**Bacillus anthracis** Secretes Proteins That Mediate Heme Acquisition from Hemoglobin

Anthony W. Maresso, Gabriella Garufi, Olaf Schneewind*

Department of Microbiology, University of Chicago, Chicago, Illinois, United States of America

Abstract

Acquisition of iron is necessary for the replication of nearly all bacterial pathogens; however, iron of vertebrate hosts is mostly sequestered by heme and bound to hemoglobin within red blood cells. In *Bacillus anthracis*, the spore-forming agent of anthrax, the mechanisms of iron scavenging from hemoglobin are unknown. We report here that *B. anthracis* secretes IsdX1 and IsdX2, two NEAT domain proteins, to remove heme from hemoglobin, thereby retrieving iron for bacterial growth. Unlike other Gram-positive bacteria, which rely on cell wall anchored Isd proteins for heme scavenging, *B. anthracis* seems to have also evolved NEAT domain proteins in the extracellular milieu and in the bacterial envelope to provide for the passage of heme.

Introduction

Vegetative forms of *Bacillus anthracis* replicate in vertebrate tissues and form spores once their host has succumbed to anthrax infection [1]. Spore contamination of food sources for vertebrates ensures pathogen dissemination to new hosts and reiterative replication cycles [2]. A hallmark of anthrax is its low infectious dose (25–50 spores can kill an animal) and explosive replication of vegetative forms that accumulate to 10^10 colony forming units (CFU) per gram of host tissue [3]. Spores are taken up by phagocytes and germinate in the phagosome [4,5]. Upon phagosome lysis, vegetative forms first multiply in the cytoplasm, however, once released into body fluids, bacilli resist phagocytosis and replicate in extracellular spaces [6].

Several key features enable the invasion and replication strategies of *B. anthracis*. First, spores are metabolically inert and survive in the environment for long periods of time until taken up by a new host [7]. To escape phagocyte killing, bacilli secrete lethal toxin and edema toxin that subvert the host immune system and implement host killing [8]. Elaboration of the dense poly-D-glutamic acid (PDGA) capsule endows vegetative forms with the characteristic trait of resisting phagocytosis [9]. PDGA is attached to peptidoglycan [10], which functions as an exoskeletal scaffold for immobilization of proteins, carbohydrates and the S-layer, a two-dimensional crystalline protein array that encases vegetative forms [11,12].

Heme scavenging has been studied in *Staphylococcus aureus*, a Gram-positive pathogen phylogenetically related to *B. anthracis*, albeit that the envelope structure of staphylococci is comprised entirely of cell wall peptidoglycan with associated protein, teichoic acid and carbohydrate polymers [13,14]. Staphylococci elaborate neither PDGA capsule nor S-layers and their ability to retrieve heme from hemoglobin/haptoglobin relies on Isd proteins that are anchored to cell wall peptidoglycan [15,16]. The *S. aureus* isd locus (isdA-isdB-isdCDEF srtB isdG) is comprised of genes that encode cell wall anchored surface proteins (IsdA, IsdB, IsdC), membrane protein (IsdD), ABC transporter for import of heme (IsdEF) as well as heme mono-oxygenase (IsdG) [17,18]. The NEAT domain (near iron transporter) of staphylococcal envelope proteins (IsdA, IsdB, IsdC) enables scavenging of heme and passage of the iron containing compound across the cell wall envelope [15,16,19,20,21]. Heme passage relies further on sortase A-mediated deposition of IsdA and IsdB at the bacterial surface as well as sortase B-mediated immobilization of IsdC within the cell wall envelope [15,22,23]. The *B. anthracis* isd locus (isdC isdX1 isdX2 isdE1 isdE2 isdF srtB isdG) is comprised of eight open-reading frames with three putative transcriptional units, each flanked by a Fur-box consensus sequence (Fig. 1A) [24,25]. The smallest gene, isdX1, harbors a NEAT domain and is conserved in all members of the *Bacillus cereus* group but absent from staphylococci, listeria and clostridia (Fig. 1B). The largest gene, isdX2, is also conserved and contains five NEAT domains.

Here we report the first identification of a secreted heme-scavenging protein, IsdX1, from Gram-positive bacteria. Further, we demonstrate that IsdX1 and IsdX2 acquire heme directly from hemoglobin and that this activity enables bacilli to scavenge iron from host hemoglobin under iron-limiting conditions. These findings indicate that unlike staphylococci, which rely on cell wall anchored Isd proteins for heme scavenging, *B. anthracis* seems to have also evolved NEAT domain proteins in the extracellular milieu and in the bacterial envelope to provide for the passage of heme.
**Results**

*Bacillus anthracis* secretes IsdX1

The presence of cleavable N-terminal signal peptides and the absence of membrane or cell wall anchoring signals suggested that IsdX1 and IsdX2 may be secreted. To test this, *B. anthracis* was grown in the presence or absence of iron and bacterial cultures were fractionated to separate proteins secreted into the medium (S) from those targeted to the cell wall envelope (C) or located in membrane and cytoplasm lysate (L) (Fig. 2A). When analyzed by immunoblotting with rabbit antiserum raised against purified recombinant IsdX1, 15 kDa and 100 kDa (including some degradation products) immunoreactive species were detected under iron-limiting conditions. Wild-type bacilli secreted both the 15 and 100 kDa proteins, which represent IsdX1 (predicted molecular mass 14,579) and IsdX2 (predicted molecular mass 99,610), as ΔisdX1 and ΔisdX2 mutant strains failed to express the former or the latter species, respectively (Fig. 2A). Cross-reactivity of IsdX1 was not observed for other NEAT domain proteins, suggesting that IsdX1 and IsdX2 may share unique structural and functional properties (data not shown). A portion of IsdX2, but not of IsdX1, was found in the cell wall fraction [24% (±9%) of the total], suggesting that IsdX2 may be partially associated with the envelope of bacilli. As a control, immunoblotting with antibodies against cell wall anchored (IsdC), membrane (SrtB) and cytoplasmic (L6) proteins was used to ensure proper fractionation of *B. anthracis* cultures (Fig. 2A). The amount of IsdX1 or IsdX2 secretion was similar when bacilli were grown at 30°C or 37°C (Fig. S1). Taken together, these data indicate that IsdX1 and IsdX2 are synthesized and secreted when bacilli are exposed to iron-limiting conditions, as occurs during infection of vertebrate hosts. isdX1 with a C-terminal hexahistidyl tag was cloned under control of the IPTG inducible Pspac promoter in pLM5 and recombinant plasmid was transformed into bacilli. Affinity blotting of fractionated cultures revealed that bacilli harboring pisdX1-H6, but not bacteria harboring pLM5 vector control, secreted IsdX1-H6 into the extracellular milieu (Fig. 2B).

**Figure 1. Bacillus anthracis isdX1 and isdX2.** (A) The *B. anthracis* isd locus contains eight open reading frames, including genes for sortase B (srtB), IsdC (a NEAT domain protein and sortase B substrate), IsdE1-IsdE2-IsdF (ABC membrane transporter), IsdG (heme mono-oxygenase), and two NEAT domain proteins of unknown function (IsdX1 and IsdX2). (B) Alignment of amino acid sequences of *B. anthracis* IsdX1 (Ba - BAS4443) with homologs from *B. cereus* (Bc - BC4548), *B. thuringiensis* (Bt - RBTH03454), and *B. weihenstephanensis* (Bw - KBAB44137). Arrow indicates the predicted signal peptide cleavage site. Black dots refer to amino acids that are not absolutely conserved. Amino acids 27–152 of IsdX1 represent a NEAT (near iron transporter) domain. Tyrosine residues 136 and 140, which are conserved in many NEAT proteins, are underlined.

doi:10.1371/journal.ppat.1000132.g001
Host immune responses to *B. anthracis* IsdX1 and IsdX2

To test whether *B. anthracis* synthesize IsdX1 and IsdX2 during infection, we analyzed the serum of guinea pigs that had survived anthrax infections. Following subcutaneous infection with spores of *B. anthracis* strain Ames, guinea pigs suffer lethal anthrax infections over seven days, even when animals are inoculated with low doses of spores [26]. To ensure survival of guinea pigs, animals were treated with ciprofloxacin five days following infection, at a time when spores had germinated and vegetative bacilli replicated throughout host tissues. Two weeks following infection, animals were bled and serum samples examined for the presence of antibodies against purified recombinant GST-IsdX1, GST-IsdX2 or a GST control. Immune sera from infected animals reacted with GST-IsdX1, and GST-IsdX2, but not with the GST control (Fig. 2C). These data suggest that *B. anthracis* secretes IsdX1 and IsdX2 during infection when vegetative forms encounter iron-restrictive conditions, thereby stimulating specific host immune responses against these proteins.

IsdX1 binds heme

Several NEAT-domain containing proteins have been shown to bind heme, including *B. anthracis* IsdC (B-IsdC) [15,20,21,25]. To determine whether IsdX1 and IsdX2 display a similar property, both genes were cloned as translational fusions to the 3' end of glutathione-S-transferase (gst) and GST-IsdX1/-IsdX2 purified from *E. coli* lysate by affinity chromatography (Fig. 3AC). Both GST-IsdX1 and GST-IsdX2 eluted with red-brown color, indicative of an association with endogenous iron-porphyrin from *E. coli* (Fig. 3AC insets) [25]. We estimate that about 10% of purified GST-IsdX1/-IsdX2 was bound to heme [27]. GST-
IsdX1 was dialyzed to remove heme, cleaved with thrombin and IsdX1 purified (Fig. 3A). Binding of added heme to IsdX1, as analyzed by spectrophotometry (Soret absorbance at 404 nm) [28], was dose-dependent and quantifiable (Kd 5.40 ± 0.85 × 10⁻⁶ M) (Fig. 3B). Heme binding was only marginally increased by an increase in temperature (Fig. S2). IsdX2 also bound heme in a dose-dependent manner and did so more efficiently than IsdX1 (Fig. 3D). The heme binding curve of IsdX2 yielded multiple inflection points, suggesting IsdX2 contains multiple binding sites for heme, presumably provided by its five NEAT domains. The complexity of the associations between IsdX2 and heme did not allow us to calculate a dissociation constant (Fig. 3D). Together these findings indicate that IsdX1 and IsdX2 bind heme and may be involved in iron scavenging during anthrax infections.

IsdX1 removes heme from hemoglobin

Hemoglobin (Hb) is the most abundant hemoprotein of mammals and several bacterial pathogens target this molecule to obtain iron during infection [29,30]. To examine whether hemoglobin serves as a source of heme for the presumed iron-scavenging activity of IsdX1, we developed a simple experimental protocol. Glutathione-Sepharose loaded with GST-IsdX1 was incubated with hemoglobin. The resin was then sedimented by centrifugation, separated from supernatant containing hemoglobin, washed and GST-IsdX1 eluted (Fig. 4A). As a control (C), hemoglobin was incubated with glutathione-Sepharose that had been charged with GST and compared with GST-IsdX1 treated samples (T) (Fig. 4BC). Following incubation with GST-IsdX1, the heme-specific absorbance of hemoglobin at 404 nm was diminished, indicating that GST-IsdX1 had removed heme from hemoglobin (Fig. 4B). GST-IsdX1 mediated removal of heme could also be observed by inspection of hemoglobin: the red-brown color of hemoglobin is cleared in GST-IsdX1 treated, but not in GST control samples (inset, Fig. 4B). When analyzed by spectrophotometry, GST-IsdX1 displayed an increase in absorbance at 404 nm following its incubation with hemoglobin (Fig. 4C). Inspection of glutathione Sepharose sediment revealed red-brown pigmented GST-IsdX1, whereas GST control samples remained clear (inset, Fig. 4C). When analyzed by spectrophotometry, GST-IsdX1 displayed an increase in absorbance at 404 nm following its incubation with hemoglobin (Fig. 4C). The abundance of hemoglobin in the supernatant samples was unchanged in the treated versus control reactions, indicating that
the observed color and spectral changes were caused by heme transfer to IsdX1 (Coomassie stained SDS-PAGE, Fig. 4B).

We sought to develop a second measure for GST-IsdX1 removal of heme from hemoprotein. Apo-hemoglobin (hemoglobin lacking heme) was loaded with $^{55}$Fe heme and radiolabeled hemoglobin was purified. $^{55}$Fe hemoglobin was incubated with GST or GST-IsdX1 bound to glutathione sepharose. As before, glutathione sepharose was sedimented by centrifugation and transfer of $^{55}$Fe heme from hemoglobin was measured by scintillation counting as an increase in $^{55}$Fe ionization (Fig. 4D).

Addition of increasing amounts of GST-IsdX1, but not of GST, to $^{55}$Fe-hemoglobin led to increased $^{55}$Fe ionization in sediment samples, until eventually all $^{55}$Fe heme had been removed from hemoglobin (Fig. 4E) and transferred to GST-IsdX1 (Fig. 4D).

Comparison of *B. anthracis* IsdX1 and *S. marcescens* HasA

*Serratia marcescens* HasA represents the best established paradigm of bacterial hemophores [31]. Following its secretion via the *Serratia* type I pathway, 19 kDa HasA binds heme ($K_a = 5 \times 10^{10}$ M$^{-1}$) [32,33]. Due to its high affinity, HasA retrieves heme from hemoglobin and, in turn, transfers heme to the HasR outer membrane receptor for heme transport across the bacterial envelope and into the cytosol [34]. To validate our heme-transfer assay as a method to measure heme transfer between proteins, we compared the ability of IsdX1 to acquire heme from hemoglobin with that of HasA. We purified GST-HasA from lysates of recombinant *E. coli* by affinity chromatography. Glutathione-sepharose was charged with each GST-HasA, GST-IsdX1 or GST and then incubated with hemoglobin. Resin was sedimented by...
centrifugation, separated from supernatant containing hemoglobin, washed and bound proteins eluted (Fig. 5). Eluate was analyzed for heme binding by measuring the absorption spectrum of GST-HasA, GST-IsdX1 or GST for heme. GST-HasA and GST-IsdX1 displayed a similar ability to remove heme from hemoglobin. Thus, it seems plausible that IsdX1 functions as a hemophore for *B. anthracis* heme scavenging.

IsdX1 binds hemoglobin, but not apo-hemoglobin

When analyzed by spectrophotometry for absorption at 404 nm, IsdX1 bound heme with an affinity significantly lower than the affinity of apo-hemoglobin for heme (K_d > 10^{11} M^{-1}) [35]. We therefore considered the possibility that IsdX1 may retrieve heme from hemoglobin by a mechanism that involves physical contact between both proteins [36]. Surface plasmon resonance (SPR) spectroscopy was used to measure the presumed physical association between IsdX1 and hemoglobin [37,38]. Infusion of IsdX1 over heme coated chips produced a large spike in the local light refraction index (RU), indicative of a physical interaction between IsdX1 and hemoglobin. This association was saturated within ~180 seconds and, when deprived of further IsdX1 infusion (arrow), decayed to near baseline RU values (Fig. 6, + heme). Infusion of IsdX1 over chips coated with apo-hemoglobin failed to reveal a physical association between both proteins (Fig. 6, − heme). Following removal of heme from hemoglobin by IsdX1, additional infusion of heme over apo-hemoglobin produced holo-hemoglobin (data not shown), suggesting the inability of IsdX1 to associate with apo-hemoglobin is not caused by the unfolding of this polypeptide. Physical interaction between IsdX1 and hemoglobin occurred in a dose-dependent manner that could be saturated as the concentration of IsdX1 increased (Fig. S3). Dissociation constants for the interaction between IsdX1 and hemoglobin are 7.33 ± 6 x 10^{-14} M (holo-hemoglobin) and 9.43 ± 6 x 10^{-23} M (apo-hemoglobin). Thus, IsdX1 appears to bind directly to hemoglobin and, upon transfer of heme, dissociates from apo-hemoglobin.

Hemophore function and specificity in the Isd pathway

To examine the specificity of IsdX1 and IsdX2 for host hemoproteins, GST-IsdX1/-IsdX2 were incubated with excess hemoglobin and myoglobin, a monomeric globin abundantly present in muscle tissue [39]. As compared to hemoglobin, GST-IsdX1/-IsdX2 displayed little hemophore activity towards human myoglobin (Fig. 7), and similar results were observed when bovine or equine myoglobin was examined (data not shown). These data suggest that during *B. anthracis* infection IsdX1 and IsdX2 most likely prefer hemoglobin over myoglobin as a heme source.

Almost the entire IsdX1 polypeptide is comprised of its NEAT domain (Fig. 3A). To test whether other NEAT domain proteins also display hemophore activity, GST fusions to *S. aureus* IsdC and *B. anthracis* IsdC were purified and compared to GST-IsdX1/-X2 (Fig. 8). All four hybrids were able to remove heme from hemoglobin. IsdX2, which contains 5 NEAT domains, was 3.4 fold more efficient than IsdX1 and 7.25 or 12.6 fold more effective than *B. anthracis* IsdC or *S. aureus* IsdC. Also, hemoglobin was not sedimented in any of the reactions, suggesting a transient association similar to that observed for IsdX1 (Fig. 8, inset). Thus,
the direct acquisition of heme from hemoglobin appears to be a general property of some NEAT domain proteins, albeit that IsdX1 and IsdX2, when compared to IsdC, clearly display superior activity. This finding is compatible with their localization to the extracellular milieu, a site expected to optimize their interaction with hemoglobin.

**B. anthracis** IsdX1 scavenges heme from hemoglobin in vivo

To examine whether the in vitro biochemical activity ascribed to IsdX1 and IsdX2 correlated with in vivo biological function, wild-type, ΔisdX1, ΔisdX2, and ΔisdX1/ΔisdX2 mutant *B. anthracis* strains were analyzed for growth in iron defined media (IDM) with hemoglobin as the only source of iron [40]. In the absence of added hemoglobin, all strains grew very poorly in IDM (Fig. 9). The addition of increasing amounts of hemoglobin allowed wild-type *B. anthracis* to grow with increasing rates (Fig. 9), indicating that bacilli can utilize hemoglobin as a source of iron. All three mutant strains (ΔisdX1, ΔisdX2, and ΔisdX1/ΔisdX2) displayed a growth defect under iron-depleted conditions with hemoglobin as the sole iron source (Fig. 9). Whereas deletion of individual genes, *isdX1* or *isdX2*, caused a reduction in growth, these defects were exacerbated for the double mutant strain, which is unable to secrete IsdX1 or IsdX2 (Fig. 9 and Fig. S4). These data suggest

---

**Figure 7. Specificity of IsdX1 and IsdX2.** Heme acquisition when equimolar amounts of hemoglobin (Hb) or myoglobin (Mb) (800 μM) were incubated with GST-IsdX1 or GST-IsdX2 (60 μM). Mean and standard deviation of three independent experiments are recorded.

doi:10.1371/journal.ppat.1000132.g007

**Figure 8. Transfer of heme from hemoglobin to NEAT domain proteins.** Purified GST hybrids with (1) *S. aureus* IsdC (S-IsdC), (2) *B. anthracis* IsdC (B-IsdC), (3) IsdX2, (4) IsdX1, or (5) GST control were incubated with hemoglobin and heme transfer measured as in Fig. 4. Mean and standard deviation of three independent experiments are recorded. Inset reveals the mobility of purified proteins on Coomassie stained SDS-PAGE.

doi:10.1371/journal.ppat.1000132.g008
isdX1 and isdX2 perform partially overlapping functions in the heme scavenging pathway of bacilli. Growth defects of ΔisdX1 and ΔisdX2 mutants were restored when bacilli were transformed with plasmids providing for IPTG inducible expression of each respective gene. Finally, all strains examined grew equally well in iron-replete media (Fig. 9, far right columns). Collectively, these experiments suggest that B. anthracis IsdX1 and IsdX2 function as secreted hemophores for heme-scavenging from hemoglobin.

Discussion

The ability of mammalian organisms to sequester iron and limit its availability serves as a defense against microbial infection [41]. Iron is stored intracellularly, where ferric iron is complexed by ferritin or incorporated by ferrochelatase into porphyrin. The resulting product, heme, is bound by hemoproteins, e.g. hemoglobin or myoglobin [42]. Dedicated traffic systems for ferric iron (transferrin) or heme (hemopexin) transport iron in body fluids between tissues. A key feature that enables bacteria to replicate within their hosts is the production of siderophores, iron-sequestering compounds that scavenge iron from transferrin, and synthesis of cognate siderophore transport systems for the bacterial envelope [43]. Vertebrates, in turn, evolved defense mechanisms that exploit the bacterial requirement for iron by producing lipocalin, siderocalin or related proteins which sequester iron [44].

B. anthracis employs two siderophores to retrieve ferric-iron during infection, bacillibactin and petrobactin (anthrachelin) [45,46]. Petrobactin, enzymatically derived from 3,4-dihydroxybenzoate, spermidine and citrate via products of the asbA-F locus, is essential for B. anthracis growth, as mutations in asbA-F cause significant defects in the pathogenesis of anthrax [40,47,48]. Interestingly, this siderophore is resistant to sequestration by siderocalin, an immune protein which binds siderophores as a bacterial defense strategy [49]. B. anthracis has also evolved a scavenging pathway for heme that is encoded by the isd locus (isdC-isdX1-isdX2-isdE-isdE2-isdF-srtB-isdG) [25]. IsdC, a NEAT domain protein with C-terminal sorting signal, is anchored to cell wall peptidoglycan by sortase B (SrtB) [25]. IsdE-IsdE2-IsdF membrane transporter is thought to import heme into bacterial cells, while IsdG, a cytoplasmic monoxygenase, cleaves the tetrapyrrol of heme, thereby liberating iron [24].

Heme scavenging strategies of B. anthracis must take into account the unique envelope attributes of this pathogen. Bacilli evolved a thick murein sacculus comprised of peptidoglycan with attached envelope polymers: poly-D-glutamic acid (PDGA) capsule, carbohydrate polysaccharide, teichoic acid and proteins [12]. Further,
bacilli elaborate S-layers, two-dimensional crystalline arrays of proteins bearing SLH domains that are immobilized by interaction with pyruvylated cell wall polysaccharide [11,50]. It is not certain that bacilli elaborate all envelope components at each stage of infection [31]. Nevertheless, explosive growth of \textit{B. anthracis} and the accompanying need for nutrients likely demand that heme scavenging pathways must engage all structural components of the bacterial envelope. Here we report that \textit{B. anthracis} secretes two polyepitides, IsdX1 and IsdX2, into the extracellular milieu. The absence of a canonical sortase recognition motif in the C terminus of IsdX2 suggests it is not anchored to the cell wall by a sortase. Both proteins remove heme from hemoglobin, thereby enabling \textit{B. anthracis} growth under conditions when hemoglobin is the sole source of iron. These findings, along with the data presented in Figures 4-8, suggest one of the functions of the NEAT domain is the direct acquisition of heme from hemoglobin. How IsdX1 and IsdX2 bind heme is currently unknown; however, studies from other NEAT proteins suggest that heme-iron is ligated by a conserved tyrosine with high spin, five-coordinate geometry [20,52,53].

It seems unlikely that IsdX1 or IsdX2 deliver heme directly to the bacterial membrane, as the cell wall envelope cannot be penetrated by proteins. Instead, IsdX1 and IsdX2 probably transfer heme to other NEAT domain proteins at strategic positions throughout the bacterial envelope, a hypothesis consistent with their secretion into the surrounding milieu. In agreement with this conjecture, in silico analysis of the \textit{B. anthracis} genome identified several genes encoding NEAT domain proteins with variable envelope locations: peptidoglycan linked IsdC [25,54], BasJ positioned in the plasma membrane [55], and BldK, an S-layer protein [56]. In contrast to the complex features of the envelope in bacilli, staphylococci, listeria and clostridia are much simpler and cannot elaborate a large capsule or S-layer [18]. Not surprisingly, these microbes are capable of scavenging heme with NEAT domain proteins that are exclusively immobilized in cell wall peptidoglycan.

Heme scavenging pathways in Gram-negative bacteria have been studied in great detail. \textit{Shigella flexneri} employs a type I secretion machine (HasDEF) and recognition of a C-terminal secretion signal to transport HasA across the bacterial double membrane envelope [32,57,58]. By virtue of its unique structure and affinity for ligand (\(K_d \approx 5 \times 10^{-8} \text{M}^{-1}\)), HasA retrieves heme from hemoglobin, myoglobin or hemepoxin [30,59,60,61] and delivers the compound to HasR, the outer membrane receptor. Although HasR has much lower affinity for heme (\(K_d \approx 5 \times 10^{-6} \text{M}^{-1}\)), the outer membrane receptor receives heme from HasA by a mechanism involving physical interactions between both proteins [60,61]. TonB(HasB)-ExbB-ExbD dependent relay then transfers heme from HasR across the periplasm, initiating subsequent import into the cytoplasm [62]. HasA production and secretion are regulated by an ECF type sigma factor (HasI) and its cognate anti-sigma factor (HasS) [63]. Biological activities of HasI/HasS are informed by reciprocal associations between HasA, HasR and heme [64]. Hemophore systems with similar design exist in \textit{Haemophilus influenzae} [65], \textit{Yersinia enterocolitica} [66], and \textit{Pseudomonas aeruginosa} [67,68,69]. Pathogenic \textit{Neisseria spp.}, on the other hand, elaborate outer membrane proteins that not only bind hemoproteins but also remove heme. IsdX1 represents the first secreted hemophore in Gram-positive bacteria, a finding that invites a functional comparison with HasA, the secreted hemophore of Gram-negative microbes [31]. Unlike HasA, which acquires heme from diverse hemoproteins such as myoglobin, IsdX1 appears to be specific for hemoglobin [30]. Further, whereas HasA seems to acquire heme from hemoglobin by virtue of its higher affinity for heme [31,60], IsdX1 directly associates with hemoglobin for extraction of the heme. Finally, the structure of HasA is quite distinct from that of other NEAT-domain proteins [20,53,70,71]. These findings suggest that the molecular mechanism whereby IsdX1 acquires heme from hemoglobin must be distinct from that of HasA. While HasA delivers heme to outer-membrane receptors [60,61], secreted components of the sld locus encoding NEAT domain proteins, such as IsdX1, provide a versatile strategy for stealing heme that can be adapted to unique microbial envelope structures of Gram-positive pathogens. Whether these specific adaptations are important during infections caused by Gram-positive pathogens, e.g. \textit{B. anthracis}, is a topic currently being explored in our laboratory.

**Materials and Methods**

**Bacterial strains and reagents**

\textit{B. anthracis} strain Sterne 34F2 [72] and \textit{E. coli} strains (DH5\(\alpha\), XL1-Blue or K1077) were grown in Luria-broth (LB) or brain-heart infusion (BHI) (Table S1). Antibiotics were used for plasmid selection (ampicillin 50 \(\mu\)g/ml, kanamycin 20 \(\mu\)g/ml). All reagents were purchased from Sigma unless otherwise noted. \textit{B. anthracis} chromosomal DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). The \textit{isdX1} gene (BAS4445) of \textit{B. anthracis} Sterne was deleted by allelic replacement with the temperature-sensitive plSM4 [26]. Briefly, 1,000 bp of 5’ and 3’ \textit{isdX1}-flanking sequences were PCR amplified with primer pairs \textit{isdX1}-EcoRI (5’-gatgatagatgatcttgagaagttaac-3’) and \textit{isdX1}-SacI (5’-gatgatagatgatcttgagaagttctaccc-3’) as well as \textit{isdX1}-SacI (5’-gatgatagatgatcttgagaagttctaccc-3’) and \textit{isdX1}-KpnI (5’-gatgatagatgatcttgagaagttctaccc-3’). Following ligation, the 2-kb inset was cloned between the EcoRI/KpnI sites of plSM4 to create plLM4-\textit{ΔisdX1}. After transformation into BAS7, bacilli were grown first at 30°C (permissive temperature) on LB/Km and then shifted to 43°C (restrictive temperature), followed by growth at 30°C to induce plasmid loss, thereby generating BAS8. DNA was analyzed for the presence of \textit{isdX1} by PCR and deletions confirmed by DNA sequencing. Deletion of \textit{isdX2} (BAS4442) was achieved as previously reported [25]. The deletion of both \textit{isdX1} and \textit{isdX2} in the same strain was achieved via the procedure described above for \textit{ΔisdX1} using the \textit{isdX1} 5’ flank primers and the following 3’ flank primers: \textit{isdX2}-SacI (5’-gatgatagatgatcttgagaagttctaccc-3’) and \textit{isdX2}-KpnI (5’-gatgatagatgatcttgagaagttctaccc-3’). Plasmid DNA was amplified in \textit{dam} mutant \textit{E. coli} strain K1077 prior to electroporation of bacilli [73].

**Protein purification**

Signal peptides of IsdX1, IsdX2, B-IsdC, and Sa-IsdC were replaced with glutathione S-transferase (GST) and recombinant proteins were purified by GST-affinity chromatography (see Protocol S1).

**IsdX1 secretion**

Overnight \textit{B. anthracis} cultures were inoculated into 2 ml of BHI (+ Fe) or chelex-treated BHI (– Fe) supplemented with Ca\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), and Zn\(^{2+}\) and incubated at 37°C for further growth [25]. Bacilli were sedimented by centrifugation at 10,000 x g, washed twice with 1 ml of PBS (pH 7.4) and fractionated as previously reported [25]. Samples were analyzed by immunoblot with \(\lambda\)Lb, \(\lambda\)StB, \(\lambda\)IsdC, or \(\lambda\)IsdX1 specific rabbit antiserum (1:1,000), followed by mouse anti-rabbit HRP-linked antibody (1:10,000) and ECL (enhanced chemiluminescence, Pierce, Rockford, IL). By comparing the amount of secreted IsdX1 and IsdX2 to a known amount of recombinant purified IsdX1/X2 via
immunoblot, we estimate that a 3 mL culture of B. anthracis containing an optical density of 1.0 will secrete 0.52±0.25 μg of total IsdX1 in 12 hours. This compares to 0.55±0.07 μg of total IsdX2 secreted under the same conditions.

Heme binding to IsdX1 and IsdX2

IsdX1 (20 μM) or IsdX2 (1 μM) were incubated in 50 mM Tris-HCl, pH 8.0, with or without hemin chloride (0.01–40.0 μM in 0.1 M NH₄OH) for 5 minutes at 25°C, followed by spectrophotometry (300–700 nm) in a Varian Cary 50Bio instrument. Peak absorbance at 404 nm, characteristic of heme, was measured at 404 nm, characteristic of heme binding, was monitored following subtraction of a hemin-only reference cuvette value at each concentration.

IsdX1 acquisition of heme from hemoglobin

GST-IsdX1 (60 μM) or PBS (control) was incubated with 50 μL of glutathione-sepharose (Amersham) for 30 min at 25°C, followed by 3 washes of 200 μL with PBS. Bovine hemeoglobin (Sigma H2500) was added to 60 μM (monomer) and the X1/Hb mixture was incubated for 30 min at 25°C. Reactions were centrifuged at 13,000 x g to sediment glutathione-sepharose/GST-IsdX1 complexes, reactions washed three times with 200 μL of PBS and GST-IsdX1 eluted in 50 μL of 600 mM reduced glutathione (pH 8.0). Sediment (GST-IsdX1) and supernatant (hemoglobin) were analyzed by spectrophotometry and heme binding quantified by measuring absorbance at 404 nm. For [153Fe]heme transfer, reactions were prepared as indicated above except that the amount of GST-IsdX1 added varied from 0.1–140 μM (see Protocol S1). The amount of [%Fe]heme in the sediment (GST-IsdX1/resin) or supernatant (hemoglobin) was quantified in a Beckman LS-6000IC instrument (Beckman-Coulter, Fullerton, CA). Per cent amount of heme was calculated by dividing the counts in the sediment or supernatant by the total number of counts in each reaction multiplied by 100. For the experiment presented in Fig. 7, the heme transfer assay was utilized with the concentrations of hemeoglobin and myoglobin (Sigma M0630) at 800 μM. Heme acquisition was calculated as follows: [%GST-IsdX1 (Abs.404nm)] minus [%glutathione-sepharose (background)]/absorbance of total heme at 404 nm times 100.

SPR analysis

IsdX1-hemoglobin interactions were measured with a BIAcore 3000 biosensor (GE Healthcare) via surface plasmon resonance (SPR) [37,38]. Hemoglobin, 180 pmol in HBS (10 mM HEPES, pH 7.4, 0.15 M NaCl, 50 mM EDTA, 0.05% Tween 20), was amine coupled to CM5 sensor chip at 25°C in a flow rate of 5 μL/min [74]. Hemoglobin injection was stopped once response was fit to a model of equimolar IsdX1-hemoglobin association with X1/Hb mixture by 3 washes of 200 μL with PBS. Bovine hemoglobin (Sigma H2500) was added to 60 μM (monomer) and the X1/Hb mixture incubated for initial incubation at 25°C. Reactions were centrifuged at 13,000 x g to sediment glutathione-sepharose/GST-IsdX1 complexes, reactions washed three times with 200 μL of PBS and GST-IsdX1 eluted in 50 μL of 600 mM reduced glutathione (pH 8.0). Sediment (GST-IsdX1) and supernatant (hemoglobin) were analyzed by spectrophotometry and heme binding quantified by measuring absorbance at 404 nm. For [%Fe]heme transfer, reactions were prepared as indicated above except that the amount of GST-IsdX1 added varied from 0.1–140 μM (see Protocol S1). The amount of [%Fe]heme in the sediment (GST-IsdX1/resin) or supernatant (hemoglobin) was quantified in a Beckman LS-6000IC instrument (Beckman-Coulter, Fullerton, CA). Per cent amount of heme was calculated by dividing the counts in the sediment or supernatant by the total number of counts in each reaction multiplied by 100. For the experiment presented in Fig. 7, the heme transfer assay was utilized with the concentrations of hemeoglobin and myoglobin (Sigma M0630) at 800 μM. Heme acquisition was calculated as follows: [%GST-IsdX1 (Abs.404nm)] minus [%glutathione-sepharose (background)]/absorbance of total heme at 404 nm times 100.

B. anthracis growth with hemoglobin

Bacilli from overnight cultures in 2 ml of LB+Km at 30°C were inoculated into IMD+Km [40], grown for 12 hours at 30°C, bacteria harvested by sedimentation at 10,000 x g, washed twice and then suspended in 1 ml IMD (O.D. 4.0). Aliquots (5 μL) were inoculated into 150 μL IMD, with or without Hb (20, 100, or 500 μM) using 96-well U-bottom plates (Corning, Corning, NY). After 16 hours of incubation at 30°C, growth was assayed by plating 5 μL of a 1:40 dilution of bacterial culture onto LB+Km agar plates and colony forming units per mL (CFUs/mL) determined. For plasmid complementation, 1.5 mM IPTG (final concentration) was added to culture media. A list of accession numbers (NCBI) for genes in this study are as follows: isdX1 = YP_030690, isdX2 = YP_030689, b-isdC = YP_030691, isdC = YP_001332076.

Supporting Information

Figure S1 Expression of IsdX1 and IsdX2 at different temperatures
Found at: doi:10.1371/journal.ppat.1000132.s001 (0.19 MB DOC)

Figure S2 Heme binding to IsdX1 at different temperatures
Found at: doi:10.1371/journal.ppat.1000132.s002 (0.09 MB DOC)

Figure S3 Association of IsdX1 and hemoglobin
Found at: doi:10.1371/journal.ppat.1000132.s003 (0.13 MB TIF)

Figure S4 Expression of IsdX1, IsdX2, and B-IsdC in ΔisdX1 /isdX2 B. anthracis
Found at: doi:10.1371/journal.ppat.1000132.s004 (0.08 MB DOC)

Protocol S1 Supplementary Materials and Methods, References, and Figure Legends
Found at: doi:10.1371/journal.ppat.1000132.s005 (0.05 MB DOC)

Table S1 Bacterial strains used in this study
Found at: doi:10.1371/journal.ppat.1000132.s006 (0.04 MB DOC)

Acknowledgments

We thank Travis Chappa for experimental assistance, Elena Solomaha and the University of Chicago Biophysics Facility for SPR, Dr. Cecile Wandersman for HasA reagents, and laboratory members for comments.

Author Contributions

Conceived and designed the experiments: AWM OS. Performed the experiments: AWM GG CU. Analyzed the data: AWM GG OS. Contributed reagents/materials/analysis tools: AWM GG. Wrote the paper: AWM OS.

References

1. Koch R (1876) Die A¨tiologie der Milzbrand-Krankheit, begru¨ndet auf die Entwicklungsgeschichte des Bacillus anthracis. Beiträge zur Biologie der Pflanzen 2: 277–310.
2. Dixon TC, Meselson M, Guilemin J, Hanna PC (1999) Anthrax. N Engl J Med 341: 815–826.
3. Mock M, Fouet A (2001) Anthrax. Annu Rev Microbiol 55: 647–671.
4. Guidi-Rontani C, Levy M, Ohayon H, Mock M (2001) Fate of germinated Bacillus anthracis spores in primary murine macrophages. Mol Microbiol 42: 911–928.
5. Dixon TC, Fadl AA, Koehler TM, Swanston JA, Hanna PC (2000) Early Bacillus anthracis-macrophage interactions: intracellular survival survival and escape. Cell Microbiol 2: 453–463.
6. Candela T, Fouet A (2006) Poly-γ-glutamate in bacteria. Mol Microbiol 60: 1091–1098.
7. Friedlander AM (2001) Tackling anthrax. Nature 414: 160–161.
8. Collier RJ, Young JA (2003) Anthrax toxins. Annu Rev Cell Dev Biol 19: 45–70.
9. Drysdale M, Heninger S, Hutt J, Chen Y, Lyons CR, et al. (2005) Capsule synthesis by Bacillus anthracis is required for dissemination in murine inhalation anthrax. Embo J 24: 221–227.
10. Candela T, Fouet A (2005) Bacillus anthracis CapD, belonging to the N-acetylglucosaminyltransferase family, is required for the covalent anchoring of capsule to peptidoglycan. Mol Microbiol 57: 717–726.
11. Mesnage S, Fontaine T, Mignot T, Delepierre M, Mock M, et al. (2000) Bacterial SLH domain proteins are non-covalently anchored to the cell surface.
via a conserved mechanism involving wall polyacrylazide pyridine. Embo J 19: 4473–4484.

12. Fouet A, Mesnage S (2002) Bacillus anthracis cell envelope components. Curr Top Microbiol Immunol 271: 87–113.

13. Nataro JP, Swaminathan B (1998) Surface proteins of Gram-positive bacteria and the mechanisms of their targeting to the cell wall envelope. Microbiol Mol Biol Rev 63: 174–229.

14. Marasco AW, Schneewind O (2006) Iron acquisition and transport in Staphylococcus aureus. J Bacteriol 189: 191–203.

15. Mazmanian SK, Skaar EP, Gaspar AH, Humes M, Gromnicki P, et al. (2003). Passage of heme-iron across the envelope of Staphylococcus aureus. Science 299: 906–909.

16. Torres TJ, Picchiani G, Humayun M, Schneewind O, Skaar EP (2006) Staphylococcus aureus IsdA is a hemoglobin receptor for heme required for heme iron utilization. J Bacteriol 188: 8421–8429.

17. Skaar EP, Gaspar AH, Schneewind O (2004) IsdA and IsdG, heme degrading enzymes in the circulation of Staphylococcus aureus. J Biol Chem 279: 436–443.

18. Skaar EP, Schneewind O (2004) Iron-regulated surface determinants (Isd) of Staphylococcus aureus: stealing iron from heme. Microbes Infect 6: 390–397.

19. Andrade MA, Ciccarielli FD, Perez-Iturra C, Bork P (2002) NEAT: a domain duplicated in genes near the components of a putative Fe^{3+} siderophore transporter from Gram-positive pathogenic bacteria. Genome Biol 3: RE-SEARCH047.

20. Grigg JG, Vermeiren CL, Heinrichs DE, Murphy ME (2007) Heme recognition by the S-layer protein IsdA. Mol Microbiol 63: 139–149.

21. Vermeiren CL, Pluym M, Mack J, Heinrichs DE, Stillman MJ (2006) Characterization of the heme binding properties of Staphylococcus aureus IsdA. Biochemistry 45: 12967–12973.

22. Marraffini LA, Schneewind O (2000) Bacterial heme sources: the role of heme, heme-based siderophores and the Isd system in heme scavenging. TIBS 25: 453–458.

23. Skaar EP, Schneewind O (2004) IsdG and IsdI, heme degrading monooxygenase. J Bacteriol 186: 8421–8429.

24. Marraffini LA, Schneewind O (2005) Anchor structure of staphyloccocal surface proteins. X. Anchor structure of the sortase B substrate IsdC. J Biol Chem 280: 16326–16371.

25. Skaar EP, Gaspar AH, Schneewind O (2006) Bacillus anthracis IsdG, a hemeregulating monooxygenase. J Bacteriol 188: 1071–1080.

26. Marasco AW, Chapa TJ, Schneewind O (2006) Surface protein IsdC and sortase B are required for heme-iron scavenging of Bacillus anthracis. J Bacteriol 188: 8143–8152.

27. Izadi N, Henry Y, Haladjian J, Goldberg ME, Wandersman C, et al. (1997) Purification and characterization of an extracellular heme-binding protein, HasA, involved in heme iron acquisition. Biochemistry 36: 7050–7057.

28. Niemantsverdriet J, Nienhaus GU (2005) Probing the protein-ligand interactions by UV/visible absorption spectroscopy. Methods Mol Biol 305: 215–242.

29. Wandersman C, Stojilkovic I (2000) Bacterial heme sources: the role of heme, heme-protein receptors and hemeophores. Curr Opin Microbiol 3: 215–220.

30. Wandersman C, Delepelaire P (2004) Iron acquisition systems from siderophore to hemophores. Annu Rev Microbiol 58: 611–647.

31. Cesca S, Cowman A, Letoffe S, Delepelaire P, Wandersman C, et al. (2007) Heme acquisition by hemophores. Biochemicals 202: 603–613.

32. Letoffe S, Ghigo JM (1994)分泌突起間の赤血球介在性のBacillus anthracis haemophore protein by an ABC transporter. J Bacteriol 176: 5372–5377.

33. Letoffe S, Ghigo JM, Wandersman C (1994) Iron acquisition from heme and hemoglobin by a Staphylococcus aureus extracellular protein. Proc Nat Acad Sci USA 91: 9076–9080.

34. Izadi-Pruneyre N, Huché F, Lukat-Rodgers GS, Lecroisey A, Gilli R, et al. (2007) Characterization of the heme acquisition system of Bacillus cereus. Mol Microbiol 60: 504–515.

35. Delepelaire P (2004) Type I secretion in gram-negative bacteria. Biochim Biophys Acta 1694: 161–191.

36. Delepelaire P, Wandersman C (1998) The SecB chaperone is involved in the secretion of the Serratia marcescens HasA protein through an ABC transporter. Embo J 17: 936–944.

37. Deniau C, Gilli R, Izadi-Pruneyre N, Letoffe S, Delepire P, et al. (2003) Thermodynamics of heme binding to the HasASM hemophore: effect of mutations at three key residues for heme uptake. Biochemistry 42: 10627–10633.

38. Letoffe S, Nato F, Goldberg ME, Wandersman C (1999) Interactions of HasA, a bacterial haemophore, with haemoglobin and with its outer membrane receptor HasR. Mol Microbiol 33: 546–555.

39. Letoffe S, Deniau C, Wolf N, Dassa E, Delepelaire P, et al. (2001) Haemophore-mediated bacterial haem transport: evidence for a common or overlapping site for haem-free and haem-loaded haemophore on its specific outer membrane receptor. Mol Microbiol 41: 439–450.

40. Stojilkovic I, Hanke K (2004) Transport of heme across the cytoplasmic membrane through a haem-specific periplasmic binding-protein-dependent transport system in Yersinia enterocolitica. Mol Microbiol 33: 719–732.

41. Rossi M, Pasqualin A, Ghigo JM, Wandersman C (2003) Haemophore-mediated signal transduction across the bacterial cell envelope in Serratia marcescens: the inducer and the transported substrates are different molecules. Mol Microbiol 48: 1467–1480.

42. Biville F, Cowherm C, Letoffe S, Rossi MS, Drouet V, et al. (2004) Haemophore-mediated signalling in Serratia marcescens: a new mode of regulation for an extra cytoplasmic function (ECF) sigma factor involved in heme acquisition. Mol Microbiol 53: 1267–1277.

43. Hanou M, Pelzel SE, Lutmer J, Muller-Eberhard U, Hansen EJ (1992) Identification of a genetic locus of Porphyromonas gingivalis that is required for the binding and utilization of heme bound to human hemopexin. Proc Nat Acad Sci USA 89: 1973–1977.

44. Rossi M, Ferathon JD, Letoffe S, Carmeli E, Perry RD, et al. (2001) Identification and characterization of the heme-dependent heme acquisition system of Yersinia pestis. Infect Immun 69: 6707–6717.

45. Letoffe S, Redeker V, Wandersman C (1998) Isolation and characterization of an extracellular haem-binding protein from Pseudomonas aeruginosa that shares function and sequence similarities with the Serratia marcescens HasA haemophore. Mol Microbiol 28: 1225–1236.

46. Letoffe S, Omori K, Wandersman C (2000) Functional characterization of the HasA/PF hemeophore and its truncated and chimeric variants: determination of a region involved in binding to the hemeophore receptor. J Bacteriol 182: 4409–4419.

47. Darboeir L, Wandersman C (2004) Hemophore-dependent heme acquisition systems. In: Croza JL, Mey AR, Payne SM, ed. Iron transport in bacteria. Washington, D.C.: ASM Press. pp 38–47.
71. Arnoux P, Haser R, Izadi N, Lecroisey A, Delepierre M, et al. (1999). The crystal structure of HasA, a hemophore secreted by *Serratia marcescens*. Nat Struct Biol 6: 516–520.

72. Sterne M (1937). Avirulent anthrax vaccine. Onderstepoort J Vet Sci Animal Ind 21: 41–43.

73. Schurter W, Geiser M, Mathe D (1989). Efficient transformation of *Bacillus thuringiensis* and *B. cereus* via electroporation: transformation of acrystalliferous strains with a cloned delta-endotoxin gene. Mol Gen Genet 218: 177–181.

74. Johnsson B, Lofas S, Lindquist G (1991). Immobilization of proteins to a carboxymethyl-dextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. Anal Biochem 198: 268–277.