Passively Released Heme from Hemoglobin and Myoglobin Is a Potential Source of Nutrient Iron for *Bordetella bronchiseptica*[▽]

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Colonization by *Bordetella bronchiseptica* results in a variety of inflammatory respiratory infections, including canine kennel cough, porcine atrophic rhinitis, and a whooping cough-like disease in humans. For successful colonization, *B. bronchiseptica* must acquire iron (Fe) from the infected host. A vast amount of Fe within the host is sequestered within heme, a metalloporphyrin which is coordinately bound in hemoglobin and myoglobin. Utilization of hemoglobin and myoglobin as sources of nutrient Fe by *B. bronchiseptica* requires expression of BhuR, an outer membrane protein. We hypothesize that heme is acquired by *B. bronchiseptica* in a BhuR-dependent manner after spontaneous loss of the metalloporphyrin from hemoglobin and/or myoglobin. Sequestration experiments demonstrated that direct contact with hemoglobin or myoglobin was not required to support growth of *B. bronchiseptica* in an Fe-limiting environment. Mutant myoglobins, each exhibiting a different affinity for heme, were employed to demonstrate that the rate of growth of *B. bronchiseptica* was directly correlated with the rate at which heme was lost from the hemoprotein. Finally, *Escherichia coli* cells expressing recombinant BhuR had the capacity to remove heme from solution. Collectively, these experiments provided strong experimental support for the model that BhuR is a heme receptor and *B. bronchiseptica* likely acquires heme during infection after passive loss of the metalloporphyrin from hemoglobin and/or myoglobin. These results also suggest that spontaneous heme loss by hemoglobin and myoglobin may be a common mechanism by which many pathogenic bacteria acquire heme and heme-bound Fe.

Pathogenic bacteria exhibit an essential requirement for iron (Fe). Although acquisition of this metal is essential for successful colonization by a pathogen, Fe does not usually occur in the form of the free metal in the host. Rather, Fe is compartmentalized in the fluids and tissues by Fe-binding proteins (e.g., lactoferrin, transferrin, etc.) or globins (14, 16, 20) or is sequestered within the cytoplasm of cells in the form of cytochromes, Fe/S-containing enzymes, and other Fe storage molecules. As a result, free Fe in the host is maintained at a concentration less than 10^{-18} M, which is far below the minimal concentration required to support bacterial growth (14, 20). In response to this strong selective pressure, bacteria have evolved a myriad of systems for acquiring Fe from the host's Fe-binding proteins.

Heme, the Fe-bound form of protoporphyrin IX, is the most abundant of the various Fe-containing molecules in the human body. As such, this metalloporphyrin is potentially a rich source of Fe for an invading pathogen. Yet, as free heme is toxic to cells (14, 20), the molecule is not allowed to circulate in the body. Rather, heme is bound in a variety of proteins, including hemoglobin (Hb), myoglobin (Mb), cytochromes, and catalase. In these hemoproteins, heme serves as a reversible O₂ binding site and redox center (23–27). Under certain conditions, however, heme is found transiently in the fluids and tissues of animals. Heme is frequently released from degraded

globins, rapidly partitions into low- and high-density lipoprotein complexes in the serum, and then is ultimately transferred to serum albumin and hemopexin for degradation recycling or excretion into bile by the liver (4, 14, 19, 20). Dilute Hb released by damaged erythrocytes rapidly dissociates into dimers, which in turn are scavenged by haptoglobin (4, 41). These Hb dimers (free or bound to haptoglobin) autooxidize rapidly and then spontaneously lose the metalloporphyrin at a rate that is ~40 times higher than that of intact Hb tetramers (26). As a result, small amounts of heme are released from Hb dimers even after scavenging by haptoglobin, and this heme is normally removed from the serum by hemopexin (4). On the cellular level, free heme, free Hb, and Hb-haptoglobin complexes (64) are cleared from serum by interaction with macrophages and epithelial cells via CD163, a surface receptor for these ligands (1, 5, 28, 47, 50, 57). Once internalized by CD163-expressing cells, heme is degraded by heme oxygenase (HO-1) (1, 50) into bilirubin (4, 62, 64). Inflammation, hemolysis, and vascular damage cause significant amounts of cellular damage (5, 13, 36, 47). In these cases, the heme-scavenging systems are overwhelmed and the concentrations of heme in the serum and in mucosal fluids rise to low but biologically significant levels (5, 13).

Two general classes of heme acquisition mechanisms have evolved in bacterial pathogens: contact-dependent systems and contact-independent systems (19). These uptake systems are both specific and promiscuous with respect to the hemoprotein ligands which are recognized. In contact-dependent systems, hemoproteins are directly bound by cell surface receptors. *Haemophilus influenzae* expresses multiple systems for heme acquisition, as first shown by Stull (56). Hb and Hb-haptoglo-

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TABLE 1. Strains and plasmids

Strain or plasmid	Description	Source or reference
<i>E. coli</i> strains		
DH5 α F'Kan	Cloning strain, Kan ^r	Invitrogen
BL21(DE3)	Expression strain	Stratagene
<i>B. bronchiseptica</i> strains		
RB50	Wild-type strain	J. Miller
RBB4	Internal <i>bhuR</i> truncation in RB50	S. K. Armstrong (unpublished)
Plasmids		
pGEM-T	PCR fragment cloning vector, Amp ^r	Promega
pET21a	Expression vector, Amp ^r	Novagen
pMOX13	PCR-amplified DNA fragment encoding <i>bhuR</i> ligated into pGEM-T	This study
pMOX14.1	NdeI/XhoI fragment from pMOX13 encoding <i>bhuR</i> ligated into pET21a	This study

bin are bound by Hbp proteins (17, 22, 38, 39); hemin is released and transported into the cell by several outer membrane hemin receptors, such as TdhA (3), Hup (39), and Hxu (51). *Moraxella catarrhalis* exhibits a contact-independent method for hemin uptake in which HmuA, an outer membrane receptor, has direct binding affinity for the metalloporphyrin (18). *Serratia marcescens* utilizes a combination of contact-dependent and contact-independent systems for heme acquisition (11, 29, 32, 33). In *S. marcescens*, heme is removed from host hemoproteins by HasA, a secreted protein hemophore. The HasA-heme complex is subsequently bound by the cognate HasR surface-associated hemin receptor. Alternatively, *S. marcescens* may acquire hemin directly by interaction of the metalloporphyrin with HasR (11, 61). Other pathogens secrete proteases that degrade hemoproteins to release heme. *Porphyromonas gingivalis* secretes gingipain proteases which digest various hemoproteins to release heme for uptake by HmuR (43, 45). To satisfy its hemin Fe requirement, *Prevotella intermedia* also degrades Hb using a presumed secreted protease (21).

Inflammatory responses are often credited with eliciting release of hemoproteins at the site of a bacterial infection. *Bordetella bronchiseptica* infections are characterized by an acute inflammatory response which is modulated by initiating Th17 inflammatory immune reactions. This modulation elicits an acute sustained inflammation with an associated increase in secretion of interleukin-17 by macrophages (52). This acute response is exacerbated through the recruitment of secondary inflammatory agent-producing cells. *B. bronchiseptica* also expresses BopC, a toxin that has been shown to elicit erythrocyte lysis in vitro (31). In *Bordetella pertussis*, adenylate cyclase toxin suppresses immune responses by selectively targeting macrophages and other monocytes which express CD163 (10). Lower expression of CD163 would be expected to suppress the clearance of heme and hemoproteins from the site of infection. Adenylate cyclase toxin, also secreted by *B. bronchiseptica*, should have a similar effect on macrophages and monocytes during infection by this pathogen. Thus, *B. bronchiseptica* likely induces release of cellular hemoproteins into the local site of infection, suppresses their clearance, and utilizes the released heme as an iron source.

B. bronchiseptica (58–60) utilizes both hemin and Hb as sources of nutrient Fe. Acquisition of hemin and Hb by *B. bronchiseptica* is dependent upon expression of *bhuR*, a gene

encoding an outer membrane protein (58–60). Although it is well established that *B. bronchiseptica* utilizes hemin and Hb as sources of nutrient Fe, the precise mechanism by which this bacterium acquires hemin from these hemoproteins in a BhuR-dependent manner has not been elucidated. While it is possible that BhuR binds both Hb and hemin, this model is in conflict with the common observation that most receptors either fail to recognize more than one type of ligand or interact with only a small number of structurally similar ligands. An alternative model is that BhuR binds to Hb by interacting directly with one or more of the four coordinately bound hemes. This model, however, is also unlikely since only the propionates of heme are surface exposed; most of the metalloporphyrin is buried in the interior of the globins and is not directly accessible to BhuR.

The apparent conundrum is potentially resolved when the stabilities of Hb and Mb are considered. Within erythrocytes and myocytes, heme remains bound to Hb and Mb, respectively, in a concentration-dependent equilibrium. The stability of the coordination bonds between heme and the globins is optimized by cellular redox enzymes which retain the Fe within the heme in a ferrous oxidation state (20, 26, 35). Release of Hb and Mb into plasma or mucosal fluids by cell lysis exposes the hemoproteins to an oxidative environment where heme is oxidized to hemin (20, 23–27, 35, 37). The affinity of Hb and Mb for hemin is significantly lower than the affinity of either protein for heme (23–27). Thus, release of Hb and Mb into plasma or into mucosal fluids from lysed cells promotes spontaneous dissociation of hemin from both hemoproteins.

In this study, growth experiments were conducted using Hb, Mb, and a unique pair of Mb mutants with markedly different rates of hemin release to demonstrate that *B. bronchiseptica* exploits the natural instability of Hb or Mb as an indirect means of acquiring hemin-bound Fe during infection. No direct contact between the organism and Hb is needed to sustain growth. Rather, only extracellular dissociation of heme from the hemoproteins is required.

MATERIALS AND METHODS

Strains, plasmids, and reagents. Bacterial strains and plasmids are described in Table 1. Antibiotics were obtained from Becton Dickinson (Sparks, MD) and Sigma Biochemicals (St. Louis, MO). Restriction enzymes were purchased from Fermentas Life Sciences (Hanover, MD).

Growth with various hemoproteins. Single-colony isolates of *B. bronchiseptica* RB50 and RBB4 (a gift from S. K. Armstrong) (Table 1) were cultured in 3 ml of brain heart infusion (BHI) (Becton Dickinson) broth at 37°C. A 1:100 dilution of each subculture was incubated for 12 h in 10 ml of BHI broth supplemented with 50 μ M deferrated ethylenediamine-*D*-(*o*)-hydroxyphenylacetic acid (EDDHA) (The Complete Green Company, El Segundo, CA) to Fe stress the bacteria. Bacteria were considered to be Fe stressed if the final culture density was ~50% of that of cells grown in Fe-replete BHI broth. One hundred microliters of Fe-stressed bacteria was used to inoculate 10 ml of Fe-restricted BHI broth containing the appropriate antibiotics and 500 μ M EDDHA to which hemin, recombinant wild-type Mb, Mb^{H64L} (23, 24), Mb^{V68T} (23, 24), human Hb (Sigma Biochemicals), rabbit Hb (Sigma Biochemicals), porcine Hb (Sigma Biochemicals), bovine liver catalase (Sigma Biochemicals), or canine cytochrome *c* (Sigma Biochemicals) was added to a final concentration of 5 μ M with respect to the Fe content. Three replicate cultures were prepared for each condition. The optical densities at 600 nm (OD₆₀₀) of the cultures were measured at various time intervals as a relative measure of growth using a Beckman Coulter DU640 spectrophotometer (Fullerton, CA) or a Titertek Multiskan Plus enzyme-linked immunosorbent assay plate reader (Thermo Electron Corp., Milford, MA). Culture densities were calculated as follows: final density = endpoint culture optical density – initial culture density.

Sequestration experiments. Six-centimeter strips cut from Spectra/Por molecular porous membrane tubing (18 by 11.5 mm; 3,000-molecular-weight cutoff; Spectrum Laboratories, Rancho Dominguez, CA) were immersed in 200 ml of deionized H₂O and autoclaved for 20 min at 121°C. Fe-stressed bacteria were cultured as described above. Two hundred microliters of an overnight Fe-stressed culture was used to inoculate 20 ml of BHI broth containing appropriate antibiotics and 500 μ M EDDHA. A 1-ml sterile solution of BHI broth with 500 μ M EDDHA containing 105 μ M (relative to the content of Fe) of hemin, recombinant sperm whale Mb (24, 25, 27), or human Hb was added to a dialysis bag, which was sealed at both ends with sterile dialysis tubing clamps. When the bag was immersed in a 20-ml culture of Fe-stressed *B. bronchiseptica*, a final hemin concentration of 5 μ M would be attained in the culture at theoretical equilibrium. The OD₆₀₀ of the cultures were determined at various time intervals.

Cloning and expression of *bhuR*. A fragment of DNA encoding *bhuR* of *B. bronchiseptica* RB50 was amplified by PCR from chromosomal DNA using the following two amplifiers (Integrated DNA Technologies, Inc., Coralville, IA): forward primer 5'-CCCAAGCTTAAAGGAGATATACATATGGTTTACGCTT GCGATCAG-3' (HindIII site underlined; consensus *Escherichia coli* Shine-Dalgarno sequence in bold type) and reverse primer 5'-TCCGCCCTCGAGTC TAGATCAATACTGGTAGGTCAACGAT-3' (XhoI site underlined; XbaI site in bold type). The PCR conditions were as follows: a reaction mixture containing 1 pM each of the forward and reverse primers, 10 mM mixed deoxyribonucleotide triphosphates (Fermentas), 10% dimethyl sulfoxide (DMSO) (Sigma Biochemicals), and 2.5 U of Easy-A polymerase (Invitrogen, Grand Island, NY); and 30 cycles of 95°C for 30 s for denaturation, 50°C for 45 s for annealing, and 72°C for 4.5 min for extension. PCR amplification products were ligated into pGEM-T (Promega Corporation, Madison, WI), and the ligation mixtures were used to transform DH5 α F' Kan (Invitrogen). Transformants containing plasmids were selected by plating the transformed cells on LB agar containing 50 μ g/ml kanamycin and 200 μ g/ml ampicillin. One of the transformants contained a plasmid, later designated pMOX13, which harbored the amplified *bhuR* gene, which was subsequently confirmed by nucleotide sequencing (Table 1). An NdeI/XhoI DNA fragment of pMOX13 encoding *bhuR* was directionally cloned into pET21a (Novagen, EMDBiosciences, La Jolla, CA). After verification by restriction mapping and nucleotide sequencing, pMOX14.1 (Table 1) was transformed into BL21(DE3), an expression strain of *E. coli* (Stratagene, Cedar Creek, TX).

To express recombinant BhuR, BL21(DE3)(pMOX14.1) was cultured for 8 h at 37°C in LB broth supplemented with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) (Sigma Biochemicals). Expression of recombinant BhuR from BL21(DE3)(pMOX14.1) was confirmed by immunoblotting using an anti-BhuR antiserum (see below).

Antiserum production, polyclonal antibody purification, and immunoblotting. Expression of BhuR in *B. bronchiseptica* or of recombinant BhuR in BL21(DE3)(pMOX14.1) was detected in whole cells and outer membranes using cross-reactive peptide-specific rabbit antibodies (Sigma Genosys, St. Louis, MO) directed against the amino acid sequence N-GENSTKKTTRERVSVDYQY N-C, which is found in BhuR of *Bordetella avium*. This amino acid sequence is highly conserved in BhuR expressed by *B. bronchiseptica*. Anti-BhuR polyclonal antiserum was enriched for BhuR-specific antibodies using a Sulfolink (Pierce, Rockford, IL) peptide affinity column. Agarose beads coupled to the *B. avium* BhuR peptide were packed into a 4-ml column. Two milliliters of antiserum,

diluted and dialyzed in phosphate-buffered saline (PBS) (pH 7.4), was passed through the column, and the bound enriched peptide-specific antibodies were eluted with 3 M glycine (pH 3.0). Eluates were neutralized with 1 M Tris-HCl (pH 8.0) and dialyzed against PBS (pH 7.4). Goat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies (Southern Biotech, Birmingham, AL) were used at a 1:8,000 dilution. Mouse monoclonal antibodies to maltose-binding protein (MBP) were used at a 1:2,000 dilution (New England BioLabs, Ipswich, MA). Rabbit anti-mouse immunoglobulin G (heavy and light chain) horseradish peroxidase-conjugated secondary antibodies were used at a 1:8,000 dilution (Southern Biotech).

Binding of hemin to BhuR. BL21(DE3) cells expressing BhuR of *B. bronchiseptica* RB50 encoded in pMOX14.1 were cultured with agitation at 37°C until early log phase (approximately 2 h), at which time the broth was supplemented with IPTG at a final concentration of 1 mM. Cultures were incubated for an additional 8 h to induce maximal expression of BhuR. Induced cells pelleted by centrifugation (12,096 $\times$ g, 10 min, 4°C) were washed three times by centrifugation in 30 ml of PBS (pH 7.4) to remove BHI broth and debris. The OD₆₀₀ of the resuspended cells was adjusted to 1.1 to 1.3 by addition of PBS (pH 7.4). Two hundred microliters of a 20 μ M solution of hemin prepared in 10% DMSO was added to 800 μ l of the cell suspension, which was gently shaken at room temperature for 1.0 to 1.25 h. After incubation, the supernatant was separated from the cells by centrifugation (16,000 $\times$ g, 10 min, 4°C). The amount of hemin remaining in the supernatant after adsorption by the cells, as determined spectrophotometrically at 400 nm (OD₄₀₀), was considered to be a relative measure of hemin binding (43).

Preparation and enrichment of outer membranes. BL21(DE3) recombinants transformed with pMOX14.1 or pET21a were cultured overnight after IPTG induction for maximum BhuR expression. Cells in 20 ml of a bacterial culture were pelleted by centrifugation (12,096 $\times$ g, 10 min, 4°C). The cell pellet was resuspended in 25 ml of 10 mM HEPES buffer (pH 7.4) (American Biochemicals, Inc., Niagara Falls, NY). To the resuspended cells, polymethylsulfone (Sigma Biochemicals) was added to a final concentration of 100 mM. Cells were sonicated on ice three times for 3 min using a Branson 450 Sonifier (Bronson, Plainview, NY) with a microtip (power setting 7, 80% duty cycle). Additional polymethylsulfone was added between sonications to obtain a final concentration of 10 mM. Sonicates were centrifuged twice in 50-ml Oak Ridge tubes (12,096 $\times$ g, 10 min, 4°C) to pellet undisturbed cells. The clarified sonicates were centrifuged (100,000 $\times$ g, 1 h, 4°C) to pellet total membranes, which were treated for 1 h at room temperature with 1% sodium dodecyl sulfate (SDS) (Sigma Biochemicals)-10 mM HEPES (Ameresco, Solon, OH) buffer to dissolve plasma membranes and enrich outer membranes. After the outer membranes were pelleted by centrifugation (100,000 $\times$ g, 1 h, 4°C), the pellet was treated for 1 h at room temperature with SDS-HEPES buffer and centrifuged (100,000 $\times$ g, 1 h, 4°C) to pellet the outer membranes. The washing procedure was repeated once more to remove any residual plasma membrane components from the outer membranes. The enriched outer membranes were resuspended in 200 μ l of 10 mM Tris (pH 8.0) and stored at -20°C. The amount of protein in the outer membranes was measured using a Micro BCA protein assay kit (Pierce) and a bovine serum albumin standard included with the kit. Ten micrograms of total protein from each fraction obtained during purification (whole cells, clarified sonicates, total and enriched outer membranes) were immunoblotted using a 1:500 dilution of anti-BhuR polyclonal antiserum to confirm the presence or absence of BhuR.

Protease treatment of recombinant cells expressing BhuR. To confirm that BhuR was associated with the outer membrane, protease digestion methods in which whole cells of BL21(DE3)(pMOX14.1) were digested with increasing amounts of trypsin or proteinase K were employed.

In brief, a 1,000-U/ml activity stock solution of bovine trypsin (Sigma Biochemicals) was prepared in 10 mM Tris (pH 8.0) and serially diluted to obtain 100, 10, and 1 U/ml of activity in 10 mM Tris buffer. Solutions were filter sterilized using a 0.22- μ m syringe tip filter (Whatman, Dassel, Germany). Recombinant cells were pelleted by centrifugation (16,000 $\times$ g for 10 min) and washed three times in PBS (pH 7.4) by repeated centrifugation in 30 ml of PBS (pH 7.4) to remove BHI broth and debris. The OD₆₀₀ of the resuspended cells was adjusted to 0.8 by addition of PBS (pH 7.4). A 1-ml aliquot of these cells was transferred to a 2-ml microfuge tube and centrifuged to remove the supernatant. The cell pellet was resuspended in 1 ml of 1-, 10-, 100-, or 1,000-U/ml trypsin buffer and incubated in a 37°C water bath for 15 min. Protease inhibitor cocktail (Bio-Rad, Hercules, CA) was added to a final concentration of 1 mM, and the cells were pelleted by centrifugation and washed in PBS (pH 7.4). The digested cells were pelleted again by centrifugation (16,000 $\times$ g, 10 min, 4°C) and washed three times in PBS (pH 7.4) by repeated centrifugation to remove debris. The digested cells were resuspended in 1 ml of PBS (pH 7.4) with protein

FIG. 1. *B. bronchiseptica* RB50 utilizes a variety of hemoproteins as sources of nutrient Fe. Fe-stressed strain RB50 and the isogenic *bhuR* mutant RBB4 were inoculated into BHI broth supplemented with 500 μ M EDDHA containing 5 μ M hemin or an equimolar concentration (with respect to hemin) of hemoproteins. Relative cell densities of the cultures grown to stationary phase were determined spectrophotometrically by measuring the absorbance at 600 nm. All conditions were replicated in triplicate. The error bars indicate one standard deviation from the mean. Fe+, untreated BHI broth; Fe-, BHI broth supplemented with 500 μ M EDDHA; pHb, porcine Hb; rHb, rabbit Hb; hHb, human Hb; swMb, sperm whale Mb; cCyto c, canine cytochrome c; bCat, bovine catalase.

electrophoresis sample buffer containing 2-mercaptoethanol (Sigma Biochemicals). Twenty microliters of solubilized cells was resolved in a 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) gel by electrophoresis. Resolved proteins were transferred to nitrocellulose (Whatman-Schleicher and Schuell, Dassel, Germany) for immunoblotting. Recombinant cells were also immunoblotted for expression of endogenous *E. coli* periplasmic MBP using a 1:2,000 dilution of anti-MBP monoclonal antibody (New England Biolabs) to confirm that treatment with trypsin did not release periplasmic or cytoplasmic proteins via cell lysis.

Recombinant cells were also treated with 1 mg/ml of proteinase K (Sigma Biochemicals) to denude the bacterial surface of all protein components. In brief, recombinant cells expressing pMOX14.1 (Table 1) were induced with IPTG, incubated for maximal expression of BhuR, and prepared for hemin-binding experiments, as previously described. Three replicates (1 ml) of optical density-adjusted recombinant cells were utilized. The cells were resuspended in 1 ml of a 1- μ g/ml solution of proteinase K prepared in 10 mM Tris (pH 8.0) buffer. Recombinant cells were incubated for 30 min in a 37°C water bath in 2-ml microcentrifuge tubes. Protease inhibitor cocktail (Bio-Rad) was added to a final concentration of 1 mM, and the cells were centrifuged to pellet them and washed in PBS (pH 7.4). The washed proteinase K-digested cells were resuspended in 800 μ l of PBS (pH 7.4). To the resuspended cells, 200 μ l of a 20 μ M hemin stock solution containing 10% DMSO was added and allowed to bind. Following 1 h of incubation to allow binding of hemin, the OD₄₀₀ of the supernatants were determined to measure the amount of hemin which was adsorbed by the recombinant cells.

Statistical analysis. Data were compared by use of analysis of variance and *t* tests (Tukey), using Graph Pad Prism 3.0 and Graph Pad InStat software (GraphPad Software, Inc., San Diego, CA).

Nucleotide sequencing. Nucleotide sequencing of pMOX13 and pMOX14.1 was performed by the Roswell Park Cancer Institute Biopolymer Facility.

RESULTS

***B. bronchiseptica* utilizes Hb and Mb to satisfy a nutritional requirement for Fe.** Although bordetellae are capable of utilizing Hb, Mb, and hemin to satisfy the bacteria's Fe requirement (12, 40, 58–60), the means by which Fe is obtained from these molecules have not been closely examined. Since *B. avium* exhibited the capacity to grow in culture medium containing hemin, Mb, and Hb from avian, human, and bovine hosts as sources of nutrient Fe, it was deemed likely that *B. bronchiseptica* would also utilize these molecules for growth. To examine which of these molecules were capable of sustaining growth of *B. bronchiseptica*, bacteria were cultured in Fe-restricted BHI broth which was supplemented with equimolar amounts with respect to Fe of hemin or various hemoproteins. As expected, *B. bronchiseptica* RB50 grew robustly when hemin

was offered as the sole source of nutrient Fe and when the broth was supplemented with human Hb, porcine Hb, or rabbit Hb (Fig. 1), in agreement with the observation of Vanderpool and Armstrong that *B. bronchiseptica* could utilize human Hb as a heme Fe source (58). Extending these observations, RB50 was also cultured in BHI broth supplemented with recombinant sperm whale Mb. Growth of RB50 was also supported by this hemoprotein. In contrast, growth of RB50 was not supported by supplementation of the BHI broth with canine cytochrome *c* or bovine liver-derived catalase (Fig. 1).

To establish that the acquisition of Fe from heme and from the hemoproteins required BhuR, growth experiments were repeated using RBB4, an isogenic mutant of RB50 in which the gene encoding *bhuR* has been functionally inactivated (a gift from S. K. Armstrong). Growth of RBB4 was not supported by any of the sources of Fe which supported growth of RB50 (Fig. 1). Although it has been previously reported that *B. bronchiseptica* exhibited the capacity to utilize Hb as a source of nutrient Fe (58), these studies established that this bacterium also has the capacity to utilize Mb. Furthermore, the acquisition of Fe from heme, Hb, and Mb required expression of *bhuR*.

The observation that the various Hb, Mb, cytochrome *c*, and catalase differentially supported growth of *B. bronchiseptica* suggested a mechanism by which the bacterium might acquire heme. In Hb and Mb, heme is coordinately and reversibly bound to a histidine, designated HisF8, which is located at position 8 along the F-helix within the hydrophobic heme pocket (23–27). Oxidation of the Fe atom and exposure of the heme pocket to water hydrolyze the HisF8–Fe coordination bond. This reaction promotes a spontaneous loss of heme from the hemoprotein (23–27). Hemin release from Hb and Mb occurs more readily in dilute aqueous solutions. In the case of Hb, dilution has the additional effect of promoting dissociation of the Hb tetramer into dimers. These $\alpha\beta$ dimers exhibit a much higher rate of autooxidation and heme loss than the intact Hb tetramer (19, 23–27). In contrast, heme in cytochrome *c* is covalently attached to the protein by thioether linkages, which prevent heme loss even after denaturation of the protein. In catalase, heme is completely embedded within the protein and is very tightly bound by a series of coordination

FIG. 2. Direct cellular contact with Hb or Mb is not required to support growth of RB50. Flasks containing 20 ml of Fe-depleted BHI broth supplemented with 500 μ M EDDHA were inoculated with a 1:100 dilution of stationary-phase, Fe-stressed RB50. A 1-ml aliquot of a solution containing an equimolar amount (5 μ M with respect to Fe) of hemin, human Hb (hHb), or sperm whale Mb (Mb) or a control solution without Fe supplementation (500 mM EDDHA) (–Fe) was added to a 3,000-molecular-weight cutoff dialysis bag which was suspended in the culture. After overnight incubation at 37°C, the cell density of each culture was determined spectrophotometrically by measuring the absorbance at 600 nm. All conditions were replicated in triplicate. The error bars indicate one standard deviation from the mean.

bonds (7, 15, 48). The observation that *B. bronchiseptica* utilizes Hb and Mb, but not cytochrome *c* or catalase, as sources of nutrient Fe (Fig. 1) strongly suggested that this bacterium exploits the propensity of Hb and Mb to spontaneously release heme as a passive means to acquire heme-bound Fe for growth. In such a case, it would not be expected that direct contact between the bacterium and the hemoproteins would be required by *B. bronchiseptica* for acquisition of heme-bound Fe.

Direct contact with hemoproteins is not required to satisfy the Fe requirement of *B. bronchiseptica*. To determine whether direct contact between Hb or Mb and the bacteria was required for utilization of hemin Fe, equivalent molar amounts (with respect to Fe) of hemin, Hb, and Mb were sequestered within 3,000-molecular-weight cutoff dialysis bags which were immersed into Fe-starved cultures of RB50. While hemin (molecular mass, 634 Da) would be expected to freely diffuse across the dialysis membrane, Mb, Hb, and other molecules larger than 3 kDa would be retained within the bags. The membrane would also be a barrier to preclude entry into the bags of peptide hemophores or proteases with molecular masses greater than 3 kDa. After overnight incubation, robust growth of RB50 was observed in cultures incubated with hemin-containing bags but not in control cultures incubated with bags in which hemin-bound sources of Fe were absent (Fig. 2). Growth was also observed in cultures of RB50 incubated with bags containing either Hb or Mb. Although less robust than the growth in cultures incubated with bags containing hemin, the growth of RB50 in the presence of Hb or Mb was much greater than the growth in the absence of all sources of heme (Fig. 2) ($P < 0.0001$). As a control experiment to confirm the integrity of the bags, mock cultures were incubated with bags containing Hb and Mb. After overnight incubation, aliquots of the supernatants outside the bags were analyzed for hemoprotein. These assays confirmed that the supernatants contained ≤ 1 nM hemoprotein, a level of hemoprotein which was below the mini-

mal concentration of Hb or Mb required to support growth of RB50 (data not shown).

These experiments demonstrated that direct cellular contact with Hb or Mb was not required for acquisition of Fe from these hemoproteins. The results also strongly indicated that *B. bronchiseptica* likely utilizes hemin which is spontaneously released by natural processes from the two hemoproteins. The results also indicated that, unless the molecules have molecular masses less than 3 kDa, *B. bronchiseptica* does employ hemophores or proteases to remove heme from Hb or Mb (19, 32, 33).

Growth of RB50 is correlated with the rate of hemin loss from Mb. Since direct contact with Hb and Mb was not required for acquisition of heme, additional experiments were performed to confirm that *B. bronchiseptica* exploits the propensity of these hemoproteins to spontaneously release heme. Two sperm whale Mb mutants, which differ in their capacity to retain hemin (24), were employed as sources of nutrient Fe in growth experiments. The order of the rates of hemin loss from the Mbs is Mb^{V68T} < wild-type Mb < Mb^{H64L} (24, 25, 27). Overnight Fe-stressed RB50 cells were subcultured in BHI broth supplemented with 5 μ M of various hemin sources, including hemin, wild-type sperm whale Mb, Mb^{H64L}, and Mb^{V68T}, as the sole sources of Fe. The cell densities of the cultures were determined spectrophotometrically at 600 nm at various time points as a relative measure of growth. These experiments revealed that the growth rate of RB50 was highest when free hemin was employed as the sole source of Fe (Fig. 3). The growth rate of RB50 in broth supplemented with Mb^{H64L} was higher than the growth rates of cultures containing wild-type Mb. In contrast, cultures supplemented with Mb^{V68T} exhibited less growth than cultures of the bacterium grown in broth supplemented with wild-type Mb. Neither of the cultures containing mutant Mb attained the stationary-phase culture density that was attained in a culture supplemented with an equivalent amount of hemin. This response

FIG. 3. Hemin-dependent growth of RB50 is dependent upon spontaneous loss of hemin from Mb. Ten milliliters of Fe-depleted BHI broth supplemented with 500 μ M EDDHA containing either no supplement ($-Fe$), hemin, wild-type Mb (Mb), Mb^{H64L}, or Mb^{V68T} was inoculated with a 1:100 dilution of stationary-phase Fe-stressed RB50. The relative affinities of the Mb for heme were Mb^{V68T} > Mb > Mb^{H64L} (24, 25, 27). The cell densities of the stationary-phase cultures were determined spectrophotometrically at a wavelength of 600 nm at various time points. All conditions were replicated in triplicate. The error bars indicate one standard deviation from the mean.

was likely due to formation of Mb into Heinz-like bodies which precipitated from solution after long periods of incubation that decreased the total Mb-bound hemin available to the bacteria.

To demonstrate that the reductions in growth in these experiments were not due to differential death of the cells in cultures containing wild-type or mutant Mb, stationary-phase cells grown in the presence of the hemoproteins were spiked with 5 μ M hemin and analyzed for restoration of growth. In all cases, growth of the stationary-phase cultures was restored by hemin supplementation (data not shown).

Taken together, these observations were consistent with a model in which *B. bronchiseptica* utilizes soluble hemin which is spontaneously lost by Mb. Since Hb has a similar chemistry with respect to heme loss and retention, it is hypothesized that *B. bronchiseptica* also utilizes heme released by this hemoprotein. Furthermore, the higher rates of growth observed in the sequestration experiments for cells incubated with Hb than for cells incubated with wild-type Mb correlate with the 10- to 30-fold-higher rates of hemin dissociation from dilute and oxidized Hb than from oxidized Mb (Fig. 2) (26).

Recombinant BhuR binds hemin. Growth experiments using RB50 and RBB4 and growth experiments in which Hb and Mb were confined within dialysis bags confirmed that *B. bronchiseptica* (i) requires BhuR for utilization of hemin, Hb, and Mb (Fig. 1), (ii) does not require direct contact with Hb or Mb for Fe acquisition, and (iii) likely obtains hemin which is spontaneously lost from the two hemoproteins (Fig. 2). Thus, it was hypothesized that BhuR directly binds hemin. Attempts to visually detect binding of hemin to Fe-starved RB50 in a *bhuR*-dependent manner failed, presumably due either to rapid transport of the metalloporphyrin into the cell or to our failure to identify conditions for stably retaining bound heme to the receptor (data not shown). Also, hemin was found to nonspecifically bind to *B. bronchiseptica* cells, which made it difficult to

measure specific binding of hemin to the cell surface. As an alternative strategy, an indirect hemin adsorption method was employed (43), using recombinant BhuR expressed in a heterologous host cell. Preliminary experiments to screen a variety of strains of *E. coli* revealed that BL21(DE3) exhibited the lowest level for nonspecific binding of heme (data not shown). BL21(DE3) expressing recombinant BhuR from pMOX14.1 was incubated in a solution containing 20 μ M hemin. BL21(DE3) containing only the vector plasmid (pET21a) was used as a negative control. Following incubation, cells were pelleted by centrifugation, and the amount of soluble hemin remaining in the cleared supernatant was determined spectrophotometrically by analyzing the absorbance of the solution at a wavelength of 400 nm. Expression of recombinant BhuR was found to correlate with the capacity to remove hemin from solution (Fig. 4A).

Subsequent experiments were performed to confirm that the capacity to remove heme from solution was associated with surface expression of recombinant BhuR in BL21(DE3) (pMOX14.1). Immunoblotting experiments performed with total and outer membranes isolated from BL21(DE3)(pMOX14.1) demonstrated that recombinant BhuR was expressed in the outer membrane fraction of the recombinant cells (Fig. 4B). As a follow-up to these experiments, BL21(DE3)(pMOX14.1) cells were digested with increasing concentrations of bovine trypsin. The amount of immunoreactive BhuR expressed by the cells decreased in a dose-dependent manner with the amount of trypsin employed (0 to 1,000 U/ml) (Fig. 4C). To confirm that trypsin was not lysing the cells and exposing cytoplasmic (inclusion bodies) or periplasmic (partially transported) BhuR to proteolytic digestion, BL21(DE3)(pMOX14.1) was probed with an antibody specific for MBP, a constitutively expressed periplasmic protein of *E. coli*. Regardless of the amount of trypsin employed, the immunoreactive signal from MBP was not altered in the cells, thus confirming

FIG. 4. Recombinant BhuR is surface exposed in BL21(DE3)(pMOX14.1) and adsorbs hemin from solution. (A) Absorption of hemin from solution by recombinant BhuR. Stationary-phase BL21(DE3)(pET21a) and BL21(DE3)(pMOX14.1) were added to a solution of 20 μ M hemin dissolved in 10% DMSO and PBS (pH 7.4) (final DMSO concentration, 2%). After 1 h of incubation, cells were removed from the suspension, and the amount of hemin remaining in solution was determined spectrophotometrically at a wavelength of 400 nm. The data are the averages of three independent experiments with three replicates per experiment ($n = 9$). The differences in the absorbance values of BL21(DE3)(pET21a) and BL21(DE3)(pMOX14.1) are highly significant (three asterisks, $P < 0.001$). (B) Recombinant BhuR is expressed in the outer membrane of BL21(DE3)(pMOX14.1). Ten micrograms of membranes isolated from BL21(DE3)(pET21a) and BL21(DE3)(pMOX14.1) was resolved in a 12.5% SDS-PAGE gel. An immunoblot of the gel was probed with a 1:500 dilution of anti-BhuR antiserum. Lane 1, total membranes of BL21(DE3)(pET21a); lane 2, total membranes of BL21(DE3)(pMOX14.1); lane 3, enriched outer membranes of BL21(DE3)(pET21a); lane 4, enriched outer membranes of BL21(DE3)(pMOX14.1). The arrow indicates the position of BhuR. Molecular masses (in kilodaltons) are indicated on the left. (C) BhuR expressed by BL21(DE3)(pMOX14.1) is susceptible to digestion by extracellular trypsin. BL21(DE3)(pMOX14.1) cells were digested with increasing amounts of trypsin (1, 10, 100, and 1,000 U). Recombinant cells adjusted to an OD₆₀₀ of 0.8 were pelleted and resuspended in 1-ml portions of the trypsin solutions. Cells were digested at 37°C for 15 min. The trypsin in the cell suspensions was neutralized with a protease inhibitor cocktail. After the reaction mixtures were diluted with electrophoresis sample buffer, equivalent amounts of solubilized cells were resolved on a 12.5% SDS-PAGE gel, and the proteins were immunoblotted with a 1:500 dilution of anti-BhuR antiserum. (D) Treatment of BL21(DE3)(pMOX14.1) with trypsin did not degrade periplasmic MBP. BL21(DE3)(pET21a) and BL21(DE3)(pMOX14.1) cells were digested with increasing amounts of trypsin (1, 10, 100, and 1,000 U) for 15 min at 37°C. Proteins in the cells were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a 1:2,000 dilution of anti-MBP antiserum (New England Biolabs).

that trypsin treatment did not expose intracellular BhuR to proteolytic degradation (Fig. 4D). To further confirm that hemin binding was dependent upon expression of surface-exposed recombinant BhuR, a second and more active protease was employed. Treatment of BL21(DE3)(pMOX14.1) cells with proteinase K also significantly decreased the ability of BL21(DE3)(pMOX14.1) to absorb hemin (data not shown). Collectively, these data demonstrated that the capacity of BL21(DE3)(pMOX14.1) to remove hemin from solution was highly correlated with expression of BhuR on the surface of the recombinant cell.

DISCUSSION

A myriad of systems have evolved in bacterial pathogens to acquire Fe either as a free inorganic ion, in an Fe-siderophore complex, or as hemin which is coordinately bound within various hemoproteins (19). In some bacteria, hemin is obtained by secretion of hemophores which directly bind hemin in solution and deliver the molecule to receptors that initiate its intracellular transport (11, 19, 32, 33, 61). In other bacteria, hemin is directly bound by specific cell surface receptors which trigger subsequent intracellular uptake via TonB-dependent, ABC transporter systems (2, 19). Specific hemoprotein-binding and hemin-binding receptors have been described for numerous

gram-negative pathogens, including *H. influenzae* (22, 38, 39), *Haemophilus ducreyi* (3), *P. gingivalis* (19, 43, 45, 53, 54), *Vibrio anguillarum* (34), *Vibrio vulnificus* (16), *Pasteurella multocida* (8), *Actinobacillus pleuropneumoniae* (46), *Campylobacter jejuni* (49), and for *B. bronchiseptica* (58–60), *B. pertussis* (58–60), and *B. avium* (12, 40). The studies reported herein were designed to evaluate the mechanisms by which *B. bronchiseptica* acquires heme. Although the *B. bronchiseptica* BhuR-dependent acquisition system for hemin and Hb has been investigated in terms of expression and regulation (58–60), the molecular mechanism(s) by which heme is acquired and the sources of hemin which are utilized in vivo have not been fully characterized. Since free hemin does not usually circulate in the host at concentrations sufficient to sustain bacterial growth, an alternative mechanism for obtaining heme by *B. bronchiseptica* was hypothesized, which depended upon spontaneous hemin loss from Hb and Mb, two abundant hemoproteins in human and animal cells.

Two potential mechanisms were initially proposed to describe the means by which *B. bronchiseptica* acquires heme from Hb and Mb: (i) BhuR directly binds Mb and Hb, which facilitates release of hemin from the hemoproteins by either a nondestructive or destructive process; or (ii) BhuR binds hemin after the metalloprophyrin is spontaneously released from

extracellular Hb and Mb. The former model seems less likely, as it has not been possible to demonstrate direct binding of Hb to cells using either of two high-resolution technologies (flow cytometry and immunoblotting) (data not shown). In addition, genes encoding globin proteases similar to those expressed by *P. gingivalis* for degradation of Hb were not identified in the genome of *B. bronchiseptica*. Rather, experiments reported here provide direct and unambiguous support for the second model, i.e., that *bhuR*-dependent heme uptake by *B. bronchiseptica* depends upon passive release of the metalloporphyrin from Hb and Mb.

The capacity of Hb and Mb to spontaneously release heme is an inherent property of the biochemistries of the two hemoproteins. Hb is packaged at remarkably high concentrations (~32 to 35 g/100 ml or ~20 mM monomer units) within erythrocytes, which ensures tetramer formation (42). The rates of autooxidation and heme loss are low for tetrameric Hb (4, 23–27, 35, 37). Erythrocytes also contain reductases which maintain heme iron in a reduced state, which is more tightly bound than the oxidized or heme form. Maintenance of Hb-bound heme is essential for erythrocyte homeostasis and for prevention of highly reactive Heinz bodies which cause oxidative damage and cell lysis. Oxidation weakens the coordination bonds between the Fe in heme and the F8 histidine in the heme-binding pockets of Hb. At low concentrations in the oxidizing environment of plasma or in mucosal fluids, tetrameric Hb disassociates into two $\alpha\beta$ chain dimers (23, 26). The dissociation event is closely followed by oxidation and release of heme from the dimers. The loss of heme from Mb occurs by a similar process. Mb is present at high concentrations in myocytes, which favors retention of the heme by the hemoprotein (44). As is the case for erythrocytes, myocytes also express reductases to help maintain Mb in the more stable reduced state (63). When liberated from muscle cells, Mb rapidly autooxidizes, which in turn promotes spontaneous loss of heme (23–25, 27, 44, 63).

Five experimentally testable predictions were employed to evaluate the model that *B. bronchiseptica* utilizes BhuR to acquire heme which is spontaneously released from hemoproteins as a source of nutrient Fe. First, *B. bronchiseptica* should exhibit a capacity to utilize both Hb and Mb but not cytochrome *c* or catalase as sources of nutrient Fe (Fig. 1). Second, direct contact between the hemoproteins and the bacterial cell should not be required for acquisition of heme (hemin) from Hb or Mb (Fig. 2). Third, a mutant Mb with a low rate of heme dissociation should be a poorer source of nutrient Fe for *B. bronchiseptica* than wild-type Mb (Fig. 3). Fourth, a mutant Mb with a high rate of heme loss should be a better source of nutrient Fe for the bacterium (Fig. 3). Finally, *E. coli* expressing surface-exposed BhuR should exhibit a capacity to remove heme from solution (Fig. 4). All five of these predictions were confirmed in this study, thus providing strong support for the hypothesis that *B. bronchiseptica* acquires heme from Mb and Hb in a BhuR-dependent manner.

Yet this model presents another conundrum. *B. bronchiseptica* infects the ciliated epithelia of the upper respiratory tract (55), an environment in which Hb and Mb are generally not present. By what means, therefore, does *B. bronchiseptica* acquire heme-bound Fe during an infection? *B. bronchiseptica* secretes and/or releases extracellular toxins and hemolysis into

the extracellular milieu. Adenylate cyclase toxin is known to elicit cell necrosis (6, 30). BopC, a type III secretion system effector protein, has been demonstrated to lyse erythrocytes (31). Additionally, *B. bronchiseptica* modulates various inflammatory immune responses (52), which has the potential for damaging the respiratory epithelium. During an active infection, these or other reactive molecules produced by *B. bronchiseptica* might degrade cells located within the capillaries and other tissues of the upper respiratory tract. Erythrocytes released into the infected area from damaged capillaries or myocytes would be lysed by the hemolysins and/or the secreted toxins, which would release Hb and Mb into the microenvironment, where these proteins would be autooxidized. Autooxidation would promote loss of heme from the hemoproteins into the local environment (31). Unfortunately, the upper respiratory system is not highly vascularized or muscularized. Thus, large numbers of erythrocytes or myocytes are not routinely found in the tissues in this system. An alternative model is that heme may be derived from Hb and Mb that is released from cells located lower in the respiratory tract which are damaged by bacterium-associated inflammatory responses. Inflammatory responses may elicit capillary leakage and cell lysis from these deeper tissues, which might release hemoproteins into the mucosal fluid. These hemoproteins or heme released from these hemoproteins might be mechanically transported to the upper respiratory tract by coughing or by a residually active mucociliary respiratory escalator. If the latter model is correct, BhuR-dependent heme acquisition by *B. bronchiseptica* would likely be most important to the bacterium during the latter stages of an infection (9). Murine models of infection are currently being employed in our lab to investigate this hypothesis.

The experiments reported here provide support for the model that (i) BhuR is a heme-binding receptor, (ii) Hb and Mb are rich potential sources of heme-associated nutrient Fe to support growth of *B. bronchiseptica*, and (iii) acquisition of heme by *B. bronchiseptica* depends, at least in part, on spontaneous release of the metalloporphyrin from Mb and Hb. It is likely that other bacterial pathogens which utilize both hemoproteins and heme as sources of nutrient Fe exploit the natural instability of Hb and Mb for Fe and/or heme acquisition.

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REFERENCES

1. Abraham, N. G., and G. Drummond. 2006. CD163-mediated hemoglobin-heme uptake activates macrophage HO-1, providing an anti-inflammatory function. *Circ. Res.* **99**:911–914.
2. Adams, H., G. Zeder-Lutz, I. Schalk, F. Pattus, and H. Celia. 2006. Interaction of TonB with the outer membrane receptor FpvA of *Pseudomonas aeruginosa*. *J. Bacteriol.* **188**:5752–5761.
3. Afonina, G., I. Leduc, I. Nepluev, C. Jeter, P. Routh, G. Almond, P. E. Orndorff, M. Hobbs, and C. Elkins. 2006. Immunization with the *Haemophilus ducreyi* hemoglobin receptor HgbA protects against infection in the swine model of chancroid. *Infect. Immun.* **74**:2224–2232.
4. Ascenzi, P., A. Bocedi, P. Visca, F. Altruda, E. Tolosano, T. Beringhelli, and M. Fasano. 2005. Hemoglobin and heme scavenging. *TUBMB Life* **57**:749–759.

5. Balla, J., H. S. Jacob, G. Balla, K. Nath, J. W. Eaton, and G. M. Vercellotti. 1993. Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage. *Proc. Natl. Acad. Sci. USA* **90**:9285–9289.
6. Basler, M., J. Masin, R. Osicka, and P. Sebo. 2006. Pore-forming and enzymatic activities of *Bordetella pertussis* adenylate cyclase toxin synergize in promoting lysis of monocytes. *Infect. Immun.* **74**:2207–2214.
7. Blanco, E., J. M. Ruso, G. Prieto, and F. Sarmiento. 2007. Different thermal unfolding pathways of catalase in the presence of cationic surfactants. *J. Phys. Chem. B* **111**:2113–2118.
8. Bosch, M., M. E. Garrido, A. M. P. de Rozas, I. Badiola, J. Barbe, and M. Llagostera. 2004. *Pasteurella multocida* contains multiple immunogenic haemin- and haemoglobin-binding proteins. *Vet. Microbiol.* **99**:103–111.
9. Brickman, T. J., C. K. Vanderpool, and S. K. Armstrong. 2006. Heme transport contributes to in vivo fitness of *Bordetella pertussis* during primary infection in mice. *Infect. Immun.* **74**:1741–1744.
10. Carbonetti, N. H., G. V. Artamonova, N. Van Rooijen, and V. I. Ayala. 2007. Pertussis toxin targets airway macrophages to promote *Bordetella pertussis* infection of the respiratory tract. *Infect. Immun.* **75**:1713–1720.
11. Cescau, S., H. Cwerman, S. Letoffe, P. Delepaire, C. Wandersman, and F. Biville. 2007. Heme acquisition by hemophores. *Biomaterials* **20**:603–613.
12. Connell, T. D., A. Dickenson, A. J. Martone, K. T. Militello, M. J. Filiatraut, M. L. Hayman, and J. Pitula. 1998. Iron starvation of *Bordetella avium* stimulates expression of five outer membrane proteins and regulates a gene involved in acquiring iron from serum. *Infect. Immun.* **66**:3597–3606.
13. Delanghe, J. R., and M. R. Langlois. 2001. Hemopexin: a review of biological aspects and the role in laboratory medicine. *Clin. Chim. Acta* **312**:13–23.
14. Eskew, J. D., R. M. Vanacore, L. Sung, P. J. Morales, and A. Smith. 1999. Cellular protection mechanisms against extracellular heme: heme-hemopexin, but not free heme, activates the N-terminal c-Jun kinase. *J. Biol. Chem.* **274**:638–648.
15. Fita, I., and M. G. Rossmann. 1985. The active center of catalase. *J. Mol. Biol.* **185**:21–37.
16. Fouz, B., R. Mazoy, F. Vazquez, M. L. Lemos, and C. Amaro. 1997. Isolation of hemin and hemoglobin binding outer membrane protein of *Vibrio vulnificus* biotype 2 (serogroup E). *FEMS Microbiol. Lett.* **156**:187–191.
17. Frangipane, M. E., D. J. Morton, J. A. Wooten, J. M. Pozsgay, and T. L. Stull. 1994. Binding of human hemoglobin by *Haemophilus influenzae*. *FEMS Microbiol. Lett.* **118**:243–248.
18. Furano, K., and A. A. Campagnari. 2004. Identification of a hemin utilization protein of *Moraxella catarrhalis* (HumA). *Infect. Immun.* **72**:6426–6432.
19. Genco, C. A., and D. W. Dixon. 2001. Emerging strategies in microbial haem capture. *Mol. Microbiol.* **39**:1–11.
20. Griffiths, W. J. H., A. L. Kelly, S. J. Smith, and T. M. Cox. 2000. Localization of iron transport and regulatory proteins in human cells. *Q. J. Med.* **93**:575–587.
21. Guan, S.-M., H. Nagata, S. Shizukuishi, and J.-Z. Wu. 2006. Degradation of human hemoglobin by *Prevotella intermedia*. *Anaerobe* **12**:279–282.
22. Hanson, M. S., C. Slaughter, and E. J. Hansen. 1992. The *hbpA* gene of *Haemophilus influenzae* type b encodes a heme-binding lipoprotein conserved among heme-dependent *Haemophilus* species. *Infect. Immun.* **60**:2257–2266.
23. Hargrove, M. S., D. Barrick, and J. S. Olson. 1996. The association rate constant for heme binding to globin is independent of protein structure. *Biochemistry* **35**:11293–11299.
24. Hargrove, M. S., and J. S. Olson. 1996. The stability of holomyoglobin is determined by heme affinity. *Biochemistry* **35**:11310–11318.
25. Hargrove, M. S., E. W. Singleton, M. L. Quillin, L. A. Ortiz, J. George, N. Phillips, and J. S. Olson. 1994. His64-Tyr apomyoglobin as a reagent for measuring rates of hemin dissociation. *J. Biol. Chem.* **269**:4207–4214.
26. Hargrove, M. S., T. Whitaker, J. S. Olson, R. J. Vali, and A. J. Mathews. 1997. Quaternary structure regulates hemin dissociation from human hemoglobin. *J. Biol. Chem.* **272**:17385–17389.
27. Hargrove, M. S., A. J. Wilkinson, and J. S. Olson. 1996. Structural factors governing hemin dissociation from metmyoglobin. *Biochemistry* **35**:11300–11309.
28. Hvidberg, V., M. B. Maniecki, C. Jacobsen, P. Hojrup, H. J. Moller, and S. K. Moestrup. 2005. Identification of the receptor scavenging hemopexin-heme complexes. *Blood* **106**:2572–2579.
29. Izadi-Pruneyre, N., F. Huche, G. S. Lukat-Rodgers, A. Lacroix, R. Gilli, K. R. Rodgers, C. Wandersman, and P. Delepaire. 2006. The heme transfer from the soluble HasA hemophore to its membrane-bound receptor HasR is driven by protein-protein interaction from a high to a lower affinity binding site. *J. Biol. Chem.* **281**:25541–25550.
30. Johansson, D., P. Bergstrom, R. Henriksson, K. Grankvist, A. Johansson, and P. Behnam-Motlagh. 2006. Adenylate cyclase toxin from *Bordetella pertussis* enhances cisplatin-induced apoptosis to lung cancer cells *in vitro*. *Oncol. Res.* **15**:423–430.
31. Kuwae, A., T. Masuzawa, N. Ishikawa, H. Abe, T. Nonaka, H. Fukada, S. Imajoh-Ohmi, and A. Abe. 2006. BopC is a novel type III effector secreted by *Bordetella bronchiseptica* and has a critical role in type III-dependent necrotic cell death. *J. Biol. Chem.* **281**:6589–6600.
32. Letoffe, S., J. M. Ghigo, and C. Wandersman. 1994. Iron acquisition from heme and hemoglobin by a *Serratia marcescens* extracellular protein. *Proc. Natl. Acad. Sci. USA* **91**:9876–9880.
33. Letoffe, S., V. Redeker, and C. Wandersman. 1998. Isolation and characterization of an extracellular haem-binding protein from *Pseudomonas aeruginosa* that shares function and sequence similarities with the *Serratia marcescens* HasA hemophore. *Mol. Microbiol.* **28**:1223–1234.
34. Mazoy, R., and M. L. Lemos. 1996. Identification of heme-binding proteins in the cell membranes of *Vibrio anguillarum*. *FEMS Microbiol. Lett.* **135**:265–270.
35. Miller, Y. I., and N. Shaklai. 1999. Kinetics of hemin distribution in plasma reveals its role in lipoprotein oxidation. *Biochim. Biophys. Acta* **1454**:153–164.
36. Moestrup, S. K., and H. J. Moller. 2004. CD163: a regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response. *Ann. Med.* **36**:347–354.
37. Monzani, E., B. Bonafe, A. Fallarini, C. Redaelli, L. Casella, L. Minchiotti, and M. Galliano. 2001. Enzymatic properties of human hemalbumin. *Biochim. Biophys. Acta* **1547**:302–312.
38. Morton, D. J., L. L. Madore, A. Smith, T. V. Wagoner, T. W. Seale, P. W. Whitby, and T. L. Stull. 2005. The heme-binding lipoprotein (HbpA) of *Haemophilus influenzae*: role in heme utilization. *FEMS Microbiol. Lett.* **253**:193–199.
39. Morton, D. J., A. Smith, Z. Ren, L. Madore, T. M. V. Wagoner, T. W. Seale, P. W. Whitby, and T. L. Stull. 2004. Identification of a haem-utilization protein (Hup) in *Haemophilus influenzae*. *Microbiology* **150**:3923–3933.
40. Murphy, E. R., R. E. Sacco, A. Dickenson, D. J. Metzger, Y. Hu, P. E. Orndorff, and T. D. Connell. 2002. BhuR, a virulence-associated outer membrane protein of *Bordetella avium*, is required for the acquisition of iron from heme and hemoproteins. *Infect. Immun.* **70**:5390–5403.
41. Nagel, R. L., and Q. H. Gibson. 1971. The binding of hemoglobin to haptoglobin and its relation to subunit dissociation of hemoglobin. *J. Biol. Chem.* **246**:69–73.
42. Ohlson, M. A., D. Cederquist, E. G. Donelson, R. M. Leverton, G. K. Lewis, W. A. Himwich, and M. S. Reynolds. 1944. Hemoglobin concentrations, red cell counts and erythrocyte volumes of college women of the north central states. *Am. J. Physiol.* **142**:727–732.
43. Olczak, T., D. W. Dixon, and C. A. Genco. 2001. Binding specificity of the *Porphyromonas gingivalis* heme and hemoglobin receptor HmuR, gingipain K, and gingipain R1 for heme, porphyrins, and metalloporphyrins. *J. Bacteriol.* **183**:5599–5608.
44. Ordway, G. A., and D. J. Garry. 2004. Myoglobin: an essential hemoprotein in striated muscle. *J. Exp. Biol.* **207**:3441–3446.
45. Paramasvaran, M., K.-A. Nguyen, E. Caldon, J. A. McDonald, S. Najdi, G. Gonzaga, D. B. Langley, A. DeCarlo, M. J. Crossley, N. Hunter, and C. A. Collyer. 2003. Porphyrin-mediated cell surface heme capture from hemoglobin by *Porphyromonas gingivalis*. *J. Bacteriol.* **185**:2528–2537.
46. Pawelek, P. D., and J. W. Coulton. 2004. Hemoglobin-binding protein HgbA in the outer membrane of *Actinobacillus pleuropneumoniae*: homology modelling reveals regions of potential interactions with hemoglobin and heme. *J. Mol. Graphics Model.* **23**:211–221.
47. Philippidis, P., J. C. Mason, B. J. Evans, I. Nadra, K. M. Taylor, D. O. Haskard, and R. C. Landis. 2004. Hemoglobin scavenger receptor CD163 mediates interleukin-10 release and heme oxygenase-1 synthesis: anti-inflammatory monocyte-macrophage responses *in vitro*, in resolving skin blisters *in vivo*, and after cardiopulmonary bypass surgery. *Circ. Res.* **94**:119–126.
48. Prajapati, S., V. Bhakuni, K. R. Babu, and S. K. Jain. 1998. Alkaline unfolding and salt-induced folding of bovine liver catalase at high pH. *Eur. J. Biochem.* **255**:178–184.
49. Ridley, K. A., J. D. Rock, Y. Li, and J. M. Ketley. 2006. Heme utilization in *Campylobacter jejuni*. *J. Bacteriol.* **188**:7862–7875.
50. Schaer, C. A., G. Schoedon, A. Imhof, M. O. Kurrer, and D. J. Schaer. 2006. Constitutive endocytosis of CD163 mediates hemoglobin-heme uptake and determines the non-inflammatory and protective transcriptional response of macrophages to hemoglobin. *Circ. Res.* **99**:943–950.
51. Seale, T. W., D. J. Morton, P. W. Whitby, R. Wolf, S. D. Kosanke, T. M. V. Wagoner, and T. L. Stull. 2006. Complex role of hemoglobin and hemoglobin-haptoglobin binding proteins in *Haemophilus influenzae* virulence in the infant rat model of invasive infection. *Infect. Immun.* **74**:6213–6225.
52. Siciliano, N. A., J. A. Skinner, and M. H. Yuk. 2006. *Bordetella bronchiseptica* modulates macrophage phenotype leading to the inhibition of CD4⁺ T cell proliferation and the initiation of a Th17 immune response. *J. Immunol.* **177**:7131–7138.
53. Simpson, W., T. Olczak, and C. A. Genco. 2000. Characterization and expression of HmuR, a TonB-dependent hemoglobin receptor of *Porphyromonas gingivalis*. *J. Bacteriol.* **182**:5737–5748.
54. Simpson, W., T. Olczak, and C. A. Genco. 2004. Lysine-specific gingipain K and heme/hemoglobin receptor HmuR are involved in heme utilization in *Porphyromonas gingivalis*. *Acta Biochim. Pol.* **51**:253–262.
55. Stefanelli, P., P. Mastrantonio, S. Z. Hausman, M. Gulinao, and D. Burns.

1997. Molecular characterization of two *Bordetella bronchiseptica* strains isolated from children with coughs. *J. Clin. Microbiol.* **35**:1550–1555.
56. **Stull, T. L.** 1987. Protein sources of heme for *Haemophilus influenzae*. *Infect. Immun.* **55**:148–153.
57. **Ugocsai, P., S. Barlage, A. Dada, and G. Schmitz.** 2006. Regulation of surface CD163 expression and cellular effects of receptor mediated hemoglobin-haptoglobin uptake on human monocytes and macrophages. *Cytometry* **69A**:203–205.
58. **Vanderpool, C. K., and S. K. Armstrong.** 2001. The *Bordetella bhu* locus is required for heme iron utilization. *J. Bacteriol.* **183**:4278–4287.
59. **Vanderpool, C. K., and S. K. Armstrong.** 2003. Heme-responsive transcriptional activation of *Bordetella bhu* genes. *J. Bacteriol.* **185**:909–917.
60. **Vanderpool, C. K., and S. K. Armstrong.** 2004. Integration of environmental signals controls expression of *Bordetella* heme utilization genes. *J. Bacteriol.* **186**:938–948.
61. **Wandersman, C., and I. Stojikovic.** 2000. Bacterial heme sources: the role of heme, hemoprotein receptors and hemophores. *Curr. Opin. Microbiol.* **3**:215–220.
62. **Wessling-Resnick, M.** 1999. Biochemistry of iron uptake. *Crit. Rev. Biochem. Mol. Biol.* **34**:285–314.
63. **Wittenberg, J. B., and B. A. Wittenberg.** 2003. Myoglobin function reassessed. *J. Exp. Biol.* **206**:2011–2020.
64. **Zuwala-Jagiello, J.** 2006. Haemoglobin scavenger receptor: function in relation to disease. *Acta Biochim. Pol.* **53**:257–268.

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