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Mechanisms of iron import in anthrax

Erin Sarah Honsa · Anthony William Maresso

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Abstract During an infection, bacterial pathogens must acquire iron from the host to survive. However, free iron is sequestered in host proteins, which presents a barrier to iron-dependent bacterial replication. In response, pathogens have developed mechanisms to acquire iron from the host during infection. Interestingly, a significant portion of the iron pool is sequestered within heme, which is further bound to host proteins such as hemoglobin. The copious amount of heme–iron makes hemoglobin an ideal molecule for targeted iron uptake during infection. While the study of heme acquisition is well represented in Gram-negative bacteria, the systems and mechanism of heme uptake in Gram-positive bacteria has only recently been investigated. *Bacillus anthracis*, the causative agent of anthrax disease, represents an excellent model organism to study iron acquisition processes owing to a multifaceted lifecycle consisting of intra- and extracellular phases and a tremendous replicative potential upon infection. This review provides an in depth description of the current knowledge of *B. anthracis* iron acquisition and applies these findings to a general understanding of how pathogenic Gram-positive bacteria transport this critical nutrient during infection.

E. S. Honsa · A. W. Maresso (✉)
Department of Molecular Virology and Microbiology,
Baylor College of Medicine, One Baylor Plaza, Houston,
TX 77030, USA
e-mail: maresso@bcm.edu

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Introduction

The bacterial necessity for iron

Host iron is required for essential bacterial processes including DNA replication and respiration (Crosa et al. 2004). Ironically, free iron is insoluble and toxic to biological cells; therefore mammalian hosts sequester iron in both intracellular and extracellular proteins with high affinity. Bacterial pathogens have evolved two general systems to assimilate host iron. The first system utilizes low-molecular weight molecules termed siderophores to acquire iron from circulating proteins such as transferrin. After their secretion into the surrounding milieu, siderophores bind ferric iron with high affinity and are subsequently bound to the bacterial surface for iron import. This functionality represents a substantial strategy to attain molecular iron.

However, upwards of 80% of mammalian iron [as Fe(II)] is bound to iron-protoporphyrin IX, or heme (Fig. 1). Heme is further confined within hemoproteins such as the oxygen-carrier protein hemoglobin (Hb), which in turn is compartmentalized within red blood cells. The current model for heme uptake consists of four events: (1) erythrocytes are lysed by bacterial enzymes, allowing exposure of Hb, (2)

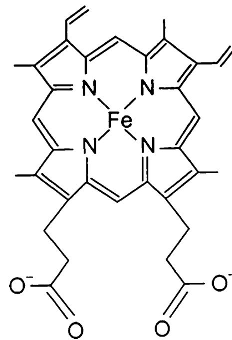

Fig. 1 Structure of the iron-porphyrin IX (heme) coordinating a single ferrous iron atom

secreted hemophores or cell surface protein receptors scavenge heme from host Hb, (3) extracted heme is transferred into the cytosol by transporters in the bacterial envelope, and (4) iron is liberated from the heme porphyrin by the action of bacterial heme monooxygenases in the cytoplasm.

Gram-negative heme acquisition systems have been studied in great detail and several excellent reviews are available (Crosa et al. 2004; Wandersman and Delepelaire 2004; Letoffe et al. 2004, Cescau et al. 2007; Tong and Guo 2009). Such systems have been described in pathogens such as *Escherichia coli* (Otto et al. 2005, Suits et al. 2006; Suits et al. 2009; Hagan and Mobley 2009); *Vibrio cholerae* (Mey and Payne 2001; Wyckoff et al. 2004; Wyckoff et al. 2006; Wyckoff et al. 2007); *Shigella* (Mills and Payne 1995; Mills and Payne 1997; Wyckoff et al. 2005); *Pseudomonas aeruginosa* (Vasil and Ochsner 1999; Tong and Guo 2007; Vasil 2007; Yukl et al. 2010; Jepkorir et al. 2010; Cornelis 2010); *Serratia marcescens* (Huche et al. 2006; Izadi-Pruneyre et al. 2006; Czjzek et al. 2007; Benevides-Matos et al. 2008; Letoffe et al. 2008; Caillet-Saguy et al. 2008); *Yersinia pestis* (Thompson et al. 1999; Rossi et al. 2001; Perry et al. 2003; Mattle et al. 2010) and *Legionella pneumophila* (O'Connell et al. 1996; Pope et al. 1996). Heme uptake in Gram-positive bacteria is less well studied. Unlike Gram-negatives, Gram-positive microbes contain a thick cell wall and no outer membrane, thereby implying the mechanisms of heme transport are distinct from their Gram-negative counterparts. To overcome this structural obstacle, Gram-positive bacteria often covalently attach proteins to the surface peptidoglycan, with the purpose of channeling molecules, including heme, through the cell wall (Mazmanian et al. 2003; Maresso and Schneewind 2006).

Recent studies on *Staphylococcus aureus* and *Bacillus anthracis* have detailed an iron-regulated surface determinant (Isd) network that utilizes bacterial "hemoprotein-receptors" on the cell surface to actively scavenge heme from host Hb or haptoglobin. This network of proteins delivers heme to cell membrane ABC transporters via a specialized protein module, the NEAr-iron transporter (NEAT) domain. Crystal structures of NEAT-containing proteins has allowed a basic understanding of NEAT function but their exact role during infection and their mechanism of action remains to be elucidated. The goal of this review is to provide an integrated analysis of NEAT protein function in the context of a bacterial infection with special emphasis on the development of mechanistic models that maybe be tested or validated for the development of antinfective strategies.

B. anthracis: a model organism to study Gram-positive iron acquisition

Bacillus anthracis is a Gram-positive pathogen with a fascinating, dynamic infectious lifecycle. During intra-and extracellular replication, the bacteria must utilize efficient iron-acquisition systems to chelate and scavenge iron from the host. This "developmental infection", in combination with distinct cell envelope architecture that includes a cell wall and possibly a crystalline S-layer (Fouet et al. 1999; Mesnage et al. 1999a; Mesnage et al. 1999b; Couture-Tosi et al. 2002; Candela et al. 2005); makes the study of iron-uptake in this pathogen significant. Table 1 lists the *B. anthracis* genes whose protein products are implicated in the acquisition and utilization of iron. The abundance and diversity of these genes suggests that the attainment of iron is an important process during *B. anthracis* replication within a host. Characterizing the iron acquisition systems of *B. anthracis* will help us understand the inner workings of this pathogen, lead to the development of antiinfectives, and provide a reference point for understanding iron uptake in related Gram-positive pathogens.

B. anthracis **pathogenesis and dynamic lifecycle**

Bacillus anthracis is a Gram-positive, spore-forming *Bacillus* and the causative agent of anthrax. The spores of *B. anthracis* have been reported to act as a potential

Table 1 *Bacillus anthracis* genes implicated in the acquisition and utilization of iron

Gene(s)	Cellular localization	Protein product function	Reference(s)
isdC	Cell wall-anchored	Accept heme from IsdXI transfer heme to IsdE (?)	Maresso and Schneewind (2006), Maresso et al. (2006)
isdX1	Secreted	Scavenge heme from Hb transfer heme to lsdX2 and IsdC	Maresso et al. (2008); Fabian et al. (2009)
isdX2	Secreted/cell wall-associated	Scavenge heme from Hb accept heme from IsdXI	Maresso et al. (2008); Fabian et al. (2009)
isdEFD	Cell membrane	Putative heme ABC transporter	Maresso and Schneewind (2006)
isdG	Cytosol	Heme-degrading monooxygenase	Skaar et al. (2006)
bas0520	Unknown	Unknown, proposed heme-binding protein	Carlson et al. (2009)
bslK	Cell surface-associated	Heme-binding protein, transfer heme to IsdC	Tarlovsky et al. (2010)
asbABSDEF	Secreted/cytosol	Petrobactin (siderophore) synthesis	Koppisch et al. (2005), Pfleger et al. (2008)
bacACEBF	Secreted/cytosol	Bacillibactin (siderophore) synthesis	Cendrowski et al. (2004); Wilson et al. (2010)
fpuA	Cell membrane	Proposed cell surface receptor for petrobactin	Zawadzka et al. (2009a, b), Carlson et al. (2009, 2010)
fur	Cytosol	Transcriptional regulator that represses iron-acquisition genes in high iron environments	Maresso et al. (2006), Skaar et al. (2006), Gat et al. (2008)

? not known

biological weapon; therefore this pathogen is a serious public health concern (Inglesby et al. 2002). Anthrax manifests in three pathologies which is determined by the site of inoculation of the *B. anthracis* spores (Ross 1955; Abramova and Grinberg 1993). After initial entry into the host, *B. anthracis* spores are phagocytosed by resident macrophages or dendritic cells, as recently proposed by Shetron-Rama et al. (2010), wherein they germinate intracellularly en route to regional lymph nodes (Guidi-Rontani et al. 1999). The metabolically active *Bacillus* undergoes outgrowth: rapid replication inside the phagocyte, virulence factor expression and subversion of immune activity (Ross 1955; Dixon et al. 2000). This intracellular part of the lifecycle represents the first phase in which *B. anthracis* must employ iron-acquisition strategies. Ultimately, vegetative cells escape the macrophage and replicate in the circulatory and lymphatic systems of the host, rapidly reaching cell densities of 10^{10} CFU/mL blood (Mock and Fouet 2001).

During replication in the extracellular environment, *B. anthracis* secrets anthyrolysin O: a cholesterol-dependent cytolysin capable of lysing erythrocytes (Shannon et al. 2003; Klichko et al. 2003; Popova et al. 2006). This hemolysis function of anthrolysin O may be responsible for the release of Hb into the blood stream during systemic anthrax infection. It is proposed that *B. anthracis* utilizes heme–iron stores during its extracellular lifecycle by secreting specialized hemophores to assimilate heme from host Hb. Therefore, the initial intracellular niche in which *B. anthracis* begins its infections lifecycle represents the first stage of iron acquisition for survival and replication.

Siderophore-mediated iron acquisition in *B. anthracis*: a role in spore outgrowth/intracellular replication?

Virtually all bacterial pathogens encode operons whose gene products are responsible for the synthesis, processing and secretion of low-molecular weight siderophores. These molecules have a high affinity for ferric iron and are secreted via cell membrane ATP-binging cassette (ABC) transporters (Ratledge and Dover 2000; Beasley and Heinrichs 2010). By utilizing a highly electronegative environment, siderophores form a hexacoordinated complex around ferric iron chelated from host proteins such as transferrin

(Wandersman and Delepelaire 2004). The iron-bound (holo) siderophore interacts with the bacterial surface and is then transported into the cytoplasm. Ultimately, the iron is liberated from the siderophore, where it is incorporated into metabolic pathways within the bacterial cell. For an excellent overview of siderophore function, see Crosa and Walsh 2002; Faraldo-Gomez and Sansom 2003.

It is proposed that *B. anthracis* require iron at every stage of the infectious lifecycle. After spore uptake, the vegetative cells are compartmentalized within the macrophage. Interestingly, studies have shown that petrobactin, an unusual 3,4-dihydroxybenzoate catecholate siderophore, is essential for *B. anthracis* growth within mouse macrophages. The inability of petrobactin synthesis-deficient cells to attain iron from the macrophage cytosol may explain the reduction in virulence observed in a mouse Sterne model of inhalational anthrax (Cendrowski et al. 2004; Garner et al. 2004; Abergel et al. 2008). In addition to petrobactin, *B. anthracis* also secretes bacillibactin. The genes responsible for the biosynthesis of bacillibactin and petrobactin are encoded by the *bacACEBF* and *asbABCDEF* loci, respectively (Koppisch et al. 2005; Lee et al. 2007; Pfleger et al. 2008). Unlike petrobactin, bacillibactin may be expendable for anthrax survival, as the deletion of the *bac* operon did not result in attenuation in the mouse inhalational model (Cendrowski et al. 2004). Recent studies have suggested that the *B. anthracis* FpuA protein is a putative cell surface receptor for the petrobactin-iron complex. Severe outgrowth defects of *ΔfpuA* spores were observed in a murine inhalation model (Carlson et al. 2010). Additionally, the LD_{50} of these spores was more than three orders of magnitude above that for wild-type spores. Furthermore, recombinant FpuA protein specifically binds petrobactin in vitro (Zawadzka et al. 2009a; 2009b). Interestingly, studies by Abergel et al. 2006 elucidated that due to the unusual structure of petrobactin, it is capable of evading siderocalin, an innate immune molecule that binds bacterial siderophores. Analysis of the temporal production of petrobactin revealed that its synthesis begins within 5.5 h of germination, correlating with siderophore production upon germination in a phagocyte (Wilson et al. 2010). These data suggest that petrobactin may be the initial iron-scavenging molecule produced by *B. anthracis* when compartmentalized within host phagocytes.

Additionally, petrobactin and bacillibactin have a high affinity for iron, and Abergel et al. 2008 have suggested that transferrin is a host source of iron for *B. anthracis* siderophores. Since transferrin is found within mammalian macrophages, it is possible petrobactin gains access to the iron-atom by exploiting this iron source during the intracellular lifecycle (Knutson and Wessling-Resnick 2003). Of note, is the lack of characterization of ferrous iron transporters in *B. anthracis*. While homologues to the *feoB* gene from several Gram-negative pathogens exist in the Ames strain *B. anthracis*, these systems remain unstudied. Additionally, due to the differences in cell envelope architecture between Gram-positive bacteria and their Gram-negative counterparts, it is not known if the Feo system or other ferrous iron uptake transporters would function in the same context as has been previously reported for pathogens such as *E. coli* and *V. cholera* (Hantke 1987; Kammler et al. 1993; Hantke 2003; Wyckoff et al. 2006 ; Mey et al. 2008).

Together, these data suggest a model whereby *B. anthracis* produces an immune-evading siderophore soon after germination to scavenge host iron for the subsequent outgrowth and replication of vegetative cells. Once the bacilli have reached the extracellular systemic phase of their infection, *Bacilli* may then switch their iron-scavenging arsenal to target the largest extracellular iron pool: heme–iron.

Targeting heme as an iron source during the extracellular phase of anthrax infection

Both Gram-positive and Gram-negative bacteria have developed systems to acquire and utilize heme as an iron source during infection. In Gram-positive bacteria, a major mediator of heme uptake is the Isd system. First discovered in *S. aureus*, this system is a network of surface-localized proteins that may acquire heme from the host during infection (Mazmanian et al. 2002; Mazmanian et al. 2003; Maresso and Schneewind 2006). Several proteins encoded within this locus possess one or more NEAT domains, a conserved protein module present in all major Gram-positive pathogenic genera (Andrade et al. 2002). The Isd system of *S. aureus* has several key features: (1) cell-wall anchored NEAT proteins that may be heme receptors at the cell surface, (2) an

ABC-transport complex that utilizes the energy from hydrolyzed ATP to power heme import through the membrane, and (3) a cytosolic monooxygenase that oxidatively degrades heme to liberate iron. (Mazmanian et al. 2003; Dryla et al. 2003; Maresso et al. 2006; Torres et al. 2006; Pilpa et al. 2006; Dryla et al. 2007; Pilpa et al. 2009; Pishchany et al. 2009).

B. anthracis utilizes a unique Isd-like functional network for heme acquisition

While siderophores may be important for acquiring iron during the intracellular phase of the *B. anthracis* lifecycle, their mechanism of action does not explain the potential exploitation of the largest iron pool within the host, heme–iron. A search for genes that may participate in heme uptake in *B. anthracis* led to the discovery of an eight gene Isd-like locus (Maresso et al. 2006). This system is located in a single genomic region on the chromosome with *isdC-X1* × *2-EFD-srtB* expressed as one transcript, and *srtB* and *isdG* each capable of being independently transcribed (Fig. 2), (Maresso et al. 2006; Skaar et al. 2006; Gat et al. 2008). Each promoter contains a Fur box, upon which a Fur repressor binds and represses transcription in high-iron conditions. For a detailed review on Fur-mediated transcriptional repression, see Carpenter et al. 2009.

Several studies have provided support that the Isd-like system in *B. anthracis* is involved in heme acquisition and utilization during infection (Skaar et al. 2006; Maresso et al. 2008; Fabian et al. 2009). Interestingly, the *B. anthracis* Isd network possesses no genes encoding cell wall-anchored hemoprotein receptors homologous to those in the *S. aureus* system. Instead, two proteins, IsdX1 and IsdX2, which lack a cell-wall anchoring motif but contain N-terminal signal peptides, are secreted by *B. anthracis* under iron-limiting conditions (Fig. 3), (Maresso et al. 2009), suggesting IsdX1 and IsdX2 are hemophores. Although hemophores have been described in Gram-negative bacteria, there were no descriptions of an equivalent protein in a Gram-positive species.

Analysis of purified, recombinant IsdX1 and IsdX2 demonstrated they are heme-binding proteins (Maresso et al. 2008). However, since heme is not readily free in mammalian hosts, it was tested if these proteins could utilize Hb has a heme source. Indeed, surface plasmon resonance (SPR) analysis provided evidence that IsdX1 physically interacts with holo-Hb in a transient manner and triggers the release and subsequent binding of heme (Maresso et al. 2008). The exact mechanism IsdX1 employs to physically extract heme from Hb remains to be fully characterized. Since IsdX1 is secreted during a systemic anthrax infection, it can be hypothesized that the protein encounters Hb from lysed erythrocytes, further supporting the idea that IsdX1 steals heme during extracellular growth (Maresso et al. 2008).

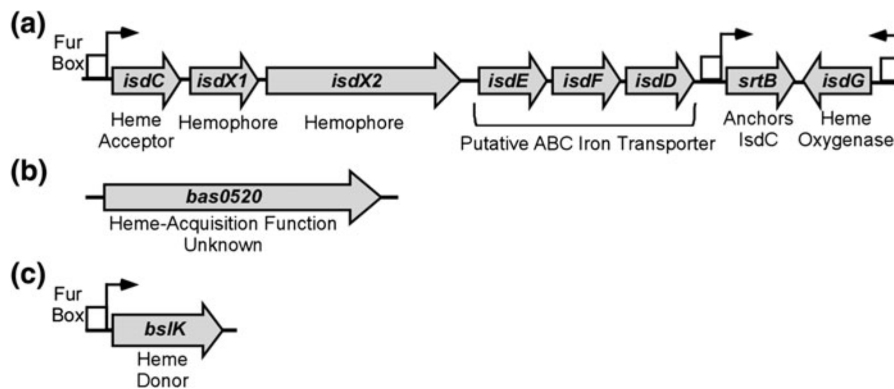

Fig. 2 Genomic organization of the Isd-like locus and distant NEAT-encoding genes in *B. anthracis* **a** The Isd-like heme-acquisition system encodes a cell wall-anchored heme-binding protein (IsdC), two secreted NEAT-hemophores, IsdX1 and IsdX2, a putative ABC-type transporter (IsdEFD), a sortase that anchors IsdC to the cell wall (SrtB), and a monooxygenase (IsdG). Fur boxes are found within each of the three promoters. **b** Recently, BslK was shown to be a heme-binding NEAT protein encoding three s-layer homology domains, suggesting it localizes to the cell surface. The promoter also contains a putative Fur box. **c** Additionally, an uncharacterized NEAT protein, Bas0520, has been implicated in possessing heme-acquisition functions. BslK and Bas0520 are both distantly located on the chromosome from the Isd-like locus

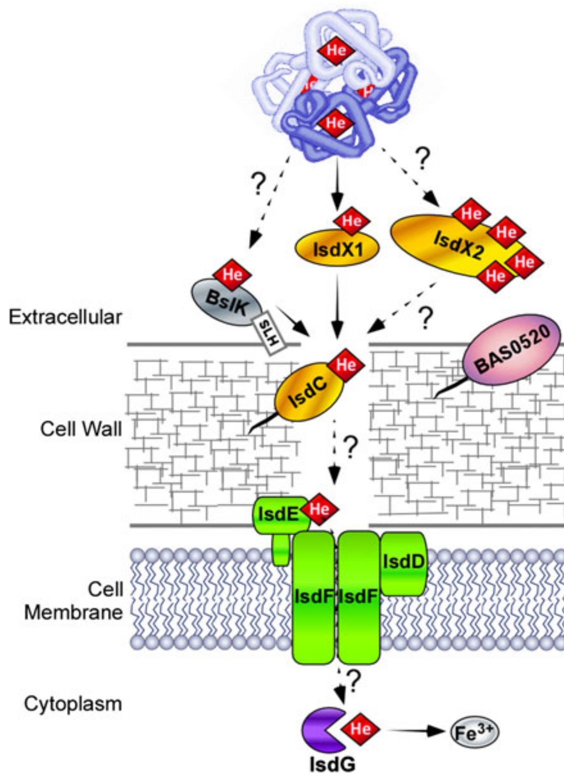

Fig. 3 Heme uptake through NEAT proteins in *B. anthracis* Secreted hemophores IsdX1 and IsdX2 (one and five NEAT domains, respectively), scavenge heme from hemoglobin, with IsdX1 capable of transferring bound heme to IsdX2 and cell wall-anchored IsdC. Additionally, a cell surface-associated protein, BslK, binds heme and directionally transfers heme to IsdC. The role of BAS0520 in this pathway is unknown, but the encoded NEAT domain is predicted to be involved in heme acquisition. IsdC may then pass the heme to IsdE within the ABC-type transporter IsdEFD, which will pump the heme through the cell membrane using energy from ATP hydrolysis. IsdG, the heme-degrading monooxygenase, degrades the heme to liberate the iron atom. *Solid arrows* indicate confirmed NEAT–NEAT interaction species, whereas *dotted lines* represent proposed, but unstudied, pathways of heme transfer between *B. anthracis* NEAT domains

Owing to its extracellular localization, it was hypothesized that IsdX1 may transfer its heme to proteins bound to the *B. anthracis* surface. Indeed, IsdX1 transfers its heme to apo-IsdC, with kinetics faster than the spontaneous dissociation of heme into solution (Fabian et al. 2009). This finding led to the proposal that heme transfer between donor and recipient NEATs utilizes an active, contact-dependent mechanism. Unexpected was the finding that IsdX1 also transferred heme to IsdX2, and, like the transfer to IsdC, did so via physical NEAT–NEAT interactions (Fabian et al. 2009). While it seems counterproductive for extracellular proteins to participate in heme transfer in this manner, the unique five-NEAT IsdX2 may be a multifaceted unit, and exactly how IsdX2 functions remains to be elucidated. It is reported that 20% of IsdX2 remains associated with the cell wall, where it may act as another cell wall-localized heme-binding protein capable of accepting multiple heme molecules (Maresso et al. 2008). Loss of IsdX1 and IsdX2 leads to a reduction in the growth of *B. anthracis* on Hb as the sole iron source, suggesting *B. anthracis* has evolved secreted hemophores to scavenge heme from Hb in the extracellular environment during host infection.

Heme transfer through the cell wall

B. anthracis IsdC specifically binds heme–iron in vitro and is covalently linked to the cell wall via sortase B-anchoring at the sortase motif NPKTG. The transpeptidase sortase B, which is encoded within the Isd locus (Fig. 2) has been shown to be essential for IsdC anchoring to the cell wall (Maresso et al. 2006). *B. anthracis* mutants lacking IsdC or SrtB expression showed defects in heme scavenging in culture, suggesting that sortase B is needed for IsdC cell wall-anchoring, and IsdC is a principal contributor to heme uptake (Maresso et al. 2006).

Although studies thus far have focused on Isd systems that mediate heme uptake in Gram-positive bacteria, more recent studies suggest non-Isd NEAT proteins also participate in heme acquisition. For example, *B. anthracis* S-layer protein K (BslK), a polypeptide annotated as a possible surface protein in anthrax, contains three SLH domains and a single NEAT domain (Fig. 2) (Tarlovsky et al. 2010). Studies of BslK indicate it is non-covalently localized to the cell surface, most likely through the binding of its SLH domains to cell wall sugars (Bahl et al. 1997; Beveridge et al. 1997) and the NEAT domain binds heme in vitro. Interestingly, BslK is also capable of transferring heme to IsdC via protein–protein interactions, but its heme source has not been identified (Tarlovsky et al. 2010). The functional consequence of this heme transfer, and the pathogenic role of BslK during infection, remains to be determined. Finally, a fifth NEAT-domain containing protein, encoded by *bas0520* (Sterne strain designation), may also partake

in heme transport through the cell wall (Figs. 2, 3). Originally described as being upregulated under low-iron conditions, deletion of this gene raises the LD_{50} of *B. anthracis* 100-fold in an inhalational model of anthrax, suggesting its importance during infection (Carlson et al. 2009). However, its function, localization, and mechanism of action have not been described. Collectively, it is proposed these five NEAT proteins (IsdC, IsdX1/X2, BslK, and BAS0520) act as an integrated unit to acquire heme from host Hb and transport the heme across the thick cell wall (Fig. 3).

Heme transfer through the cell membrane and iron liberation

After the secreted hemophores IsdX1 and IsdX2 have delivered heme to IsdC, it is proposed that it subsequently delivers heme to IsdE, a component of the IsdEFD ABC transporter within the cell membrane. While the *B. anthracis* IsdE protein has not been studied, a homologue in *S. aureus* has been characterized (Mack et al. 2004). Using magnetic circular dichroism spectroscopy (MCD), it was determined that IsdE specifically binds both ferric and ferrous heme. Additional studies by (Grigg et al. 2007b) revealed that IsdE is a heme–iron binding lipoprotein found within an ABC transporter complex. Crystallography allowed for visualization of the heme binding site, allowing six-coordination of the heme molecule by methionine and histidine residues. Additionally, it is proposed that IsdE delivers the heme to the bacterial cytosol, although the mechanism by which this occurs is not described.

Once heme has been released into the cytosol, bacterial heme degrading enzymes, termed monooxygenases, are responsible for the degradation of heme to liberate the iron molecule (Fig. 3). The heme-monooxygenase IsdG encoded in the *B. anthracis* Isd-like locus oxidatively degrades heme to release the iron (Skaar et al. 2004, 2006). This activity is thought to be mediated by an asparagine, tryptophan, and histidine (NWH) catalytic triad that is essential for heme degradation. *B. anthracis* IsdG was shown by Skaar et al. (2006) to be essential for efficient heme utilization and protection against heme-mediated toxicity. Interestingly, the inactivation of IsdG did not affect the ability of anthrax bacteria to replicate within macrophages, suggesting heme uptake may not be essential for replication in the intracellular environment.

NEAT domains: a conserved heme-binding module

Work examining NEAT proteins from *B. anthracis* and *S. aureus* indicates the NEAT domain is a conserved structural unit mediating heme acquisition by Gram-positive pathogens during infection. The name originates from the initial discovery and classification of these modules, having been annotated in proximity to hypothetical iron-transporter genes (Andrade et al. 2002). NEAT domains are encoded in the genome of pathogens such as *Listeria monocytogenes, Streptococcus pyogenes* and *Clostridium perfringens*. It is proposed that NEAT-containing proteins act together to acquire host heme and deliver it to a cell surface heme-receptor. These domains are functionally diverse and possess several unique attributes, including heme/Hb binding and protein–protein heme transfer (Dryla et al. 2003; Mazmanian et al. 2003; Maresso et al. 2006; Pilpa et al. 2006; Torres et al. 2006; Vermeiren et al. 2006; Dryla et al. 2007; Liu et al. 2008, Maresso et al. 2008; Muryoi et al. 2008, Fabian et al. 2009, Tarlovsky et al. 2010).

Bacterial proteins may harbor one or more non-identical NEAT domains each possessing heme-acquisition-related functions. All NEAT domains consist of approximately 125 amino acids and share a conserved structural fold (Andrade et al. 2002). Crystal structures of *S. aureus* NEAT domains have elucidated that they consist of eight β-strands that form an immunoglobulin-like fold; however, their sequence has no homology to members of the immunoglobulin superfamily (Sharp et al. 2007; Grigg et al. 2007a). Currently available NEAT crystal structures indicate a hydrophobic binding pocket is necessary to coordinate the heme-porphyrin molecule (Fig. 4) (Villareal et al. 2008). Additionally, two antiparallel β-sheets form a “platform” for the ligand to rest upon. Heme is not completely buried within this pocket; rather approximately 35% remains exposed to the environment (Sharp et al. 2007; Watanabe et al. 2008). It is likely that this feature allows the NEAT domain to perform two opposing functions: sequester its ligand from host proteins and to deliver its cargo to a sequential bacterial NEAT domain.

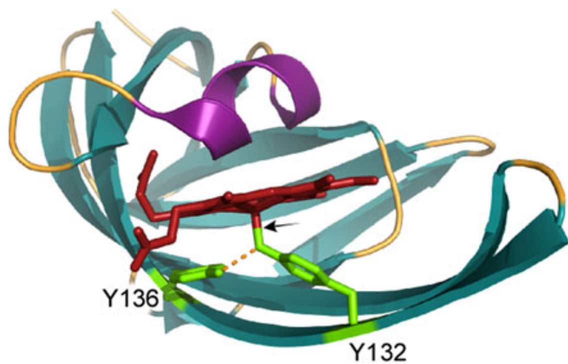

Fig. 4 Structural model of the heme-binding pocket of the NEAT domain of IsdC from *Staphylococcus aureus* The eight-*β* strand NEAT domain indicating the two tyrosine residues predicted to be essential for heme coordination and binding (*green*, Y132 and Y136). A single heme molecule is shown bound within the hydrophobic binding pocket (*red*). The flexible lip-region, suggested to be essential for heme transfer and heme scavenging, is indicated in *purple*. The *arrow* indicates the phenol bond formed between the OH group of Y132 and the iron atom within the heme molecule. A hydrogen bond connecting the OH groups of the two tyrosine residues is demonstrated as an *orange dotted line*. The structure was constructed using PyMol; pdb file 206P (Sharp et al. 2007)

NEAT-mediated heme scavenging, binding and transfer

In order to utilize heme–iron during infection, heme must be extracted from hemoproteins. *B. anthracis* has been characterized to target Hb as a source of heme during iron-starved growth. Hb binds heme–iron with high affinity, thus it is proposed that the method of heme scavenging is mediated by a physical NEAT-Hb interaction, whereby the NEAT triggers the release of heme and coordinates it within the hydrophobic binding pocket. The NEAT domain of IsdX1 transiently bound holo-Hb to facilitate heme extraction, suggesting a structural basis for these activities (Maresso et al. 2008). Furthermore, studies of *S. aureus* NEAT domains suggest that the heme–iron is high-spin, five-coordinated by a NEAT domain (Eakanunkul et al. 2005; Vermeiren et al. 2006; Pluym et al. 2008; Villareal et al. 2008). Two tyrosines, four residues apart, are proposed to be necessary for heme coordination (Fig. 4). The first conserved tyrosine coordinates the iron within heme via a phenol interaction using the OH group on the side chain (Sharp et al. 2007). Additionally, the second tyrosine hydrogen bonds with the first tyrosine via R-groups to help strengthen heme coordination (Grigg et al. 2007a). However, the roles of these residues in heme scavenging and transfer, and their overall mechanism of action, are still not understood.

Once a NEAT domain secures a heme molecule, the next task is to transfer the ligand to a "downstream" NEAT recipient associated with the cell wall. This funnel-like network allows the bacteria to specifically acquire heme–iron and import it through the cell envelope into the cytosol. Specific residues within NEAT domains are proposed to be essential for heme scavenging and NEAT-NEAT heme transfer. On the distal side of the ligand binding pocket, a 3_{10}-helix, or "lip-region", is thought to clasp onto one side of the heme, further coordinating the NEAT ligand (Fig. 4), (Sharp et al. 2007; Villareal et al. 2008). Crystal structures of apo/holo-*S.aureus* NEAT domains suggest that a displacement of amino acids around the binding pocket allows the helix to peel off the heme (Pilpa et al. 2006; Villareal et al. 2008). This switching between lip-region conformations may be responsible for the sequestration and transfer of heme; when the lip-region clasps its ligand, sequestration and binding occur. In contrast, when the helix is unwound from a locked state, the heme is released, or transferred, to an acceptor NEAT domain. Further mechanistic studies are needed to test this hypothesis.

Overall, heme transfer between NEATs seems to be mediated by a contact-dependent mechanism. Examination of the transfer between IsdX1 and IsdC was shown to be mediated by direct physical binding of each NEAT domain in a transient manner (Fabian et al. 2009). Collectively, these data allows the formulation of a general model for heme exchange between NEATs. The donor NEAT, when bound to heme, likely exists in a conformation that is conducive to association with the apo-recipient NEAT. Upon binding of the two NEATs, the lip region of the donor likely opens, thereby allowing the proximal tyrosine of the recipient to insert into the heme binding pocket of the donor to coordinate the heme. After ligand exchange, another conformational change likely lowers the affinity of local contacts between the two NEATs and dissociation occurs. However, a mechanistic appreciation of this process, and the residues involved, are lacking.

Physiological relevance of NEAT domains

Although many studies of NEAT proteins have been structural or biophysical in nature, determining the

physiological impact of NEAT-mediated heme acquisition on infection progression is an important topic of study. Several studies have demonstrated NEAT proteins are important for bacterial growth on heme or Hb as the iron source. In 2006, Maresso et al. (2008) showed that deletion of IsdC or SrtB reduced the growth of *B. anthracis* on heme. In addition, deletion of the genes that encode the secreted hemophores IsdX1 and IsdX2 compromises the growth of *B. anthracis* on Hb (Maresso et al. 2008). Similar results were observed by Gat et al. (2008) who demonstrated that *B. anthracis ΔisdC* mutants were defective in the sequestration of heme and a *ΔisdX2* deletion mutant showed a reduction in heme scavenging abilities.

Recently, several interesting studies have explored the possibility that NEAT proteins play a substantial role in bacterial pathogenesis. Somewhat conflicting results are observed in studies of *B. anthracis* NEAT-mediated heme acquisition and its contribution to virulence. While Gat et al. (2008) reported no significant difference in virulence between wild-type and *ΔisdCX1X2 B. anthracis* strains during infection of guinea pigs, Carlson et al. (2009) showed that deletion of the NEAT protein BAS0520 resulted in an ~100-fold increase of the LD_{50} of *B. anthracis* in

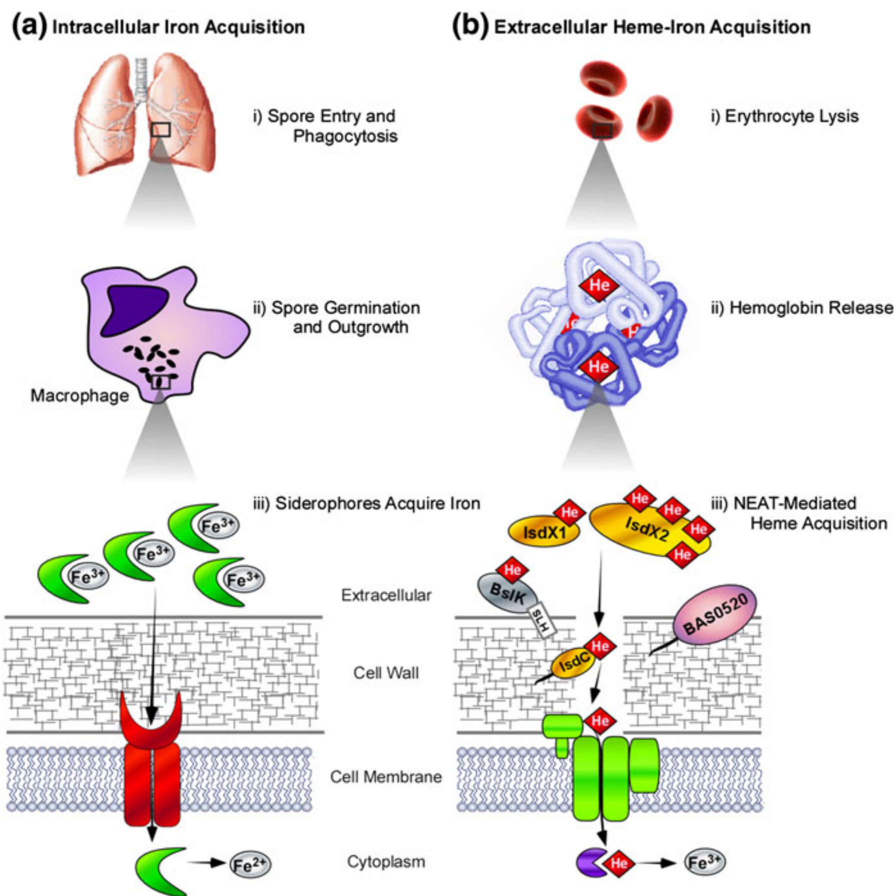

Fig. 5 Model for the utilization of two alternative iron-acquisition systems employed by *Bacillus anthracis* during infection **a** *i* Spores enter the lung to initiate inhalational anthrax disease, where they are phagocytosed by resident phagocytes. *ii* During this initial intracellular phase, *B. anthracis* vegetative cells synthesize and secrete two siderophores, petrobactin and bacillibactin. *iii* These siderophores (*green*) chelate phagocytic iron stores (*silver*) for utilization during infection. **b** *i* Once *B. anthracis* has escaped to replicate in the extracellular milieu, erythrocytes are lysed, due to hemolysins and cytolysins. *ii* Hemoglobin (Hb) is released and represents a rich iron-store for anthrax bacteria to target. *iii* Heme is then acquired by the action of NEAT proteins, the iron-porphyrin transferred into the cell, and the liberated iron used to promote bacterial replication

an inhalational model of disease. Additionally, Chitlaru et al. 2007 identified IsdX2 during a serological proteome analysis of *B. anthracis* infection in guinea pigs. IsdX2 was detected as a potent immunogen during infection, suggesting that this secreted hemophore has significance in relation to the growth and possibly pathogenesis of anthrax bacteria. Clearly, however, more studies are needed to determine what NEAT proteins are necessary for infection progression in *B. anthracis* and related pathogens.

Perhaps more intriguing are the results of recent studies suggesting *S. aureus* NEAT-proteins have efficacy as subunit vaccines, providing protection against intravenous *S. aureus* challenge (Kim et al. 2010). Rabbit antibodies directed against IsdA and IsdB generated significant protection in two murine models of infection. Passive transfer of these antibodies protected against abscess formation and lethal challenge with virulent staphylococci, with α-IsdB generating a stronger response. It is proposed that these antibodies reduced the pathogenesis of *S. aureus* by inhibiting the heme-acquisition functions of IsdA and IsdB. We suggest that this protective ability of α-NEAT antibodies could be applied to several Gram-positive pathogens utilizing NEAT-mediated heme-acquisition during infection.

Interestingly, IsdC, IsdX1 and IsdX2 have been characterized as potent immunogens during a systemic Ames infection in a murine model (Chitlaru et al. 2007; Gat et al. 2008). This suggests that these proteins are expressed during infection, and that antibodies directed against each may function to reduce heme import during systemic anthrax. Collectively, these studies indicate NEAT proteins may be viable targets for vaccine development against Gram-positive bacteria.

Conclusion

Over the past ten years, much progress has been made in understanding how Gram-positive bacteria acquire host iron, with major emphasis on the mechanism and function of NEAT proteins. We propose that during the initial intracellular lifecycle, *B. anthracis* satiates its requirement for iron by secreting siderophores that chelate iron from phagocytic iron stores (Fig. 5a). Once the bacteria enter the systemic, extracellular phase of infection, we propose that they switch their iron-acquisition system to target heme–iron by employing NEAT proteins (Fig. 5b). Interestingly, NEAT domains are versatile effectors, having the ability to not only bind heme but also extract heme from hemoglobin and transfer the heme to downstream receptors. While the NEAT-mediated heme-acquisition systems may vary in their spatial and mechanistic functions, there seems to be an overall contribution to replication in vivo, and subsequent virulence and disease progression. These heme-scavenging modules represent a promising target for antibacterial treatments against a range of medically important bacteria, whether that protection arises from antibodies, or small molecule inhibitors. Future studies will help expand our understanding of heme-acquisition in Gram-positive pathogens from both a biochemical and biological standpoint.

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