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 hemin 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 hemin from solution. Collectively, these experiments provided strong experimental support for the model that BhuR is a hemin 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 a potentially 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-
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 HumA, an outer membrane receptor, has direct binding affinity for the metalloporphyrin (21).

TABLE 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
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<tr>
<td>DH5αF*Kan</td>
<td>Cloning strain, Kan’</td>
<td>Invitrogen</td>
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<tr>
<td>BL21(DE3)</td>
<td>Expression strain</td>
<td>Stragene</td>
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<tr>
<td><strong>B. bronchiseptica strains</strong></td>
<td></td>
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<tr>
<td>RB50</td>
<td>Wild-type strain</td>
<td>J. Miller</td>
</tr>
<tr>
<td>RBB4</td>
<td>Internal bhuR truncation in RB50</td>
<td>S. K. Armstrong (unpublished)</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pGEM-T</td>
<td>PCR fragment cloning vector, Amp’</td>
<td>Promega</td>
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<tr>
<td>pET21a</td>
<td>Expression vector, Amp’</td>
<td>Novagen</td>
</tr>
<tr>
<td>pMOXI3</td>
<td>PCR-amplified DNA fragment encoding bhuR ligated into pGEM-T</td>
<td>This study</td>
</tr>
<tr>
<td>pMOXI4.1</td>
<td>NdeI/XhoI fragment from pMOXI3 encoding bhuR ligated into pET21a</td>
<td>This study</td>
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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 equilbrium. 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 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 mucosal fluids from lysed cells promotes spontaneous dissociation of heme 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 culture was incubated for 12 h in 10 ml of BHI broth supplemented with 50 μM deuterated ethylenediamine-τ-(-)-hydroxyphenylacetic acid (EEDDHA) (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 μM EDDHA to which hemin, recombinant wild-type Mb, Mb\(^{\text{VHxL}}\) (23, 24), Mb\(^{\text{VHSST}}\) (23, 24), human Hb (Sigma Biochemicals), rabbit Hb (Sigma Biochemicals), porcine Hb (Sigma Biochemicals), bovine liver catalase (Sigma Biochemicals), or canine cytochrome (Sigma Biochemicals) was added to a final concentration of 5 μM with respect to the Fe content. Three replicate cultures were prepared for each condition. The optical densities at 600 nm (OD\(_{600}\)) of the cultures were measured at various time intervals as a relative measure of growth using a Beckman Coulter DU60 spectrophotometer (Fullerton, CA) or a Tiertek 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.

Sequstration 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 H\(_2\)O 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 μM EDDHA to which whole cells of RB50 encoded in pMOX14.1 were digested with increasing protease activities in 10 mM Tris buffer. Solutions were filter sterilized using a 0.22-μm syringe tip filter (Whatman, Dassel, Germany). Recombinant cells were pelleted by centrifugation (100,000 × g, 10 min, 4°C) and the pellets were resuspended in 20 ml of PBS (pH 7.4) to remove BHI broth and debris. The OD\(_{600}\) 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 μM solution of hemin prepared in 10% DMSO was added to 800 μl of the cell suspension, which was gently shaken at room temperature for 1.0 to 2.25 h. After incubation, the supernatant was separated from the cells by centrifugation (16,000 × g, 10 min, 4°C). The amount of hemin remaining in the supernatant after adsorption by the cells, as determined spectrophotometrically at 400 nm (OD\(_{400}\)), 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 of expression. Cells bursted with 100 1-ml serial dilutions were pelleted by centrifugation (12,096 × g, 10 min, 4°C). The cell pellet was resuspended in 25 ml of 10 mM HEPES buffer (pH 7.4) (American Biorganics, 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 sonication was added to sonicate the enriched outer membranes to 100 1-ml serial dilutions to a final concentration of 10 mM. Sonicates were centrifuged twice in 50-ml Oak Ridge tubes (12,096 × g, 10 min, 4°C) to pellet undisrupted cells. The clarified sonicates were centrifuged (100,000 × 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 × g, 1 h, 4°C), the pellets were washed three times by centrifugation (12,096 × g, 10 min, 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 μl of 10 mM Tris (pH 8.0) and stored at −20°C. The amount of protein in the outer membranes was measured using BioRad protein assay (BioRad, Hercules, CA) to obtain a final concentration of 10 mg protein/ml 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-μm syringe tip filter (Whatman, Dassel, Germany). Recombinant cells were pelleted by centrifugation (100,000 × g, 1 h, 4°C), the pellets were treated with 1 h at room temperature with SDS-HEPES buffer and centrifuged (100,000 × 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 μl of 10 mM Tris (pH 8.0) and stored at −20°C. The amount of protein in the outer membranes was measured using BioRad protein assay (BioRad, Hercules, CA) to obtain a final concentration of 10 mg protein/ml 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.

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solution containing 10% DMSO was added and allowed to bind. Following 1 h density-adjusted recombinant cells were utilized. The cells were resuspended in IPTG, incubated for maximal expression of BhuR, and prepared for hemin-

Biochemicals) to denude the bacterial surface of all protein components. In

via cell lysis.

dilution of anti-MBP monoclonal antibody (New England Biolabs) to confirm

that treatment with trypsin did not release periplasmic or cytoplasmic proteins

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-µg/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 µl of PBS (pH 7.4). To the resuspended cells, 200 µl of a 20 µM hemin stock solution containing 10% DMSO was added and allowed to bind. Following 1 h of incubation to allow binding of hemin, the ODbas 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 Ferestricted 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, hemin 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 hemin 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 αβ dimers exhibit a much higher rate of autoxidation and hemin 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 hemin 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. 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 µM EDDHA containing 5 µM 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.
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 heme (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 \( \approx 1 \) nM hemoprotein, a level of hemoprotein which was below the minimal 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 heme 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 heme (24), were employed as sources of nutrient Fe in growth experiments. The order of the rates of heme loss from the Mbs is Mb\(^{V68T}\) < wild-type Mb < Mb\(^{H64L}\) (24, 25, 27). Overnight Fe-stressed RB50 cells were subcultured in BHl broth supplemented with 5 \( \mu \)M of various heme sources, including hemin, wild-type sperm whale Mb, Mb\(^{H64L}\), and Mb\(^{V68T}\), as the sole sources of heme. 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 heme 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...
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 $5 \mu$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 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 μM 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
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). 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 heme after the metalloporphyrin 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 hemin 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 heme-proteins. 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 hemin 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 αβ 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 autoxidizes, 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 hemin dissociation should be a poorer source of nutrient Fe for the bacterium (Fig. 3). Fourth, a mutant Mb with a high rate of hemin loss should be a better source of nutrient Fe for the bacterium (Fig. 4). 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 hemolysin 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 autoxidized. 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 inflamatory 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 hemin-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 heme-proteins 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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