Hal Is a Bacillus anthracis Heme Acquisition Protein

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The metal iron is a limiting nutrient for bacteria during infection. Bacillus anthracis, the causative agent of anthrax and a potential weapon of bioterrorism, grows rapidly in mammalian hosts, which suggests that it efficiently attains iron during infection. Recent studies have uncovered both heme (isd) and siderophore-mediated (asb) iron transport pathways in this pathogen. Whereas deletion of the asb genes results in reduced virulence, the loss of three surface components from isd had no effect, thereby leaving open the question of what additional factors in B. anthracis are responsible for iron uptake from the most abundant iron source for mammals, heme. Here, we describe the first functional characterization of bas0520, a gene recently implicated in anthrax disease progression. bas0520 encodes a single near-iron transporter (NEAT) domain and several leucine-rich repeats. The NEAT domain binds heme, despite lacking a stabilizing tyrosine common to the NEAT superfamily of hemoproteins. The NEAT domain also binds hemoglobin and can acquire heme from hemoglobin in solution. Finally, deletion of bas0520 resulted in bacilli unable to grow efficiently on heme or hemoglobin as an iron source and yielded the most significant phenotype relative to that for other putative heme uptake systems, a result that suggests that this protein plays a prominent role in the replication of B. anthracis in hematogenous environments. Thus, we have assigned the name of Hal (heme-acquisition leucine-rich repeat protein) to BAS0520. These studies advance our understanding of heme acquisition by this dangerous pathogen and justify efforts to determine the mechanistic function of this novel protein for vaccine or inhibitor development.

The extracellular concentration of free iron in mammals is kept very low (~10^{-18} M) (7, 9, 10, 43) as a means to prevent the formation of insoluble aggregates and metal-induced free radicals (51). Iron concentrations are regulated in the body by high-affinity iron-binding proteins such as transferrin and ferritin, which transport and store iron, respectively (1, 43, 51, 52). However, approximately 80% of the host iron pool is contained in heme (iron protoporphyrin IX) (8), which is mostly bound to hemoglobin, the major oxygen carrier protein in vertebrates (42, 47). There are two principal systems by which bacteria satisfy their need for iron. One mechanism is to secrete siderophores, small molecules that bind ferric iron through high-affinity interactions (43, 51). This mechanism is unlikely to account for iron import from heme sources owing to the strict sequestration of the iron in and the low off rates from heme and hemoproteins. In this regard, bacteria have evolved sequestered or cell surface proteins that bind and acquire either free heme or heme from host hemoglobin (43, 51). The current paradigm is that imported iron is used as a cofactor for DNA synthesis and energy production, thereby promoting bacterial infection and replication in infected hosts (20, 51). Thus, the assimilation of iron from host heme is considered to be an important step in the maintenance and persistence of a bacterial infection.

Bacillus anthracis, a Gram-positive spore-forming bacterium and the causative agent of anthrax, grows to high densities during infection of mammalian hosts, implying that this pathogen contains efficient and versatile iron-scavenging mechanisms. In 2004, Cendrowski et al. reported the identification of two siderophore systems in this pathogen, asb (anthracis siderophore biosynthesis) and bac (Bacillus anthracis catechol) (13). Functional loss of asb but not bac reduced the growth of B. anthracis in iron-depleted medium and virulence in an inhalational mouse model of anthrax disease. This defect was traced to an inability of the asb mutant spores to outgrow inside macrophages (13), leaving open the question of how bacillus attains needed iron when it reaches blood or host tissues. In 2006, Maresso et al. identified an eight-gene system with similarities to the then recently described isd (iron-regulated surface determinant) locus, with one component, IsdC, being a heme-binding protein and important for bacillus growth on heme (34). However, the deletion of isdC did not attenuate virulence in guinea pigs, which suggested that Isd may be dispensable for anthrax disease progression (23). During a search for genes that are surface anchored by transpeptidase sortases, Gaspar et al. annotated that the B. anthracis Sterne strain contained a gene (bas0520) with an LGATG sorting signal (21). This gene is predicted to encode an N-terminal near-iron transporter (NEAT) domain, several internal leucine-rich repeat (LRR) regions, and a C-terminal sortase-like cell wall anchor (Fig. 1A). Analysis of this NEAT domain revealed sequence similarity to the NEAT domains of isdC (21%), isdX1 (29%), and bsK (49%) and the 5 NEAT domains of isdX2 (~29%) (this work). Furthermore, a recent study by Carlson at al identified the Ames homolog of bas0520 (gbau0552) as being highly upregulated under conditions of iron starvation, and deletion of the gene resulted in an approximately 110-fold increase in the 50% lethal dose of B. anthracis Sterne 34F2 in an inhalational model of anthrax disease (11). Hypothesizing that BAS0520 is the missing factor needed for heme assimilation during anthrax disease, we demonstrate here that BAS0520 is a heme-binding protein that takes heme from mammalian hemoglobin and is necessary for growth on heme and hemoglobin in low-iron environments.
environments. With these new findings, we have appropriately assigned the name of Hal (heme-acquisition leucine-rich repeat protein) to BAS0520 and propose efforts to examine whether Hal is suited for vaccine or drug development.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* strains (DH5 and BL21) were used for the cloning and amplification of hal and were grown in Luria broth (LB) supplemented with 50 μg/ml ampicillin (Fisher Scientific, Pittsburgh, PA). Wild-type *B. anthracis* strain Sterne (34F2) (46) was used for the growth studies and to generate a complete deletion in hal. For this purpose, hal was deleted by allelic replacement using the temperature-sensitive plasmid pLM4 (36). Briefly, genomic DNA of *B. anthracis* strain Sterne 34F2 was extracted using a Wizard DNA purification kit (Promega, Madison, WI), and 1,000 bp of the 5′ and 3′ sequences flanking the hal gene was PCR amplified with primer pairs hal-SmaI (5′-CCCGGGCAAAGATTTGACGACAGGATTTTACG-3′) and hal-KpnI (5′-GGTACCCAGGATATTGACGACAGGATTTTACG-3′) as well as hal-KpnI (5′-GGTACCCAGGATATTGACGACAGGATTTTACG-3′) and hal-SacI (5′-GGTACCCAGGATATTGACGACAGGATTTTACG-3′), respectively. Following ligation of the two fragments at the KpnI site, the 2-kb insert was cloned between the SmaI/SacI sites of pLM4 and plasmid DNA was amplified in the dam mutant *E. coli* strain K1077 prior to electroporation into *B. anthracis*. After transformation into strain Sterne, bacilli were first grown at 30°C (permissive temperature) on LB (20 μg/ml kanamycin) and then shifted to 43°C (restrictive temperature), followed by growth at 30°C to induce plasmid loss, thereby generating a *B. anthracis* /H9004 hal strain. Bacteria were examined for kanamycin resistance by plating on LB agar, and DNA sequencing was performed to verify the presence or absence of wild-type and mutant allele nucleic acid sequences. Isogenic deletions in bslK and all eight genes of the isd system were also constructed using this procedure.

Primer pairs 5′-flank (bsk-Smal [5′-GGTACCCAGGATATTGACGACAGGATTTTACG-3′] and bsk-KpnI [5′-GGTACCCAGGATATTGACGACAGGATTTTACG-3′]) and 3′-flank (bsk-KpnI [5′-GGTACCCAGGATATTGACGACAGGATTTTACG-3′] and bsk-SacI [5′-GGTACCCAGGATATTGACGACAGGATTTTACG-3′]) were used as a combination of PROSITE, SignalP, and KEGG. The arrowhead indicates the predicted site of signal peptidase cleavage. Hal is annotated as having a NEAT domain (residues 29 to 152, gray highlighted), 16 leucine-rich repeats (bold; labeled 1 through 16), and a C-terminal Gram-positive bacterium anchor (residues 1028 to 1070; underlined). (B) The NEAT domain of Hal was aligned with the eight NEAT domains of *B. anthracis*, the NEAT domain of Iba, a protein involved in iron uptake in *B. cereus*, and Shr from *Streptococcus pyogenes* (14, 39). Hal contains two regions common to all NEAT domains, an N-terminal 310 helix of 4 to 6 amino acids and a C-terminal β hairpin (bracketed regions). It is notable that, unlike heme-binding NEAT domains, Hal lacks a conserved tyrosine residue (instead, there is a phenylalanine) in the hairpin that is known to stabilize the interaction with the heme iron (hairpin, bold residues).
TTTACATTACGAGAACG-3') were used for isl depletion. For the isl deletion, the primers were 5' flank (isd-Smal [5'-GGTCTACTGTTG
GAATTAC-3']) and isd-KpnI (5'-GCTAAATTATGGGTAGAA
G-3') and 3' flank (isd-KpnI [5'-CGGATCTGACTCCTCASE-3']) and isd-SacI (5'-CCATGACGATGCTAATCCCAAT-3').

To construct the complementation strain, the full-length hal gene was PCR amplified from the genomic DNA of B. anthracis strain Sterne 34F2 using forward (5'-GATCCATGGAGCTTACACCTG
GGAGA-3') and reverse (5'-GATCCATGGCTACCTGTGGT
GTTGGATGATCCCTCTTCCTCCATTATAA-3') primer pairs with engineered Sall and SpII restriction enzyme sites, respectively. The PCR product was digested and cloned into a B. anthracis and E. coli shuttle vector, pUTE657 (27), such that expression was under the control of the isopropyl-β-D-thiogalactopyranoside-inducible hyper-spac promoter, generating phal. Ligation products were transformed into E. coli DH5α and selected on 100-μg/ml ampicillin agar plates. Plasmid DNA was then transformed into E. coli K1077 (with dam and dcm mutations) and then electroporated into B. anthracis Δhal strain to create B. anthracis Δhal phal.

**Protein purification.** The NEAT domain (amino acids 29 to 152) of Hal (Hal2) was purified as a glutathione S-transferase (GST) fusion protein similar to that described for other NEAT-domain proteins of B. anthracis (16, 19, 28, 34, 35, 48). Genomic DNA of B. anthracis strain Sterne 34F2 and primer pair hal2-BamHI forward (5'-GATCCATGGAGCTTACACCTGGGAGA-3') and reverse (5'-GTA CGTATGGCTACCTGTGGTGTTGGATGATCCCTCTTCCTCCATTATAA-3') were used to amplify DNA encoding the NEAT domain. DNA was then cloned into the BamHI restriction site of pGEX2TK to create pGST-HalN. GST-HalN was then transformed into E. coli BL21 (DE3) and grown in 100 ml of LB plus ampicillin at 37°C overnight. Cultures were then seeded into 1.5 liters of LB with ampicillin at 37°C and rotated at 250 rpm for 2 h, at which point isopropyl-β-D-thiogalactopyranoside (final concentration, 1.5 mM; Sigma, St. Louis, MO) was added to the culture, which was incubated for an additional 2 h. Cells were next sedimented by centrifugation at 6,000 × g for 10 min and resuspended in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4), and the cells were lysed by French press. The lysate was then centrifuged at 14,000 × g for 15 min, and the supernatant was sterilized with a 0.22-μm-pore-size cellulose filter. The filtrate was then subjected to affinity chromatography using a Bio-Rad silver kit.

**Association with hemoglobin.** The interaction between Hal2 with methemoglobin was determined by surface plasmon resonance (SPR) using a BIAcore 3000 biosensor (Amersham Biosciences). Holo-bovine methemoglobin (in 50 mM Tris-HCl, pH 7.0) was covalently coupled to a CM5 sensor chip at 25°C to a density of 3,600 response units (RU's) using amine chemistry as previously described (30, 38). Hal2 (50 to 200 μM) in HBS-N (0.01 M HEPES, 0.15 M NaCl, pH 7.4) was injected at 20 μl/min for 300 s at 25°C. Data were analyzed using BIAlEvaluation (version 4.1) software (Amersham Biosciences) after fitting the data to a 1:1 Langmuir binding model (15, 30, 38). Each concentration of Hal2 was injected twice, and each experiment was performed in triplicate.

**Growth studies.** RPMI (Life Technologies, Grand Island, NY) was chelated of iron by incubation with 2.5 g of Chelex-100 per 100 ml of medium for 12 h at 4°C (cRPMI). B. anthracis Sterne 34F2 wild-type, Δhal, ΔbslK, and Δisd (all containing empty plasmid pUTE657) strains and the complemented mutant (B. anthracis Δhal phal) were subcultured in LB overnight for 12 h at 37°C, and the bacterial pellets were washed with cRPMI and inoculated into cRPMI for 12 h at 37°C. Ten microliters of this culture at an optical density of 1.0 was then inoculated into 4 ml of fresh cRPMI with or without hemin (1, 10 μM) or hemoglobin (10, 100 μM), and growth (absorbance 600 nm) at 37°C was monitored for 2 to 16 h.

**RESULTS**

**Hal annotation and NEAT-domain purification.** The gene for hal is not localized to the isl locus and thus may code for a protein that is functionally separate from the Isd system. To gain insight into the function of hal, the amino acid sequence for this protein was analyzed using a combination of PROSITE, SignalP, and KEGG domain annotation programs (31, 40, 45) (Fig. 1A). The analysis revealed the presence of a putative N-terminal signal peptide (amino acids 1 to 21) and a proposed C-terminal Gram-positive bacterium anchor (GPA) region (amino acids 1028 to 1070). Two of these findings, along with the bioinformatic identification of Hal as a surface protein (the gene is designated BA0552 in the Ames strain of B. anthracis) (21), suggest that Hal is secreted through the general secretory pathway and tethered to the surface peptidoglycan, most likely by covalent attachment by a sortase transpeptidase. Furthermore, between the signal peptide and the GPA are 16 LRRs, with each repeat containing 21 amino acids. There are two clusters of these repeats; the first cluster contains 9 repeats and the second cluster contains 7, with each cluster separated by 171 amino acids. The exact function of bacterial LRRs is still unresolved, but several reports link them to protein-protein interactions (32, 33). The NEAT domain of Hal also contains two regions common to all NEAT domains, an N-terminal 39 helix of 4 to 6 amino acids and a C-terminal β hairpin consisting of 6 to 8 residues (Fig. 1B, bracketed regions). However, unlike heme-binding NEAT domains, Hal lacks a conserved tyrosine residue (instead, it possesses a phenylalanine) in the hairpin that is known to stabilize the interaction with the heme iron (Fig. 1B, bold residue in hairpin region), thus drawing into question whether Hal is a functional heme-binding protein. Consistent with its observed expression under low-iron conditions (11), a putative 19-residue Fur box (12, 26) is located 112 bp upstream of the transcriptional start.
suggests that some HalN copurifies with heme during its purification. Following removal of the GST tag, pure HalN and the distance of the box from the transcriptional start site is noted. These data suggest that the NEAT domain of Hal is sufficient in the extraction of heme from hemoglobin.

A Hal-hemoglobin interaction may facilitate heme removal. To determine if the observed heme transfer from hemoglobin to the NEAT domain of Hal is possibly mediated by a protein-protein interaction mechanism (active process), we assessed the potential binding of HalN to hemoglobin using surface plasmon resonance spectroscopy. Hemoglobin was coupled to a carboxymethyl surface, and increasing amounts of recombinant apo-HalN were infused over the surface. After fitting the data to a 1:1 binding model (30,38), as observed in Fig. 3D, dose-dependent increases in the response units, a measure of physical association, were observed for chambers containing HalN and hemoglobin. These results suggest that the NEAT domain of Hal directly engages hemoglobin, a process that possibly mediates heme acquisition from hemoglobin.

Loss of Hal compromises growth on heme and hemoglobin. The analysis of the NEAT domain of Hal indicates that Hal is a heme-binding protein that actively acquires heme from host hemoglobin. To determine whether this protein plays a functional role in enhancing the growth of B. anthracis in low, iron, high-heme environments, we generated an isogenic strain with a knockout in hal (Δhal) and tested this strain for growth on heme and hemoglobin relative to that of a wild-type strain and two additional knockouts: one that harbors a complete deletion in all eight genes of the isd-like locus (Δisd) and one that lacks the gene encoding bslK, the only other annotated NEAT protein in B. anthracis (ΔbslK). BslK is a surface protein that binds heme with a spectroscopy. As observed in Fig. 3A, GST-HalN incubated with hemoglobin yielded a higher Soret absorbance than GST-HalN incubated with a buffer control. Performing this experiment in triplicate and quantifying the ratio of the absorbance at 403 nm (heme) to that at 280 nm (GST-HalN), indicated that the difference in heme was approximately 3-fold between each group, suggestive of significant heme transfer to GST-HalN (Fig. 3B). As analyzed by SDS-PAGE and silver stain analysis, this effect was not due to hemoglobin contamination of the GST-HalN fraction (Fig. 3C; compare the migration of the eluted proteins to that of the hemoglobin standard). Taken together, these data indicate the NEAT domain of Hal is sufficient in the extraction of heme from hemoglobin.

### Table 1

<table>
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<tr>
<th>Gene</th>
<th>Fur box</th>
<th>Distance from ATG (bp)</th>
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<tr>
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<tr>
<td>hal</td>
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<td>isdG locus 2</td>
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*The similarity of each putative Fur box for hal, bslK, and isd with a consensus Fur box sequence was determined. Deviations from the consensus sequence are shaded gray, and the distance of the box from the transcriptional start site is noted.*
very high affinity and whose exact role in *B. anthracis* iron homeostasis is not known. At low concentrations of heme (1.0 μM) and hemoglobin (10 μM), there were observable differences in the extent of growth between the wild-type and Δhal strain; however, these differences were not significant (Fig. 4C and D). When the concentrations of heme (10 μM) and hemoglobin (100 μM) were increased, significant differences in the growth of Δhal compared to that of wild-type *B. anthracis* were observed, with the Δhal strain growing poorly on both iron sources (Fig. 4A and B). Each strain grew equally well when grown in nutrient-rich LB (Fig. 4E), and the growth defect on heme or hemoglobin was partially or fully restored, respectively, by the plasmid expression of full-length *hal* in the knockout strain. Together, these results suggest that the absence of *hal* leads to a growth defect of *B. anthracis* Sterne when heme or hemoglobin is the only iron source. Interestingly, the Δhal strain showed a higher growth defect than both the Δisd and ΔbsK strains on heme or hemoglobin, a result that suggests that Hal may play a more prominent or unique role in iron uptake from heme iron than these other NEAT-containing proteins or systems. These data also highlight the importance of further studying Hal and uncovering its full function in *B. anthracis* growth and heme acquisition. In agreement with the biochemical results presented in Fig. 2 to 4, these data provide the first evidence that Hal functions as a bacillus hemoprotein that specifically acquires heme from host hemoglobin.

**DISCUSSION**

Here we report the first functional characterization of **bas0520**, hereby named **hal** (heme-acquisition leucine-rich repeat protein), a gene important for inhalational anthrax and upregulated under low-iron conditions (11). Our findings suggest that (i) the NEAT domain of Hal (Hal₅₅) can bind heme, despite lacking a stabilizing tyrosine, (ii) Hal₅₅ can acquire heme from hemoglobin, (iii) a physical complex forms between Hal₅₅ and methemoglobin, and (iv) Hal is important for growth on heme and hemoglobin, seemingly more so than Isd or BsK, which may explain the marked defect in virulence (in comparison to that of wild-type infection) observed for an inhalational anthrax murine model using a Sterne strain lacking this protein (11).

It is now recognized that Gram-positive pathogenic bacteria utilize secreted or cell surface proteins containing NEAT domains to mediate the acquisition and import of heme (24, 25, 35, 37). *B. anthracis* contains five genes that harbor one or more NEAT domains (23, 28, 34, 35, 48). Three of these (isdX1, isdX2, and isdC) are part of the Isd locus, an array of eight genes proposed to encode a system that acquires heme from hemoglobin and delivers captured heme to the cell surface and across the cell membrane into the bacterial cytosol (19, 29, 34, 35). IsdX1 and IsdX2 are secreted proteins that extract heme from hemoglobin and deliver the heme to cell wall-bound IsdC (23, 29, 35). These are highly antigenic proteins, a finding that suggests an important role in heme-iron uptake during anthrax disease (22). Interestingly, a triple mutant lacking all three genes encoding the Isd proteins IsdX1, IsdX2, and IsdC was not reduced in virulence, as assessed by a guinea pig model of infection (23). The biochemical study of IsdX1 and IsdX2, however, has led to important insights into how bacillus NEAT-domain proteins mechanistically function, including the identification of NEAT-hemoglobin and NEAT-NEAT complexes (for transfer) and amino acids that facilitate heme and hemoglobin association (16, 29). Comparison of the properties of the NEAT domains of IsdX1 and IsdX2 to those of Hal yields interesting observations when considering the mechanism of heme coordination. For example, Hal has a phenylalanine in the fifth position of the heme-binding motif (YDKEF in Hal), a five-residue stretch of amino acids common to all proteins with NEATs (Fig. 1). For heme-binding NEAT domains, the first tyrosine in this region serves as the sixth axial ligand for the hemin iron (29). The second tyrosine hydrogen bonds to the first, an interaction that stabilizes the coordination (25, 41, 44). Both tyrosines are essential for heme stabilization in the NEAT domain, and all the NEAT-containing proteins from *B. anthracis* that contain the second tyrosine efficiently bind heme (25, 29, 41, 44). For example, the second NEAT domain of IsdX2 lacks the second tyrosine and consequently does not associate with heme (28, 29).

We modeled the structure (Fig. 5) of the Hal NEAT domain using our published structure of IsdX1 (Protein Data Bank [PDB] accession number 3SZ6) as a template. The IsdX1 model demonstrated that tyrosine 112, conserved in all bacillus NEAT-containing proteins and required for heme association, is the sixth axial ligand (the iron-to-hydroxyl distance is 2.2 Å). Interestingly, phe-

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**FIG 3** The NEAT domain of Hal can acquire heme from methemoglobin. (A) GST–Hal₅₅ (7.5 μM) was incubated with or without bovine methemoglobin (2.5 μM) for 30 min at 25°C, and proteins were separated by affinity chromatography using glutathione–Sepharose. Hb, hemoglobin. (B) Heme occupancy was calculated by taking the ratio of the Soret absorbance (heme, 403 nm) to the protein absorbance (280 nm) for three independent experiments. (C) Ten microliters of each elution was applied to an SDS-polyacrylamide gel, and GST–Hal₅₅ or hemoglobin was detected by silver stain. Lane M, molecular mass markers; lane ST, standard representing an aliquot of methemoglobin to provide a reference for hemoglobin’s mobility upon SDS-PAGE. (D) Apo-Hal₅₅ (50, 100, 200 nM) was infused over methemoglobin coupled to a carboxymethyl chip, and response units were measured for 300 s, followed by buffer infusion to monitor dissociation. Each concentration of Hal₅₅ was injected twice, and each experiment was performed in triplicate. One representative experiment is shown.

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L-tyrosine 116 is within 5 Å of this tyrosine, suggesting that the aromatic ring of F116 may function in a comparable way as a tyrosine aromatic ring, as found in other similar NEAT proteins (i.e., to stabilize the coordinating hydroxyl). Interestingly, there is a tyrosine on the opposite side of the heme-binding pocket in the 310 lip helix (ESYT). Ekworomadu et al. observed this region in IsdX1 to regulate heme and hemoglobin binding (16), and others have proposed that the lip helix is important due to its location over the heme-binding pocket (41,44). In Hal, the tyrosine residue in this region is in close proximity to the heme and extends above the distal side of the heme iron. A bistyrosine linkage, one on each side of the heme face, would be a novel finding for a bacterial protein with a NEAT domain. The binding of heme by Hal provides evidence that this rule is not strictly observed, meaning that the heme-iron association of this protein is likely different than that of other NEAT-containing proteins. The model also confirmed the presence of the lip-helix tyrosine directly over the heme iron. However, the distance between the lip-helix tyrosine hydroxyl and the heme iron is approximately 5 Å, seemingly too far to act as an iron ligand. Additional studies are required to determine the role of this tyrosine in heme binding and transfer; however, it is a possibility that the lip-helix tyrosine interacts with the heme porphyrin itself, strengthening the interaction between the heme and NEAT.

A previous study by Tarlovsky et al. discovered and characterized the B. anthracis protein BslK as possessing a single NEAT domain that localizes noncovalently to the surface of B. anthracis, most likely via the three S-layer homology (SLH) regions (48). BslK was shown to bind heme with high affinity and transfers heme to IsdC. The role of this protein in B. anthracis pathogenesis or heme uptake has not yet been tested. However, it is clear that deletion of bslK does not render B. anthracis unable to grow on heme or hemoglobin (Fig. 4). Interestingly, the /H9004 bslK strain grew better than the isogenic wild-type strain on hemoglobin (and heme at later time points; Fig. 4). This may be partially explained by considering the high affinity of BslK for heme (almost no heme dissociation is observed in a 24-h period) (48). In the absence of bslK, Hal and Isd systems have access to a larger heme pool, thus
possibly allowing these two systems to function more efficiently. This hypothesis is currently being tested.

Gaspar et al. searched the sequenced genome of *B. anthracis* for putative sortase-anchored proteins and described bas0520 as coding for a protein with a proposed GPA (21). In fact, Hal contains two of the three features that are common for cell wall-attached proteins, including a hydrophobic and polybasic stretch of residues at the C terminus. However, a conserved putative proline observed in the second position of the canonical sortase A pentapeptide recognition motif (LPXTG) is missing in the sequence for Hal (the closest resemblance is LGATG). More recently, Carlson et al. noted that hal is upregulated under low-iron conditions and found the Sterne strain lacking this gene to demonstrate an approximately 100-fold reduction in virulence in a mouse model of inhalational anthrax (11). The putative GPA motif, the proposed importance of hal in virulence, the presence of a heme-binding NEAT domain, and the above-mentioned results all suggest that Hal may be an important mediator of iron uptake from blood sources. Indeed, findings in this report include data that support this hypothesis and possibly provide a link between anthrax disease progression and heme assimilation by this protein from the bacterial cell.

In addition to a NEAT domain, Hal contains 16 LRR sequences. The presence of evenly spaced repeating units of leucine allows a protein or domain to fold into a horseshoe shape with the hydrophilic residues exposed to solvent (32, 33). The internal leucines create a hydrophobic core that may enhance interactions with proteins. LRRs are found in evolutionarily diverse proteins harboring various numbers of copies of this structural motif ranging from 1 to 30 (32, 33). Functional attributes of LRRs include the modulation of cell adhesion and signal transduction, especially in eukaryotic cells. However, bacterial LRRs have not been extensively studied. A well-noted Gram-positive bacterial LRR protein is *Listeria monocytogenes* internalin (InlA), a surface protein that binds the host receptor E cadherin, a process that mediates the invasion of this pathogen into epithelial cells, which suggests the importance of the LRR region in the invasion process (4). The *Streptococcus pyogenes* protein Srl, which also contains several LRRs, binds to type 1 collagen, thereby showing that LRRs participate in bacterial adhesion to tissue components (5). LRR regions are also associated with bacterial toxins or effectors (e.g., the type III secreted protein YopM from *Yersinia pestis*) (18). These studies suggest that at least some fraction of LRR-containing proteins in bacteria promote the interaction of bacteria with host tissues, a process that likely contributes to pathogenesis. Interestingly, although LRRs may function to drive protein–protein interactions, work here suggests that Hal’s NEAT domain alone is sufficient for the interaction with hemoglobin. This interaction may drive heme transfer, although proof of this would require knowledge of the rates of heme loss from hemoglobin and transfer to Hal. We also cannot rule out the possibility that the LRRs strengthen this association or allow binding to other serum hemoproteins, such as the hemoglobin–haptoglobin complex, hemopexin, or other iron-binding proteins (14, 41). Indeed, the IskA protein from *Bacillus cereus*, which contains a NEAT domain and several LRRs, binds heme, hemoglobin, and ferritin (14). We are currently evaluating the role of the LRRs in the heme acquisition functions of Hal.

One question raised by this study is why *B. anthracis* would require three heme uptake systems that evolved separately (Isd, BslK, and Hal). There are two possible reasons for this when considering an infection with *B. anthracis*. First, each system may be differentially expressed under certain conditions, in certain tissues, or at different times and thus plays a functional role at a different point during the infection. For example, deletion of the biosynthetic operon that encodes the siderophore anthrachelin (petrobactin) in *B. anthracis* results in a loss of spore germination in macrophages (13). A second possibility is that bacillus has evolved multiple systems to increase the overall rate of heme acquisition. In this regard, each component is functionally redundant, but collectively, the presence of multiple systems ensures that the cell maximizes its heme uptake capabilities. However, we cannot rule out the possibility that there are additional unrecognized functions for these proteins that impact our results, especially when one considers that Hal and BslK both contain uncharacterized protein domains (LRRs and SLH, respectively). It is clear from the growth studies whose results are presented in Fig. 4 that the uptake of heme in this pathogen may be a complex process with inputs from multiple systems. For example, in the absence of *isd*, bacilli seem to grow better than the wild-type strain on high concentrations of heme or hemoglobin. The simplest explanation is that in the absence of one system (BslK or Isd) at these concentrations of heme or hemoglobin, less overall heme is transported into the cell, leading to less heme toxicity and subsequently greater growth. Conversely, in the absence of a system, the cells may be sensing that they are iron starved and thus increase the expression of hal, which enhances growth. We are currently devising experiments to differentiate between these possibilities.

The results presented here allow us to suggest a basic model for the function of Hal (Fig. 6). Upon entry into a mammalian host, *B. anthracis* encounters a low-iron environment and Fur-mediated modulation of cellular responses. Infection occurs in the absence of sufficient heme. Hal may enter the cell via mannose receptor, an interaction that requires both proteins. Once the hal-encoded protein is inside the mammalian cell, it must find an alternate mechanism to transport heme from hemoglobin into Hal. In this regard, each system is functionally redundant, and collectively, the presence of multiple systems ensures that the cell maximizes its heme uptake capabilities. The combination of these two systems likely drives the invasion of this pathogen into epithelial cells, which suggests the importance of the LRR region in the invasion process (4). The *Streptococcus pyogenes* protein Srl, which also contains several LRRs, binds to type 1 collagen, thereby showing that LRRs participate in bacterial adhesion to tissue components (5). LRR regions are also associated with bacterial toxins or effectors (e.g., the type III secreted protein YopM from *Yersinia pestis*) (18). These studies suggest that at least some fraction of LRR-containing proteins in bacteria promote the interaction of bacteria with host tissues, a process that likely contributes to pathogenesis. Interestingly, although LRRs may function to drive protein–protein interactions, work here suggests that Hal’s NEAT domain alone is sufficient for the interaction with hemoglobin. This interaction may drive heme transfer, although proof of this would require knowledge of the rates of heme loss from hemoglobin and transfer to Hal. We also cannot rule out the possibility that the LRRs strengthen this association or allow binding to other serum hemoproteins, such as the hemoglobin–haptoglobin complex, hemopexin, or other iron-binding proteins (14, 41). Indeed, the IskA protein from *Bacillus cereus*, which contains a NEAT domain and several LRRs, binds heme, hemoglobin, and ferritin (14). We are currently evaluating the role of the LRRs in the heme acquisition functions of Hal.
repression of the gene is relieved. As Hal is passed through the Sec secretion system, the N terminus is recognized by one of the three B. anthracis sortases, possibly sortase A, thereby covalently attaching the protein to the cell wall. Upon exposure to blood or tissues, the NEAT domain temporally associates with released hemoglobin, and the heme is removed and passed to an unidentified transporter(s) associated with the cell membrane. The LRRs may aid this protein-protein interaction or, alternatively, initiate binding to additional host proteins. The import of heme promotes bacterial replication and, eventually, anthrax disease, presumably through the use of the iron in key cellular processes.

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REFERENCES


