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Review Article

Strategic Exploitation of Heme Acquisition by Gram-positive Bacteria from Human Host

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ABSTRACT: Iron is an essential nutrient for microorganisms that plays a vital role in pathogenesis during the course of an infection. One of the primary defense strategies of mammalian hosts against bacterial infection is to limit the availability of free extracellular iron. The largest reservoir of iron in human is heme, which is complexed with hemoglobin. Numerous high-affinity heme-scavenging pathways are employed by pathogenic bacteria to acquire iron. Mutations in these pathways often result in attenuated virulence. A limited understanding of heme uptake mechanisms is available for Gram-positive bacteria as the study of heme acquisition was restricted to Gram-negative bacteria for many years. This review summarizes some of the old and new investigations covering the uptake, transport and degradation of heme in several Gram-positive bacteria.

KEYWORDS: Iron, heme acquisition, heme degradation, Gram positive bacteria.

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INTRODUCTION

With negligible exceptions in a few bacterial species, iron is indispensable for all form of life. The metal is a cofactor required by diverse cellular processes including cell proliferation, differentiation, energy storage, nucleotide biosynthesis, gas exchange and respiration, electron transport, and peroxide reduction. Iron is also important for the function of proteins that are involved in cell signaling and gene regulation. However, this vital element can also be toxic under aerobic condition. Iron can generate reactive oxygen species (ROS) that can damage to membrane lipid, protein and DNA. Therefore careful management of the cellular levels of free iron is required in all forms of life to prevent metal toxicity [1]. Most of the iron in human is sequestered by a number of proteins including transferrin, lactoferrin, and ferritin. Furthermore, almost 75% of iron in the human body is available in the form of heme, where it is complexed to the protoporphyrin IX ring and serves as the prosthetic group of hemoglobin, myoglobin and other proteins. Moreover, hemoglobin is further sequestered in erythrocytes [2]. As a result, serum concentrations of free iron and heme are bellow 10^{-18} M [3]. This compartmentalization of iron provides two benefits to human. First, by sequestering iron it is possible to reduce iron toxicity by protecting the cell integrity from ROS. Secondly, paucity of free iron can create difficulties for pathogenic bacteria to survive inside human hosts. Therefore, to prevent microbial infections, it is important for human to compartmentalize iron so that the availability of iron can be regulated more stringently. Virulent microorganisms employ diverse strategies to overcome the nutritional immunity, as well as, to maintain iron homeostasis for avoiding ROS mediated damages. Some of the general mechanisms employed by pathogenic microorganisms to procure iron are as follows:

secretion of high-affinity iron-binding receptors to obtain iron from various exterior sources, accumulation of iron in the intracellular environment to satisfy metal requirement in iron-starved condition, production of redox stress resistance systems to destroy reactive oxygen species as well as to repair the damage induced by ROS, and expression of regulatory systems to control the overall iron utilization process [1]. Invading bacteria exert numerous mechanisms to uptake iron from a variety of external sources in human. Some pathogenic bacteria acquire iron from transferrin, lactoferrin or ferritin by secreting scavenger proteins. *Neisseria gonorrhea* can obtain ferric iron (Fe^{3+}) by synthesizing the transferrin binding receptor TbpAB and lactoferrin binding receptor LbpAB [4]. Several pathogenic bacteria utilize catecholamine stress hormones to release free iron from transferrin and lactoferrin [5]. Iron can also be obtained by secreting siderophores, which are small molecules having high affinity for ferric iron. Both Gram-positive and Gram-negative bacteria synthesize siderophores to sequester iron. Siderophores are highly diverse in terms of structural biology, as well as iron binding capacity [6]. Also, pathogenic bacteria occasionally acquire ferrous iron (Fe^{2+}) to satisfy iron requirements. The ferrous iron uptake mechanism was first reported in the non-pathogenic *E. coli* K-12 strain and this mechanism has also observed in other pathogenic bacteria. In the case of Gram-negative bacteria ferrous iron can be diffused spontaneously through the outer membrane due to its smaller size and later internalized by the ABC transporter FeoABC [7]. There are also other transporter systems available to uptake ferrous iron. A recent study has reported that, Gram-positive pathogen *Bacillus subtilis* can acquire ferrous and ferric iron by using EfeUOB transporter system [8].

Heme acquisition by bacterial pathogen

Majority of iron in human is found as heme, which is further sequestered by hemoproteins. Therefore, a number of strategies are applied by pathogenic bacteria to circumvent this nutritional immunity. Bacteria synthesize hemophores for scavenging heme from host hemoproteins. These are small extracellular proteins that can be anchored to the cell surface or secreted by distinct secretion pathways. Both Gram-positive and Gram-negative bacteria utilize hemophores to procure heme from hemoproteins and deliver it to the cell surface receptors. Other proteins are also involved to assist hemophores during heme acquisition. In some bacteria, erythrocytes lysis is done by hemolysins that lead to the release of intracellular hemoglobin in the extracellular milieu. Hemophores then bind to hemoglobin ad extract heme. Finally, heme-bound hemophores interact with specific cell surface receptors that lead to the internalization of heme into the bacterial cell by an elaborate protein-based system [9]. Different types of hemophores are synthesized by pathogenic bacteria to sequester heme from variety of heme bound molecules. HasA-type hemophores are found in some Gram-negative bacteria such as- *Serratia marcescens* (HasASM), *Pseudomonas aeruginosa* (HasAPA), and *Yersinia pestis* (HasAYP). It has been reported that, heme is acquired by HasA in an affinity-driven manner without the formation of a stable protein–protein complex [9]. HxuA-type hemophores are identified in *Haemophilus influenzae* which scavenge heme from hemopexin along with two other hemophores HxuB and HxuC [10]. Unlike HasA-type hemophores, HxuA form a tight complex with hemopexin to acquire heme [11]. Hemophores, harboring a conserved near iron transport (NEAT) domains are characterized in several Gram-positive pathogenic bacteria including *Staphylococcus aureus* [12], *Listeria monocytogenes* [13], *Bacillus anthracis* [14], *Bacillus cereus* [15], and *Streptococcus pyogenes* [16]. There are approximately 125 amino acids present in these protein motifs. NEAT domains are encoded from the genes located near ABC iron transporter genes in the chromosomes. In Gram-positive pathogen, NEAT domains are present in variable copy numbers with remarkable degree of functional diversities [17].

However, heme utilization has been studied most in the Gram-negative bacteria. Surface receptors of the outer membrane bind heme and other heme-containing proteins like hemoglobin, haptoglobin, hemopexin, and myoglobin, and mediate the internalization of heme into the periplasm. Energy required for this translocation comes from proton motive force which is carried to the outer membrane receptors by TonB. In the periplasm, periplasmic binding proteins (PBP) bind to heme and mediate the transportation of heme to the cytoplasm through inner membrane ABC transporters. In the cytoplasm, cytoplasmic binding proteins (CBP) interact with heme and deliver heme to heme degrading enzymes to release iron [18]. On the other hand, for many years it was undiscovered that how Gram-positive bacteria acquire and transport heme through the thick layer of peptidoglycan. Gram-positive cell wall is a very dynamic structure consisting of peptidoglycan network, carbohydrates & teichoic acids. A variety of cell wall anchored proteins are also present which are covalently attached to the cell wall by

sortase enzymes. Some of the Gram-positive bacteria may also contain polysaccharide capsule or crystalline protein layer (S-layer). Presence of the thick peptidoglycan layers provides extreme rigidity and scaffolding for surface proteins. All these variations in cell wall structure cause difficulties in heme uptake in Gram-positive pathogens as heme molecules are unable to diffuse through the thick cell wall unlike their Gram-negative counterpart. Nevertheless, Gram-positive bacteria have developed sophisticated heme utilization pathways to promote bacterial survival and pathogenesis. A cascade of proteins present on the cell wall mediates the heme uptake and transfer process in a much orchestrated manner. This review summarizes the past researches as well as current knowledge in different strategies employed by several Gram-positive bacterial pathogens to acquire heme from human hosts.

Heme uptake by *Staphylococcus aureus*

Staphylococcus aureus, the causative agent of major nosocomial infections in the United States, is the most intensively investigated Gram-positive pathogen for iron utilization as well as heme utilization. Heme uptake system in *S. aureus* is known as iron responsive surface determinant (Isd) system which is composed of nine proteins. The cell wall anchored surface receptors in the Isd system includes IsdA, IsdB, IsdC and IsdH (also referred to as HarA). In *S. aureus*, surface receptor proteins are anchored to the cell wall by the action of sortase, like other Gram-positive bacteria. Two sortase enzymes (SrtA and SrtB) are involved in anchoring proteins to the cell wall in this system. IsdC is attached to the peptidoglycan cross-bridge by the action of SrtB, which is encoded from the *Isd* locus, whereas, the rest of the surface receptors of the Isd system anchored by Sortase A, which is coded from outside of the *Isd* locus. Interestingly, the surface proteins of the Isd system are anchored in the cell wall at varying depths. IsdB and IsdH are completely exposed to the extracellular milieu, while IsdA is partly exposed and IsdC is completely buried within the cell wall. This arrangement of proteins directs the proposed mechanism of heme uptake from the outside environment to the intracellular environment [12]. All of the surface proteins found in Isd system in *S. aureus* harbor one to three copies of NEAT domains. In *S. aureus*, IsdB harbors two sequential NEAT domains having significant (41%) sequence homology. The first NEAT domain can bind to hemoglobin, whereas, the second domain can only interact with heme. Another surface protein, IsdH contains three NEAT domains having differential binding capacity despite of high sequence homology (38-41%). Two of the domains can bind to hemoglobin and haptoglobin but do not bind to heme, whereas, the third NEAT domain can only interact with heme [19] .

The *S. aureus* ABC transporter of Isd system is composed of a substrate-binding protein (IsdE), a membrane permease (IsdF) and an ATP hydrolase. A lipid moiety at the N-terminus end of IsdE enables this lipoprotein to anchor to the exterior side of the membrane. The membrane permease, IsdF is the homodimeric integral membrane protein. The ATPase in this ABC transporter is encoded from outside of *Isd* locus [12]. Lastly, IsdG and IsdI are the heme-degrading enzymes present in the cytoplasm that mediate the heme degradation to release iron [20]. However, IsdD, an uncharacterized protein is also present in the cytoplasmic membrane near the ABC transporter of the Isd system.

Recently a model of heme transfer in *S. aureus* has been proposed based on the kinetics of heme delivery among purified Isd surface proteins. Initially, heme has been delivered from metHb to IsdB or IsdH. A bidirectional movement of heme between IsdB-N2 and IsdH-N3 has been observed. A rapid heme transfer has been occurred from holo-IsdB (or IsdH) to apo IsdA. Then heme has been shown to transport from holo-IsdA to apo-IsdC. Finally, an efficient heme transfer from IsdC to IsdE has observed [21].

It has been reported that, the Isd system is essential for growth on heme as a sole source of iron. Moreover, it is required for complete virulence in several models of pathogenesis. Mutations in the Isd components result less ability to utilize heme, as well as reduced growth in some models of infection. In a recent study, it has been reported that, the components of Isd system are highly expressed during infection [22]. It has also been demonstrated that the human body generates a significant amount of humoral immune response against Isd components. Moreover, vaccine trials with IsdA and IsdB have shown promise in animal models [23].

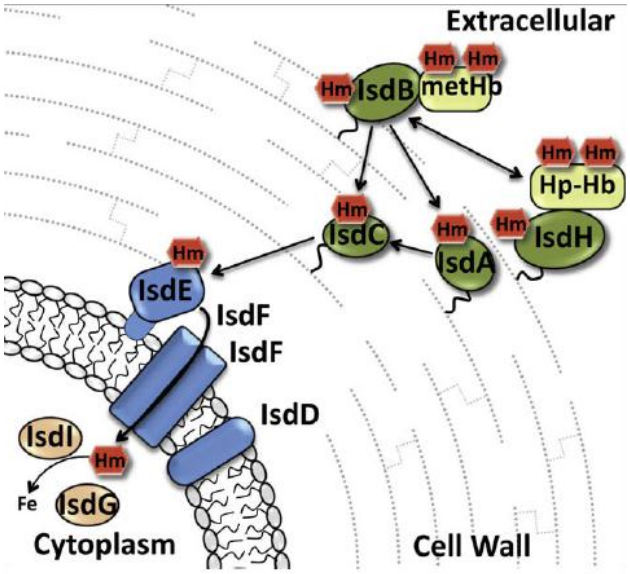


Figure 1: Proposed model for Heme acquisition in *S. aureus*; Adopted from [23]

Heme uptake by Group A Streptococcus (GAS)

Streptococcus pyogenes, also known as 'Group A Streptococcus' (GAS), can generate severe infections with diverse clinical manifestations, as well as a serious post-infection immune sequel [24]. Most frequently the upper respiratory tract or the epidermis can be affected by GAS which leads to infections including pharyngitis and impetigo. The consequences of untreated infections are rheumatic fever and acute glomerulonephritis [25]. The most severe infections caused by GAS are the invasive diseases including necrotizing fasciitis and streptococcal toxic shock syndrome which are associated with high mortality rate [26]. GAS is infamous for being highly genetically diversified pathogen, that results the transition from localized to systemic infection [24]. This common human pathogen can readily utilize hemoglobin to satisfy its iron requirements. Heme acquisition system in GAS is encoded in a 10-gene operon named as streptococcal iron acquisition (*sia*) operon. Until recently two surface proteins and a ABC transporter system encoded from this *sia* operon has showed involvement in heme acquisition in GAS. Interestingly, these two NEAT-type surface receptors, Shr and Shp are anchored to the cytoplasmic membrane instead of peptidoglycan layer unlike to other heme obtaining surface receptors in Gram-positive pathogen .

The initial player of heme uptake pathway in GAS is the streptococcal hemoprotein receptor, Shr (145 kDa protein) which is encoded from the first gene of *sia* operon. It harbors a unique N-terminal region that interacts with methemoglobin (metHb), followed by two heme-binding NEAT domains, which are alienated by a leucine-rich repeat segment [17]. A short hydrophobic tail present in the C-terminus of Shr, anchor this protein into the cell membrane and then by crossing the peptidoglycan layer Shr can be exposed on the streptococcal surface [28]. In addition to associate with hemoglobin, Shr can also bind to fibronectin and laminin, as well as, enable the attachment of streptococcal cells to the epithelial surface. Mutations in Shr exhibit attenuated virulence in murine models and decreased growth in blood [29]. Moreover, it has also been reported that, Shr can induce immunity against GAS infections in both passive and active vaccination models [30]. In a recent study it has been found that, one of the NEAT domains of Shr (NEAT2) is able to bind to the extracellular matrix components. Moreover, NEAT2 domain has the capacity to reduce iron, whereas, NEAT1 is only involved in heme acquisition. Interestingly, the axial ligand or the accessory tyrosine residues in NEAT domains are missing in GAS unlike to Isd super-family [16]. This phenomena (missing tyrosine residue in heme binding pocket) has also observed in the NEAT domain of HalA from *B. anthracis* [31].

After binding of metHB through the NTD of Shr, heme is extracted and then transferred to a NEAT-like element in Shp. It is another surface protein encoded from the second gene of *sia* operon in GAS. Shp harbors a beta-sandwich fold that is analogous to that of NEAT domains and therefore, considered a distal member of the NEAT family [17]. It has been reported that, Shp can interact with heme at the cell surface and deliver heme to SiaA (HtsA), the lipoprotein component of the ABC transporter [32].

Kinetic studies of heme delivery mechanism of GAS revealed that, the rate of heme delivery from Holo-Shr to Apo-Shp varies between two NEAT domains of Shr protein. A rapid release of heme is observed from holoNEAT1 to apoShp, whereas, heme liberation is slight and slow from HoloNEAT2 to apoShp. Until recently this is the first document of differential heme transfer between two NEAT domains of the same receptor. Moreover, heme can also be transferred between two NEAT domains of Shr protein in a quick manner depending on the concentration of the heme. In presence of high concentration of heme, holo-NEAT1 rapidly relay heme to Shp and also deliver heme to apo-NEAT 2 to accumulate. On the other hand, in low heme concentration stored heme is transferred back from NEAT2 to NEAT1 and then follow the similar pathway [33]. Finally a direct, rapid, and affinity-driven mechanism of heme transfer is mediated from Shp to SiaA [34].

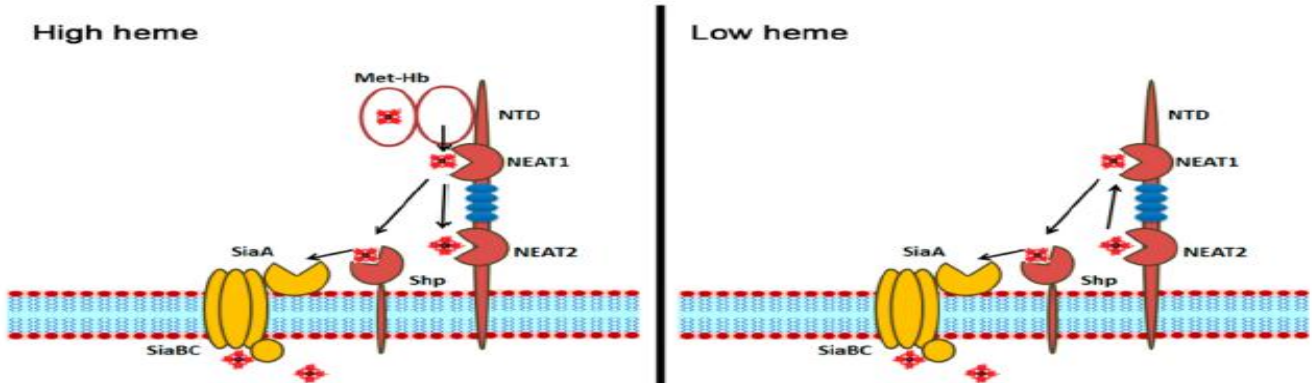
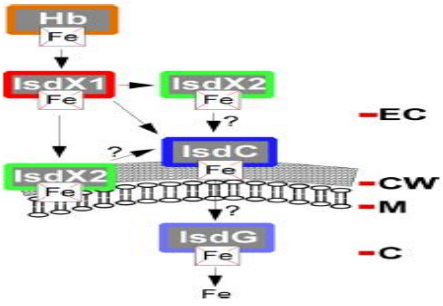


Figure 2: Proposed model for Heme acquisition in GAS**;** Adopted from [33]

Heme uptake by Bacillus anthracis

Bacillus anthracis is a good model organism to study heme acquisition in gram positive bacteria, as it can replicate in the host blood rapidly and proficiently. This anthrax causing organism contains homolog of the isd (iron-regulated surface determinants) locus to acquire heme from human hosts. Eight open reading frames are identified in the isd locus of Bacillus anthracis. Proteins that are expressed form the isd locus including sortase B (srtB), IsdC (a NEAT domain protein), IsdE1-IsdE2-IsdF (ABC membrane transporter), IsdG (heme mono-oxygenase), and, IsdX1 and IsdX2 (extracellular NEAT domain hemophores). However, IsdX1 and IsdX2 are secreted in the extracellular environment to scavenge heme from hemoglobin [35] and then deliver heme to the cell wall-anchored protein IsdC by a contact-dependent manner [36]. Variations in the number of NEAT domains, as well as, in the direction of heme transfer have been observed in IsdX1 and IsdX2.



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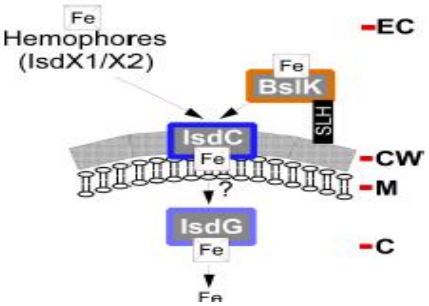


Adopted from [36]

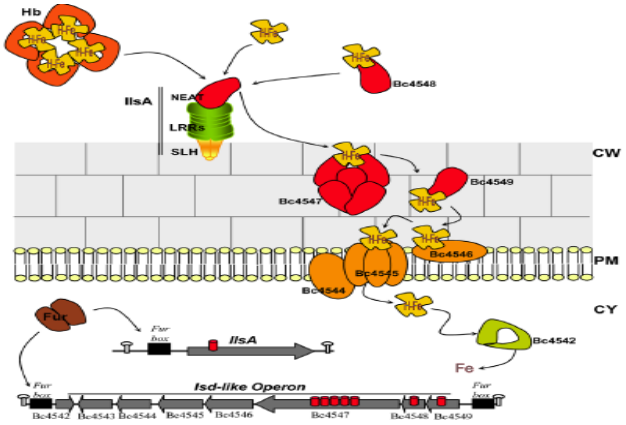
Figure 3: Proposed model for Heme acquisition in *B. anthracis*;

IsdX2 harbors five NEAT domains, whereas IsdX1 contains a single NEAT domain. Found that, all the NEAT domains in IsdX2 (except domain-2) can bind heme, while domain 5 exhibits the highest affinity for binding. However, only NEAT 1 and 5 can extract heme from hemoglobin, then NEAT 3 and 4 transfer that heme to IsdC. Interestingly, IsdX2 can also receive heme from IsdX1, indicating that IsdX2 may have other unrecognized role besides heme transfer to IsdC [37]. IsdC can also receive heme from multiple sources. A S-layer homology (SLH) protein, harboring a NEAT domain, can directly deliver heme to IsdC by a contact-dependent manner. Acquisition of heme from multiple sources reflects a functional cross talk among different NEAT proteins, possibly to facilitate heme acquisition during infection [38].

Heme uptake by Bacillus cereus

Bacillus cereus is a gram-positive, spore-forming, human opportunistic pathogen which is associated with food poisoning because of the production of diarrheal and emetic toxins [15]. Occasionally it can also cause non-gastrointestinal infections such as meningitis, pneumonia, endophthalmitis or gas gangrene like cutaneous infections [39]. Like other pathogens, B. cereus also require iron to survive and produce disease inside the human hosts. Several sources of host iron can be utilized by B. cereus to satisfy its iron requirement including hemoglobin and heme. A surface protein, IlsA has been identified in this pathogen, which is found to be essential for iron acquisition from hemoglobin and heme, as well as important for virulence. IlsA is composed of an exclusive combination of three conserved domains- an N-terminal NEAT domain, followed by thirteen Leucine Rich Repeat (LRR) domains, and three C-terminal S-Layer Homology (SLH) domains. The unique LRR domains

contribute more complexity to IlsA protein. Until recently, only a couple of LRR-containing proteins have been characterized in Gram-positive bacteria. Heme acquiring strategy in B. cereus varies from other closely related pathogen due to the presence of LLR and SLH containing NEAT protein, IlsA. Based on an in silico analysis of B. cereus ATCC 14579 strain, several NEAT domain proteins have been characterized and a model has been proposed to elucidate heme acquisition from hemoglobin. According to this putative model, cell wall anchored IlsA scavenges heme directly from hemoglobin. IlsA can also interact with other NEAT domain containing secreted hemophores to optimize heme uptake, as these hemophores can bind heme and deliver to IlsA. Then acquired heme is transferred to the cell wall anchored proteins, from where heme is conveyed to the lipoprotein receptor of a membrane permease system. Finally after internalization of heme into the cytoplasm by the transporter, a putative mono-oxygenase mediate the degradation of heme to release iron in the cytoplasm [15]. Gram-positive pathogens do not have many pathways to utilize ferritin, although it can be considered as the gold mine of iron source for pathogen inside the human hosts. It has found that, the surface protein of Bacillus cereus, IlsA has the capacity to bind to host ferritin. Association of a NEAT domain protein with ferritin for iron acquisition has been demonstrated for the first time [15]. A recent study done by Segond et al. (2014) has confirmed that IlsA is a ferritin receptor and can aggregate ferritin on the surface of bacterial cell. Moreover, in presence of IlsA protein, a significant amount of iron mobilization has observed from ferritin in that same study; which demonstrates the capacity of a bacterial protein to amend the stability of the ferritin iron core. Ferritin uptake mechanism by IlsA has not been elucidated yet. It has been speculated that, destabilization of the ferritin core is achieved by a possible interaction between ferritin and IlsA LRR domain.



Adopted from [15]

Figure 4: Proposed model for Heme acquisition in B. cereus

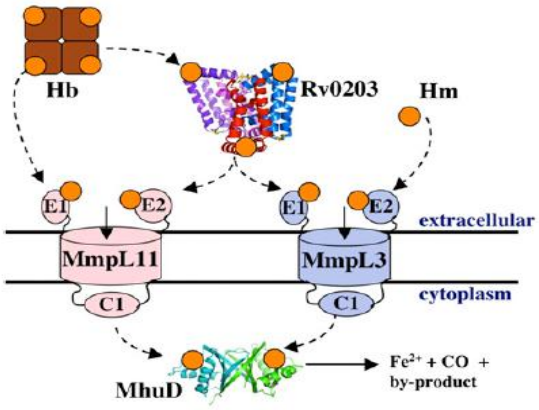
Heme uptake by Listeria monocytogenes

L. monocytogenes, a saprophytic as well as intracellular gram positive parasite require iron not only during infection inside the hosts, but also to survive in many diverse environmental conditions [40]. L. monocytogenes employ different mechanisms to obtain iron from host. A detailed study of iron acquisition sytems in L. monocytogenes was done by Jin et al. (2006) [13] (Jin, et al., 2006) for the first time. In L. monocytogenes, two Fur-regulated regions- srtB region and hupDGC region are responsible for heme uptake. Mutations at hupDGC locus diminish acquisition of heme. Moreover, deletion of hupC (ATP binding membrane permease) abolishes heme uptake as well as, reduce the virulence in mice models. A recent study done by Xiao et al. (2011) has reported that Listeria monocytogenes utilize both Sortase independent and dependent systems for acquisition of heme. One of the Fur-regulated heme acquiring system- srtB region encodes sortase-anchored proteins, a putative ABC transporter and hemophore-Hbp2. Interestingly, heme acquisition through this system is mediated depending on the concentration of heme in the extracellular environment. At low heme concentration (< 50 nM), SrtB-dependent peptidoglycan-anchored proteins (e.g. Hbp2) can bind to heme and then HupDGC complete the uptake process. However, at higher heme concentrations, heme acquisition is sortase independent. At higher heme concentration, HupDGC bind to the heme directly without taking any assistance from Hbp2 and then internalize it.

Heme uptake by Mycobacterium tuberculosis

In present days, Mycobacterium tuberculosis is being infamous for infecting and killing a large number of populations worldwide. Therefore, it is very critical to understand the pathogenesis of this successful human pathogen to come up with effective therapeutic drugs. Mycobacterium

tuberculosis is able to grow and multiply within the macrophage. M. tuberculosis can utilize both non-heme and heme iron sources from human hosts. It is possible for M. tuberculosis to come across with heme or hemoglobin in the intracellular environment when Mtb is inside the macrophage, or in the extracellular environment while it is transmitting from one site to another site of infection through blood stream [41]. Therefore, it has been speculated for long time that there should be an unrecognized heme utilizing pathway for Mtb pathogen as it can encounter heme very frequently inside the human host. Finally, a heme acquisition pathway in M. tuberculosis has been demonstrated by Tullius et al. (2011) where heme uptake is mediated by three proteins. A mycobacterial-specific heme-binding protein, Rv0203 can bind to free heme or hemoglobin. Then it can deliver heme to trans-membrane heme transfer proteins MmpL3 or MmpL11. These proteins belong to a protein family of 13 members, named as 'Mycobacterial membrane protein Large' (MmpL). Both of these trans-membrane heme transfer proteins contain two extracellular domains (E1 and E2), and one intracellular domain C1. Heme binding affinities vary between two extracellular domains of both MmpL3 and MmpL11 [42]. ATP is suggested as one of the potential energy source in this system. The crystallography of tetrameric Rv0203 discloses an exclusive fold with an atypical self-association and suggests that Rv0203 is an extracellular heme binding protein [43]. Interestingly, despite having no sequence or structural alikeness, Rv0203 harbors a similar heme-binding motif (Tyr59, His63, and His89) to that of S. marcescens hemophore HasA. This phenomenon is a document of convergent evolution of two unrelated organisms [44]. The outcome of point mutations in these three residues (Tyr59, His63, and His89) of heme binding motif suggests that Tyr59 is the critical player for the association of Rv0203 to heme in the heme-binding motif [43]. It has been reported that the rate of passive heme dissociation from Rv0203 is significantly lower than the active heme transferring rate from Rv0203 to MmpL3-E1 and MmpL11-E1 domains. However, the same study has proposed a two step heme-binding mechanism depending on the oligomerization of the extracellular domains. It has been reported that heme transfer happens very quickly and proficiently when holo- Rv0203 deliver heme to oligomerize E1 domains, whereas a slow phase of heme transfer has been observed when holo-Rv0203 transfer heme to a single E1 domain, which is telling an event of inter-action driven mechanism [42].



Adopted from [42]

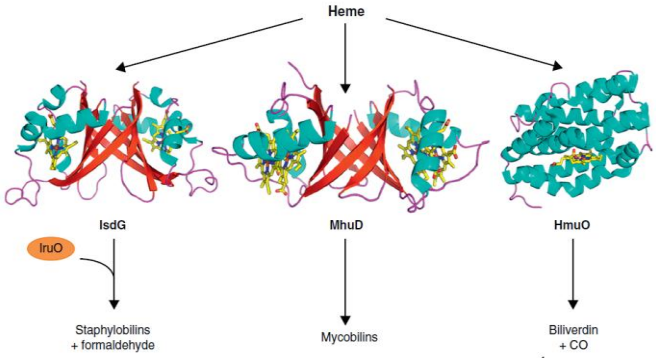
Figure 5: Proposed model for Heme acquisition in M. tuberculosis;

Bacterial cytosolic heme degradation

The final step of heme acquisition is the oxidative degradation of heme that results the liberation of iron by an enzymatic reaction in the cytoplasm. The responsible enzyme is named as 'Heme Oxygenase' (HO). Heme degradation in mammal is mediated by a conventional cytosolic heme oxygenase that leads to the release of iron, biliverdin and CO. The required electrons in this enzymatic process are provided by NADPH [45]. The first HO in bacteria was isolated from Corynebacteria diptheriae (HmuO) that showed significant homology to the canonical HOs. Similar catalytic intermediates are released by HmuO like conventional HOs. Also, in the initial oxygenation steps, the characteristics of the active forms are similar between HmuO and the mammalian HOs. However, there is variation in the heme pocket structure between these two kinds of HOs [46]. Previously it was speculated that, all kinds of life forms from human to bacteria share the heme oxygenase enzyme from same family. However discovery of a novel family of heme degrading enzymes in Staphylococcus aureus (belong to Isd system) refute that thought. Two components of Isd system in S. aureus- IsdG and IsdI are able to degrade heme [20]. There are significant structural dissimilarities in these newly characterized heme degrading enzymes with the conventional HOs enzymes. The conventional Heme oxygenase enzymes are monomeric alfa-helical proteins, whereas, Isd family enzymes are homodimers with a beta barrel formation at the dimer interface. Each monomer is able to associate with one heme molecule at the hydrophobic cleft. A catalytic triad composed of asparagine, tryptophan and histidine (Asn, Trp, His) is found at

the active site of Isd family enzymes. Also the ordered hydrogen bonding network is missing in this Isd family enzymes. The hallmark feature of this Isd-type enzymes is having the nonplanar (ruffled) heme at the active site. A significant distortion (approximately 2 Å out of plane) of the heme molecule has been observed which results the alteration in the O_2 activation chemistry on the heme molecule. As a result, instead of biliverdin, novel catalytic products are produced such as staphylobilin for S. aureus and mycobilin for M. tuberculosis. Moreover, Isd type enzymes do not produce CO, instead they produce formaldehyde. In S. aureus, genes for IsdG and IsdI remain in cluster in the Isd locus and show nearly 64% sequence homology. Both of these heme degrading enzymes are vital for heme utilization, as well as, for complete virulence in S. aureus. Participation of two enzymes in heme degradation is proposing that these are not functionally redundant enzymes. However, deviation in regulation has been reported for these two enzymes. It is found that both IsdG and IsdI are regulated by iron in a Fur-dependent manner transcriptionally, while an additional post-transcriptional regulation is observed in case of IsdG by heme. This phenomenon is possibly to fine-tune the expression of HO in a particular environment by adding an extra layer of regulation [22]. Recently this regulation mechanism has been studied in detail and reported that, when S. aureus encounter an iron-starved environment devoid of heme, it regulates the HO activity by minimizing the expression of HO. Particularly, degradation of IsdG is mediated by targeting the enzyme in a novel ATP-dependent proteolytic pathway. Moreover, this targeted degradation is accomplished by an amino acid motif located within the primary sequence, which is unique as most of the additional sequences are located either N or C terminus [47]. Very recently, an oxidoreductase (designated as iron utilization oxidoreductase- IruO) has been identified in S. aureus that can deliver electrons to IsdI and IsdG for heme degradation. The detail mechanism of the interaction between IruO and IsdG or IsdI is yet to know [48].

Homologue of this heme degrading Isd family enzymes are also recognized in other bacteria including Bacillus anthracis, Listeria monocytogenes and M. tuberculosis [49]. Mycobacterial enzyme, MhuD has comparatively high sequence homology to S. aureus HOs-IsdG and IsdI (46 and 43% sequence homology, respectively). However, structural analysis revealed that, MhuD can accommodate two heme molecules at the active site, whereas, the other Isd family enzymes as well as canonical HOs, can accommodate only one heme molecule at the active site. Interestingly, di-heme complex containing MhuD is unable to degrade heme and exhibits an inert state. The significance of di-heme molecule in MhuD is yet to discover. Another unique feature of MhuD is, production of different byproduct upon heme degradation. A chromophore named as 'Mycobilin' is produced by MhuD without the release of any CO. Until recently, MhuD is the first characterized heme degrading enzyme, that produce no CO as a result of the retaining aldehyde at the site of ring cleavage upon degradation [50].



Adopted from [51]

Figure 6: Heme degradation by different heme oxygenase;

Concluding remarks

The field of heme acquisition and utilization by Gram-positive bacteria is flourishing at an amazing rate. Characterizations of the key players involved in heme uptake pathways enable us to understand more profoundly the virulence factors as well as pathogenesis of these organisms. As a result, novel ideas are coming out to develop antibiotics against these Gram-positive pathogens by using the heme utilization pathways. Streptococcal hemoprotein Shr is one such kind which proves it's efficiency as a virulent factor (Dahesh et al., 2012). Also, in another study, it has been reported that Shr can be utilized in vaccination [29, 30]. MmpL3 in Mtb has found to be a potent target for several anti-mycobacterial compounds in a recent drug-discovery study [52]. Presently, developments of porphyrin-based therapeutic drugs are in the spot light. These metalloporphyrin-based derivatives are able to target bacterial heme-uptake pathways. Therefore, these drugs can be utilized to control the microbial infections by interfering with the pathways. Very recently it has been revealed that, gallium, a structurally analogous compound to iron(III), can be a prospective anti-microbial agent as it can bind to any complex that binds Fe(III) [53]. Although a number of novel components in

the heme uptake pathways in Gram-positive bacteria are being invented and characterized these days; there are several undiscovered vital issues needs to discover. For example, it is not clear, how these heme utilizing systems sense heme in the human hosts and how heme is acquired from hemoglobin or other heme containing sources? Further, how exactly the cascade of proteins anchored in the peptidoglycan layer, involve in the delivery of heme from outside to inside of the cell. In depth characterization of this heme utilizing pathways, in light of structural and mechanistic point of view, can generate a platform to develop novel antimicrobial therapeutic interventions.

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