methHb and transfer between methHb and IsdA, IsdC, and IsdE, indicating that methHb transfers heme to IsdB specifically (Zhu, Xie, et al., 2008). An in-depth analysis of the kinetics of heme transfer from intact IsdA to IsdC (which have a single NEAT domain) revealed a two-step process, namely complex formation followed by heme transfer and dissociation (Liu, Tanaka, et al., 2008). In contrast, a one-step kinetic model is observed for heme transfer between the isolated IsdA N1 and IsdC N1 domains, indicating that protein–protein interaction is rate limiting for heme transfer between holo-IsdA N1 and apo-IsdC N1. IsdA accepts heme from IsdH/B and variants of the proximal Tyr-166 significantly reduce the rate of heme transfer to and from IsdA, despite the fact that the off rates increased significantly on loss of the proximal Tyr (Grigg, Mao, & Murphy, 2011). The data confirmed the Tyr ligand was essential for directional transfer and a model whereby heme is directly shuffled from the Tyr ligand of the donor protein to the Tyr on the acceptor protein. Whether heme is passed off via a hexacoordinate di-Tyr intermediate between the donor and acceptor protein or rapid release from the donor followed by coordination of the Tyr in the acceptor protein has not been determined. A recent paramagnetic relaxation enhancement NMR study has proposed a weak transient “handclasp” model for heme transfer from IsdA to IsdC N1 where the binding affinity between the proteins is extremely weak and estimated to be in the low millimolar range, thus facilitating rapid transfer (Fig. 6B) (Villareal, Spirig, Robson, Liu, Lei, & Clubb, 2011).

Heme is transferred from the cell wall-anchored Isd proteins to the Isd membrane ABC transporter for transport into the cytoplasm. The ABC transporter has three components: (1) a heme-binding protein, (2) a membrane permease, and (3) an ATP hydrolase (Jones,

O'Mara, & George, 2009). The structure of the heme-binding protein IsdE of *S. aureus* like all NEAT domains has an immunoglobulin-like eight-stranded β -sandwich fold with two domains linked by an α -helix (Grigg et al., 2007). Heme binds in a cleft between the two domains where the heme is six coordinate with an axial ligand coming from each domain, Met-78 and His-229. Very little is known about the remaining components of the ABC transporter in *S. aureus*; however, IsdF shares 22% identity with the structurally characterized Vitamin B₁₂ transporter, BtuC. However, the *isd* locus of *S. aureus* does not encode an ATPase (Mazmanian et al., 2003) similar to the siderophore transporters Hts and Sir (Dale, Sebuly, & Heinrichs, 2004; Speziali, Dale, Henderson, Vines, & Heinrichs, 2006). Genetic studies have provided some evidence that these systems utilize FhuC as the ATPase portion of the ABC transporter. The same is likely the case for the Isd system. *S. pyogenes* (Bates et al., 2003), *L. monocytogenes* (Jin et al., 2006), and *C. diphtheriae* (Drazek et al., 2000) all encode putative ATPases; however, no structural data are available for any of these ABC transporter systems. Despite the lack of structural data on the transmembrane transporters, they are likely to be similar to the ABC transporter systems of gram-negative bacteria described in the previous sections.

Although the mechanism by which gram-negative and gram-positive pathogens acquire heme has fundamental differences due to the nature of the cells architecture, it is clear that common motifs in heme binding are emerging. All NEAT domains of gram-positive bacteria have an immunoglobulin-like β -sandwich fold comprised of two antiparallel β -sheets bridged by an α -helix. This architecture is very similar to gram-negative Class III PBPs ShuT, PhuT, BtuF, and FhuD that are comprised of two globular β -domains linked by a single α -helix. In both cases, heme is coordinated in a cleft between the two β -domains. Both the cell wall-anchored Isd proteins and the PBPs coordinate the heme through a Tyr axial ligand. Furthermore, the unusual His–Tyr ligation observed in the HasA hemophores of *S. marcescens* and *P. aeruginosa* has significance in the mechanism of heme capture and transfer to the OM receptor. In contrast to the gram-positive cell surface-anchored proteins, the translocation of heme through the membrane is driven primarily via bis–Histidine coordination in both the gram-negative OM receptors and ABC transporters, a theme most likely conserved in gram-positive ABC transporters.

3. TRANSMEMBRANE HEME SENSORS AND ATP-DEPENDENT EFFLUX PUMPS

3.1. Transmembrane Two-Component Heme Sensor Kinase Systems

Bacteria respond to chemical and physical environmental signals through two-component systems comprising a membrane sensor kinase and a soluble response regulator (Krell et al., 2010; Szurmant, White, & Hoch, 2007). The ability of the invading pathogen to respond to the extracellular environment is crucial to the acquisition of heme as well as the prevention of heme toxicity. Heme import is required to meet the iron needs of the pathogen; however, as heme accumulates, the propensity for toxic side effects increases. Pathogens respond to intracellular heme accumulation through such a two-component sensor kinase system that reduces intra-cellular heme by signaling expression of heme-degrading enzymes (Bibb, King, Kunkle, & Schmitt, 2005; Bibb, Kunkle, & Schmitt, 2007) or heme efflux transporters (Stauff, Torres, & Skaar, 2007; Stauff et al., 2008). In *C. diphtheriae*, two heme-dependent sensor kinases have been described the *Corynebacterium* heme response system (ChrAS) and heme response regulator (HrrAS) where both systems are required for the optimal expression of HmuO under low iron conditions. Furthermore, the ChrAS system is responsible for 80% of the activation of *hmuO* under low iron conditions and in high iron in the presence of heme appears to partially reverse the effects of iron. Recently, the ChrAS system was also shown to regulate the ATP-dependent heme-regulated transporter HrtAB

responsible for the efflux of heme from the cell (Bibb & Schmitt, 2010). Similarly, the *S. aureus* heme sensor system (HssRS) responds to elevated heme levels by increasing expression of a similar heme efflux system HrtAB (Stauff et al., 2008). The sensor kinase presumably responds through direct binding of heme to the extracytoplasmic domain of HssS, although an indirect mechanism cannot be ruled out (Stauff et al., 2007). Ligand binding to the extracytoplasmic domain of the histidine kinase HssS triggers phosphorylation of an aspartate on the cognate receptor, HssR. Phosphorylated HssR then binds to a consensus sequence within the *hrtAB* promoter upregulating transcription of the heme efflux pump. The catalytic domains of the sensor kinases, namely the ATP-binding domain and HisKA phosphorylation domain, are highly conserved with some variation in the cytoplasmic signaling and extracytoplasmic sensor domains (Szurmant et al., 2007). Based on sequence identity, HssS shares significant homology with the homodimer sensor kinases belonging to the HAMP (for histidine kinases, adenylyl cyclases, methyl-accepting proteins, and other prokaryotic signaling proteins) domain family. The HAMP domain is thought to be involved in transducing the signal from the sensor domain through rotational changes in conformation applied through a four-helix bundle composed of two helices from each monomer (Ferris et al., 2011; Hulko et al., 2006). The structure of the heme-binding domain and detailed mechanism of activation remain to be determined.

3.2. ATP-Dependent Efflux Pumps

Heme efflux transporters have been identified in *S. aureus* (Stauff et al., 2008; Stauff & Skaar, 2009), *C. diphtheriae* (Bibb & Schmitt, 2010), and *Lactococcus lactis* (Lechardeur et al., 2012). In contrast to the gram-positive *C. diphtheriae* and *S. aureus* HrtAB efflux pumps which are regulated by membrane-associated two-component sensor kinase systems in gram-negative organisms such as *L. lactis*, the efflux system responds to intracellular heme through a transcriptional regulator, HrtR. Despite advances in the regulation of the efflux pumps, there is currently no structural details available although sequence alignment suggests that the heme efflux systems are similar to the macrolide-specific ABC transporters (MacAB) (Routh et al., 2011; Xu et al., 2009, 2010). The recent identification and characterization of heme efflux systems and their role in virulence beg further structural and mechanistic analysis as potential future antimicrobial targets. To date, heme utilization as an antimicrobial target has solely been focused on uptake and utilization (see Section 5).

4. HEME UPTAKE AND UTILIZATION IN ANTIMICROBIAL DRUG DEVELOPMENT

4.1. OM Heme Receptors as Vaccine Targets

As a result of the wealth of knowledge on the surface-exposed TonB-dependent receptors of gram-negative pathogens, they have been extensively investigated as potential vaccine targets (Rohde & Dyer, 2003; Schryvers & Stojiljkovic, 1999). The surface accessibility and role in virulence of the Hb and transferrin receptors made them early candidates for vaccine development (Afonina et al., 2006; Cornelissen, 2003). The transferrin and Hb receptors of *Haemophilus influenza*, *Neisseria meningitidis*, and *Neisseria gonorrhoeae* were the early focus of such studies (Holland et al., 1996; Lewis et al., 1999; Richardson & Stojiljkovic, 1999; Stevenson, Williams, & Griffiths, 1992; Webb & Cripps, 1999). However, a potential drawback has been the high level of redundancy and phase variation in the transferrin and heme uptake systems (Ekins, Bahrami, Sijercic, Maret, & Niven, 2004; Richardson & Stojiljkovic, 1999, 2001). For example, *N. meningitidis* encodes two Hb receptors HpuAB and HmbR that undergo phase variation controlled by a translational frameshift (Lewis et al., 1999; Richardson & Stojiljkovic, 1999) and similar phase variation has also been observed in *N. gonorrhoeae* Hb receptors (Chen, Elkins, & Sparling, 1998). In addition, phase variation allows the bacteria to adjust rapidly to the host environment allowing

specific receptors to play a greater or lesser role during infection. Despite this drawback, both *N. meningitidis* and *N. gonorrhoeae* have been the focus of vaccine development (Cornelissen, 2008). In contrast, the *N. gonorrhoeae* transferrin-binding proteins TbpA and TbpB were shown to be expressed in all clinical isolates, while showing little strain to strain variation, and do not undergo high-frequency phase variation (Cornelissen, Anderson, Boulton, & Sparling, 2000; Cornelissen, Anderson, & Sparling, 1997). These proteins have shown partial success as potential vaccine candidates in animal models of infection (Cornelissen, 2008; Price, 2007; Price et al., 2007). *Hemophilus ducreyi* has three TonB-dependent receptors of which only the Hb receptor HgbA is required for acquisition of iron during infection (Leduc et al., 2008). Initial immunization studies with HgbA in a swine model for chancroid provided some protection from infection (Afonina et al., 2006). Subsequent studies have focused on identification of the core domain essential in Hb recognition in order to develop a subunit vaccine (Nepluev et al., 2009).

In the gram-positive arena, much of the focus on vaccine development has been directed toward *S. aureus* (Clarke et al., 2006). Culturing *S. aureus* in a mouse tissue cage model versus iron-rich media identified over 54 genes, 36 of which were upregulated during infection and in iron-restricted media (Allard et al., 2006). From this initial study, six proteins were selected for further evaluation including the Hb receptor, IsdB, its coreceptor IsdH, and the sortase, SrtB, which is required in anchoring IsdC to the cell wall. From this study, IsdH appeared to be the best vaccine candidate when screened in a mouse mastitis model (Ster et al., 2010). In a separate study, the IsdA and IsdB receptors were investigated as potential vaccine targets in the prevention of abscess development in mice challenged by intravenous *S. aureus* (Kim et al., 2010). The conclusion from this study indicated that antibodies against IsdB and IsdA in part protected against staphylococcal infection by interfering with heme uptake and utilization.

The significant advances in the genomics of surface-exposed receptors have identified potential vaccine candidates from *in vivo* transcriptional profiles of the surface-exposed proteins during infection (Kurz et al., 2003). While, to date, there have been no clinical trials of human vaccine candidates as a result of such studies, it is inevitable that genomics and proteomics will provide a more sophisticated and detailed picture of the role of the OM heme receptors in colonization, infection, and disease (Clarke et al., 2006). Such studies will provide a framework for the future development of vaccine development.

4.2. Metalloporphyrin-Based Inhibitors of Heme Utilization

It has been known for decades that pathogenic bacteria are susceptible to porphyrin-based compounds (Begum, Kim, Kumar, Stojiljkovic, & Wataya, 2003; Bozja, Yi, Shafer, & Stojiljkovic, 2004; Stojiljkovic, Evavold, & Kumar, 2001). The antimicrobial activity of porphyrins can arise from their photoactive properties or by specifically targeting heme uptake systems. While there are many examples of photodynamic antimicrobial porphyrin and chlorin derivatives (Embleton, Nair, Cookson, & Wilson, 2002; Farrell, Robbins, Rhys-Williams, & Love, 2010; Huang et al., 2010; Nitzan, Balzam-Sudakevitz, & Ashkenazi, 1998), we restrict the current review to metalloporphyrins that specifically target heme uptake across the OM (Bozja et al., 2004; Stojiljkovic et al., 2001). Early studies showed that noniron metalloporphyrins have broad spectrum antibacterial activity against both gram-positive and gram-negative organisms, including *S. aureus*, *Yersinia enterocolitica*, *N. meningitidis*, and *N. gonorrhoeae* (Stojiljkovic, Kumar, & Srinivasan, 1999). A series of noniron metalloporphyrins (PPIX) were tested against a panel of bacterial pathogens with several having bacteriostatic activity (Mn-PPIX, Zn-PPIX, Ga-PPIX, and Ru-PPIX). In *Y. enterocolitica*, Ga-PPIX and Ru-PPIX at higher concentrations were shown to be bactericidal and mutants devoid of the OM receptor HemR were resistant to Ga-PPIX. In contrast, the cytoplasmic heme-dependent ABC transporter system was not required for

sensitivity. In these initial studies, it was not determined if Ga-PPIX was actively transported into the periplasm via the OM heme receptor or by passive diffusion through the membrane. Furthermore, it was not definitively shown that Ga-PPIX was present in the periplasm, largely due to inconsistencies in the recovery of extractable Ga-PPIX from the cells. However, the fact that metal-free porphyrins have no antimicrobial activity and the inhibitory effect could be alleviated with increasing heme concentrations suggests that the noniron PPIXs are competing for binding to the OM receptor. In a recent study, the oral pathogen *P. gingivalis* when treated with In-PPIX showed retarded growth, which on the basis of transcriptional analysis appeared to be due to an inhibition of nucleotide metabolism and protein processing following internalization of In-PPIX through the Iht receptor system (Yukitake et al., 2011).

A model for the antimicrobial activity of noniron porphyrins has been proposed whereby exposure to such inhibitors induces production of ROS and oxidative stress (Stojiljkovic et al., 1999, 2001). This model is based on the fact that superoxide dismutase and catalase mutants have increased sensitivity to the noniron porphyrins and that active respiration is required for the antimicrobial effects. The authors propose that this generation of ROS may be due to insertion of noniron porphyrins directly into respiratory proteins by the cytochrome assembly machinery. Although it has been shown that *E. coli* overexpressing the Hb receptor of *P. gingivalis* binds both Cu- and Zn-PPIX with similar affinity as Fe-PPIX, there remains no direct evidence for active transport of noniron porphyrins into the periplasm (Olczak, Dixon, & Genco, 2001). Despite promising early studies on the OM heme receptors as Trojan horses in delivering noniron metalloporphyrin antibacterials, most of the current focus has been directed at utilizing the photodynamic properties of light-activated Sn-chlorins as antibacterials (Embleton et al., 2002, 2005; Suci, Kang, Gmur, Douglas, & Young, 2010).

5. FUTURE PERSPECTIVES

The challenges of transporting a highly reactive and toxic cofactor such as heme across the physical barrier of the cell wall and lipid membrane require specialized systems. Our current knowledge of the mechanisms by which heme is transported has been aided greatly by the wealth of structural data on the cell wall lipoproteins and OM receptors of gram-positive and gram-negative bacteria, respectively. However, as we define the common structural motifs of heme transporters, there are significant challenges remaining, such as understanding the complex nature of TonB-coupled conformational changes in driving heme transport across the gram-negative OM and the nature of the protein–protein interactions of the cell wall-anchored lipoproteins in heme transfer within the peptidoglycan cell wall. Future studies must encompass the challenges of understanding the nature of protein–protein-driven allosteric changes that trigger ligand coordination and spin-state changes required for heme translocation. Structural determination of protein complexes combined with advanced spectroscopic studies will greatly enhance our understanding of heme uptake in bacterial pathogens. The significance of heme uptake and utilization in bacterial pathogenesis and virulence combined with a structural and mechanistic understanding of such processes may provide a platform for future antimicrobial therapeutic intervention.

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Figure 1.

Heme transport across the gram-negative OM. Heme is transported across the OM by a TonB-dependent OM receptor, shuttled to an ABC transporter by the PBP, and actively transported across the inner membrane (IM). In the cytoplasm, heme is sequestered by the cytoplasmic heme-binding protein (CHBP) for transfer to HemO. In some bacteria, such as *Serratia marcescens* and *Pseudomonas aeruginosa*, a secreted hemophore sequesters heme for transport via a hemophore-dependent OM receptor.

Figure 2. Structure of the HasA-HasR complex of *Serratia marcescens*. (A) HasA docked to the HasR through the extracellular loops. (B) Enlarged view of the heme-binding site. Heme is coordinated in HasR by His-602 from extracellular loop 7 and His-189 on the N-terminal plug. Adapted from PDB file 3CSL. See the color plate.

Figure 3. Overall structural fold of the holo- and apo-ShuT. (A) Holo-ShuT with the proximal Tyr-94 highlighted. (B) Apo-ShuT with the proximal Tyr-94 pointing outward. *Adapted from PDB file 2R7A. See the color plate.*

Figure 4.

Proposed ATP-switching model for the heme ABC transporters. (A) PBP interacts with the TMD transporter causing a conformational change. (B) Conformational changes in TMD drive ATP binding and release of the ligand to the transporter channel. (C) ATP hydrolysis then couples translocation heme to the cytoplasmic heme-binding protein (CHBP). *Adapted from Burkhard and Wilks (2008).*

Figure 5.

Heme uptake system of gram-positive organisms. (A) Schematic representation of the Isd NEAT domain proteins with the secretion signal represented by a white box. The NEAT domains are numbered from the N-terminus and the heme-binding NEAT domains are shown by the dark boxes. The sortase anchoring sequence for the respective proteins are indicated at the C-terminus. (B) Heme transfer across the cell wall (CW) and periplasmic membrane (PM). IsdB/H, IsdA, and IsdC are anchored to the cell wall and IsdD, IsdE and IsdF are the heme-binding protein and permease components. Heme transported into the cytoplasm is degraded by the IsdG/I system. *Adapted from Grigg et al. (2010).*

Figure 6. The NEAT domains of IsdA and IsdC. (A) Heme-binding site of IsdA high-lighting hydrogen bond interactions of Ser-82, Lys-75, and Tyr-170 with the heme propionates and proximal Tyr-166, respectively. (B) Heme transfer from IsdA to IsdC via the "handclasp" model. Heme slides 18 Å from one binding pocket to the other. *Adapted from PDB files 3QZO and 2O6P.* See the color plate.