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Battisti, James M.; Sappington, Kate N.; Smitherman, Laura S.; Parrow, Nermi L.; and Minnick, Michael F., "Environmental Signals Generate a Differential and Coordinated Expression of the Heme Receptor Gene Family of Bartonella Quintana" (2006). Biological Sciences Faculty Publications. 143.
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Environmental Signals Generate a Differential and Coordinated Expression of the Heme Receptor Gene Family of \textit{Bartonella quintana}

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Received 14 February 2006/Returned for modification 10 March 2006/Accepted 17 March 2006

Of all bacteria, \textit{Bartonella quintana} has the highest reported in vitro heme requirement, yet an explanation for this remains elusive. To produce diseases such as trench fever, endocarditis, and bacillary angiomatosis, \textit{B. quintana} must survive and replicate in the disparate environments of the \textit{Pediculus humanus corporis} (body louse) gut and the human vasculature. We previously identified a five-member family of heme binding proteins (Hbps) synthesized by \textit{B. quintana} that bind hemin on the outer surface but share no similarity to known bacterial heme receptors. In the present study, we examine the transcription, regulation, and synthesis of this virulence factor family by cultivation of the bacterium in environments that simulate natural heme, oxygen, and temperature conditions encountered in the host and insect vector. First, quantitative real-time PCR data show that \textit{hbpC} expression is regulated by temperature, where a \(>100\)-fold increase in transcript quantity was seen at 30°C relative to 37°C, suggesting that \textit{HbpC} synthesis would be greatest in the cooler temperature of the louse. Second, cultivation at human bloodstream oxygen concentration (5% relative to 21% atmospheric) significantly decreases the transcript quantity of all \textit{hbp} genes, indicating that expression is influenced by \(O_2\) and/or reactive oxygen species. Third, a differential expression pattern within the \textit{hbp} family is revealed when \textit{B. quintana} is grown in a range of heme concentrations: subgroup I (\textit{hbpC} and \textit{hbpB}) predominates in a simulated louse environment (high heme), and subgroup II (\textit{hbpA}, \textit{hbpD}, and \textit{hbpE}) is preferentially expressed in a simulated human background (low heme). By using two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblotting, and matrix-assisted laser desorption ionization—time of flight mass spectrometry fingerprinting, we demonstrate that synthesis of \textit{HbpA} correlates with \textit{hbpA} transcript increases observed at low heme concentrations. Finally, an \textit{hbpA} promoter-\textit{lacZ} reporter construct in \textit{B. quintana} demonstrates that a transcriptional regulator(s) is controlling the expression of \textit{hbpA} through a \textit{cis}-acting regulatory element located in the \textit{hbpA} promoter region.

Trench fever, the common name for the acute febrile syndrome associated with \textit{Bartonella quintana} infection, has affected millions of people during war and is presently reemerging in inner cities throughout the world (28, 36) and in AIDS patients. Chronic manifestations of persistent infection by this \(\alpha\)-proteobacterium include protracted bacteremia, endocarditis, bacillary angiomatosis, and bacillary peliosis (20, 31). Although \textit{B. quintana} has been found in small mammals (24), ticks (11), and fleas (41), maintenance in nature is thought to be restricted to humans and body lice (\textit{Pediculus humanus corporis}). Transmission to humans occurs when louse fecal matter or a crushed louse containing the bacterium is introduced into the bloodstream by breaches in the integument, usually by the itching caused by louse infestation. Living between the clothing and the skin, body lice normally take several meals per day and acquire \textit{B. quintana} by imbibing the blood of a bacteremic host (9). Unhygienic, overcrowded conditions disseminate infected lice throughout the population and can quickly result in an epidemic.

Of all bacteria, \textit{B. quintana} has the greatest known requirement for exogenous heme (33, 34, 48). Heme consists of an iron atom contained in the center of a large heterocyclic organic ring called a porphyrin. The \(Fe^{3+}\) oxidation product of heme is called hemin. It is generally accepted that this extraordinary supplement requirement (20 to 40 \(\mu\)g/ml of medium) is similar for all \textit{Bartonella} species, and erythrocytes, hemoglobin, or hemin is essential for in vitro cultivation (8). Since combinations of iron and porphyrin cannot substitute for heme in \textit{Bartonella} cultivation, several researchers have hypothesized that high levels of heme are necessary for one or more of the following: a source of iron (10, 42), a precursor for synthesis of porphyrin-containing proteins (34), and a hydrogen peroxide-detecting system (33).

To generate disease, \textit{B. quintana} must survive immune attack, adapt to host and vector environments, and proliferate throughout the human-louse-human cycle. Free heme is quite rare in humans (6), whereas potentially toxic levels are frequently generated following blood meal digestion within the louse gut (9, 19, 37, 47). Considering its extraordinary heme requirement, it is obvious that heme acquisition mechanisms are essential for replication and, ultimately, the pathogenesis of \textit{B. quintana}.

Previously, we discovered a family of heme binding proteins (HbpA, HbpB, HbpC, HbpD, and HbpE) synthesized by \textit{B. quintana} that serves as heme receptors yet shares no similarity to known bacterial heme binding proteins (10, 32). In the present study, we examine the expression, regulation, and synthesis of this virulence factor family in conditions that reflect body louse and human environments. We report that hemin, oxygen, and temperature influence the \textit{hbp} transcript profile in a differential and coordinated manner.

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Bacteria kit with Turbo-DNaseI treatment (Ambion, Austin, TX) and a FastPrep bead homogenizer (Qi-Biogene, Carlsbad, CA) per the manufacturer’s instructions. Primers and probes used for qRT-PCR analysis of the hbpA family were previously described (32). The lacZ probe-primer set was designed with Beacon Designer version 4.0 (Bio-Rad, Hercules, CA). The dual-labeled lacZ probe was synthesized with fluorescent tags as described for the hbpA family, where 5-carboxyfluorescein and N,N,N',N'-tetracyethyl-6-carboxyrhodamine were covalently linked to the 5’ and 3’ ends, respectively (Sigma-Genosys, Woodlands, TX). The lacZ primer pair (Table 1) was synthesized by Applied Biosystems (ABI, Foster City, CA).

Plasmids and primers used in this study are described in Table 1. Standard PCR and cloning procedures were employed for the construction of plasmids (5) with the exception of the Expand Long Template PCR system (Roche Diagnostics, Indianapolis, IN), utilized for high-fidelity ampiclon production in inverse PCR-mediated cloning per the manufacturer’s instructions. For routine cloning, the Perfectprep Plasmid Mini kit (Eppendorf, Hamburg, Germany) and the QiAquick Spin kit (QiAgen, Valencia, CA) were used for plasmid isolation and DNA purification, respectively. The Wizard Midiprep kit (Promega, Madison, WI) was used to purify plasmids employed in electroporation-mediated transformation of B. quintana. Bacterial genomic DNA was prepared with a DNase Tissue kit (QIAGEN) per the manufacturer’s instructions. Quantification of nucleic acids was accomplished by spectrophotometric analysis using a Spectronic Genesys 2 (Milton Roy, Rochester, NY).

**Construction of HbpA reporter construct.** Diagrams of the reporter plasmid pHPRO + lacZ + and two control plasmids (pHPRO lacZ + and pHPRO − lacZ −) are shown in Fig. 6. Primers pHPRO FOR and pHPRO REV were designed to generate a PCR fragment containing the promoter region of hbpA (HPRO). Using pHPRO-CMV (52) as the template, the resulting ampiclon was cloned into pCR2.1-TOPO per the manufacturer’s instructions (Invitrogen, Carlsbad, CA), resulting in pCR2.1-HPRO. Sequence analysis of both strands with M13 universal primers verified that pCR2.1-HPRO contained the HPRO, 240 bp immediately 5’ to the hbpA start site, and it was identical to published sequences (1, 32).

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<th>Strains, plasmids, and primers</th>
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<td>B. quintana</td>
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<td>JK31</td>
<td>Low-passage virulent human isolate</td>
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**Plasmids**

- pHBP-CMV
- pCR2.1-TOPO
- pC2R1-HPRO
- pUJ9
- pBRR1MCS-2
- pPHRO + LACZ +
- pPHRO LACZ +
- pPHRO LACZ −

**Primers**

- HPRO FOR
- HPRO REV
- PBFR FOR + AscI
- HPROlac REV + AscI
- LINK FOR + AscI
- LINK REV + EcoRI
- HSEQ
- LACZ FOR
- LACZ REV
- LACZ PROBE
The HPRO was then recloned into the promoterless "lacZ" fusion plasmid, pU9 (12). Specifically, the 302-bp EcoRV/BamHI fragment of pCR2.1-HPRO was ligated to Smal/BamHi-restricted pU9, resulting in pU-HPRO. Orientation of HPRO relative to "lacZ" was confirmed by sequence analysis of pU-HPRO with the HPRO FOR primer. Finally, inverse PCR-mediated cloning was utilized to move the HPRO-"lacZ" fragment into the Bartonella shuttle vector, pBBR1MCS-2 (7, 22). Specifically, primers (PBRR FOR + AvrII and PBRR REV + AscI) were designed to amplify the entire pBBR1MCS-2 plasmid while introducing AvrII and AscI restriction sites into the termini of this 4,604-bp amplicon. The 2,258-bp HPRO-"lacZ" fragment was amplified from pU-HPRO with primers (HPROLAC REV + AvrII and HPROLAC FOR + AscI) that introduced the same restriction sites into the termini of this product. The pBBR1MCS-2 and HPRO-"lacZ" amplicons were digested (AvrII/AscI) and ligated, resulting in the 6,676-bp reporter construct pHPRO-"lacZ". The construct was verified by sequence analysis with primers HPRO FOR and HPROLAC REV + AvrII.

Two control plasmids were also generated with this reporter plasmid lacking either the hbpA promoter (pHPRO-"lacZ") or "lacZ" (pHPRO-"lacZ"). First, the hbpA promoter was removed from pHPRO-"lacZ" by digestion (EcoRI/AscI) and was replaced with a 15-bp linker formed by hybridization of primers (LNRK FOR + AscI and LINK REV + EcoRI) containing corresponding restriction sites. This 6,643-bp plasmid was confirmed by sequence analysis with primer HSEQ and was termed pHPRO. Restriction sites. This 6,643-bp plasmid was confirmed by sequence analysis with primers HPRO FOR and HPROLAC REV + AvrII.

The promoter was then recloned into the fusion plasmid, pBBR1MCS-2, by using the comparative method of qRT-PCR (4) using this first expressed from the gel, transferred to siliconized microcentrifuge tubes, treated with 50% acetonitrile-25 mM NaHCO3 (at 25°C until colorless), and dried in a speedvac. Sequencing Grade Modified Trypsin (Promega) was prepared per the manufacturer's instructions and diluted to 12.5 ng/μl in 25 mM NaHCO3. Dried gel fragments were resuspended in the trypsin solution (4°C, 20 min), resuspended in 25 mM NaHCO3, and digested for 16 h at 37°C. Peptides were extracted from the gel fragments with 0.1% trichloroacetic acid (TFA)-60% methanol, dried in a speedvac, and resuspended in 4 μl 2.5% TFA. Finally, peptides were cleaned and concentrated using Omix C18 pipette tips (Varian, Palo Alto, CA) per the manufacturer's instructions, analyzed using the Voyager-DE PRO MALDI-TOF BioSpectrometry Workstation (ABI), and fingerprinted with Mascot software (http://www.matrixscience.com) (38).

RESULTS

Growth of B. quintana at “louse-like” temperature results in a dramatic increase in hbpC. To determine if the hbp transcripts profiles were influenced by temperature, we compared the effects of growth at louse-like temperature (30°C) (27, 29) to those at human bloodstream temperature (37°C). B. quintana strain JK31 was grown on BA-H (0.15 mM hemin) at 37°C and 30°C. RNA was prepared from bacteria cultivated at each temperature, and qRT-PCR was performed as described above. To clarify the methods and compound calculations of the comparative method of qRT-PCR (4) using this first experiment, a brief description follows. The threshold cycle (Ct) is the cycle number at which the fluorescence signal generated by PCR crosses a threshold just above background and begins exponential increase. The detection system (MyIQ; Bio-Rad) statistically determines the threshold value during each run using a series of computations based on real-time output of fluorescent signal (data not shown). The average of three Ct values (Avg Ct) was determined for each target (16S as well as hbpA to hbpE) using RNA preparations from 30°C and 37°C and is listed in Fig. 1. The average Ct value reflects the quantity of a specific target transcript in a given RNA sample. Where the lower the Avg Ct the more abundant the specific transcript. In this experiment, the 16S transcript is the most common and hbpB is the least common in both 30°C and 37°C RNA samples. Considering only the hbp genes, the hierarchy of transcripts from the 30°C RNA sample is hbpA > hbpC > hbpD >
hbpE > hbpB, and from the 37°C preparation it is hbpA > hbpD > hbpE > hbpC > hbpB.

The Avg \( C_T \) values are useful for determination of transcript quantity in a single sample. However, when determination of relative quantities between RNA samples is necessary, the Avg \( C_T \)s and corresponding standard deviations (SDs) are used to calculate the fold difference using the comparative method. The fold difference is the amount of target mRNA normalized to an endogenous reference and relative to a calibrator. In this experiment, the amount of a specific hbp mRNA transcript in the 30°C preparations (target) was normalized to the amount of 16S rRNA (endogenous reference) and is relative to the quantity of that particular hbp mRNA transcript in the 37°C preparations (calibrator). Fold differences calculated by this method for each hbp transcript are derived from the given Avg \( C_T \)s and corresponding SDs and are listed with resultant range limits (Fig. 1). To illustrate the reproducibility of these transcript profile determinations, the bar graph portion of Fig. 1 depicts fold differences calculated from the given data set (Avg \( C_T \)s and corresponding SDs) together with a second independently derived data set where error bars show the SD between these independent fold difference determinations.

The more than 108-fold difference in hbpC transcript quantity at 30°C is the largest fold difference of any environmental condition tested in this study, and it shows that hbpC transcription is temperature regulated. Furthermore, this significant increase in hbpC expression at 30°C suggests that HbpC function may be important in the louse. The fold difference of the other four hbp transcripts is statistically insignificant, suggesting that the expression of these hbp genes is not temperature regulated and not necessarily specific to the louse.

Growth at “bloodstream” oxygen levels results in downregulation of all hbp genes. As the combination of \( O_2 \) and heme (or iron) can result in the formation of toxic reactive oxygen species (ROS) (6, 18), we were curious to see if hbp transcript profiles are influenced by oxygen. B. quintana strain JK31 was grown on BA-H (0.15 mM hemin) at 37°C in an environment containing 5% \( O_2 \) or 21% \( O_2 \). RNA was prepared from bacteria cultivated at each \( O_2 \) concentration, and qRT-PCR was performed as described above. Average \( C_T \)s and corresponding SDs are listed in Fig. 2, where the hierarchy of hbp transcripts is the same at both \( O_2 \) concentrations (hbpA > hbpD > hbpE > hbpC > hbpB). Fold differences calculated from these Avg \( C_T \) values are listed in Fig. 2 with corresponding range limits. In this experiment, the amount of a specific hbp mRNA transcript derived from 5% \( O_2 \)-grown bacteria (target) was normalized to the amount of 16S rRNA (endogenous reference) and is relative to the quantity of that partic-
ular hbp mRNA transcript from a 21% O2-grown preparation (calibrator). The bar graph portion of Fig. 2 shows the fold differences calculated from the given data set together with a second independently derived data set, where error bars show the SD between these independent fold difference determinations. The fold differences of all hbp transcripts are significantly decreased when bacteria are grown in a 5% O2 environment relative to 21% O2, indicating that hbp expression is influenced by oxygen and/or ROS.

Variable hemin levels reveal expression subgroups of the hbp genes. B. quintana must survive and replicate in environments containing markedly different heme concentrations throughout the infectious cycle. Humans maintain very low free heme levels by sequestering it within hemopexin, albumin, and hemoglobin complexes (6). On the contrary, body lice feed several times per day and digest their blood meal within minutes, exposing B. quintana to very high levels of potentially toxic heme with each meal (9, 47). Thus, we were interested in examining hbp expression at low (human) and high (louse) heme levels.

B. quintana strain JK31 was grown at 37°C on BA-H supplemented with a range of hemin concentrations (0.0, 0.035, 0.04, 0.05, 0.15, 1.0, 2.5, 5.0, and 8.0 mM), and RNA was prepared from the cultures. The control (0.15 mM), low (0.05 mM), and high (1.0, 2.5, 5.0 mM) hemin concentrations were empirically determined by relative growth rates and 16S Avg CT values. Growth was significantly affected in the extremes of this range, where growth did not occur (0.0, 8.0 mM heme) or a slower growth rate combined with a high 16S Avg CT value (0.035, 0.04 mM heme) eliminated these conditions from our experiment (data not shown).

qRT-PCR and fold difference calculations were performed as described above. Figure 3 shows the effect on hbp transcript profiles at low hemin concentration (0.05 mM) compared to the control concentration (0.15 mM). The average C_T hierarchy at low hemin (hbpA > hbpD > hbpE > hbpC > hbpB) is similar to that of the control (hbpD > hbpA > hbpE > hbpC > hbpB), where hbpA and hbpD quantities are nearly equal. However, fold differences indicate that expression of hbpA, hbpD, and hbpE are significantly increased at low hemin concentration, whereas hbpC and hbpB remain relatively unchanged. This demonstrates that hbpA, hbpD, and hbpE respond to low hemin levels and suggests that HbpA, HbpD, and HbpE may play a more significant role in the human environment relative to HbpC and HbpB. Finally, these data infer that

![Graph](image_url)

**FIG. 2.** Fold differences in hbp mRNA following growth at human bloodstream oxygen concentration (5% O2) relative to that of the routine in vitro culture environment (21% O2). At 96 h, the amount of target hbp mRNA transcript in 5% O2 preparations was normalized to the amount of 16S rRNA and is relative to the quantity of that particular hbp mRNA transcript from 21% O2 preparations. A representative experiment is shown, where C_T values were determined in triplicate. SD bars were generated by comparing fold differences to those obtained from a second independent triplicate determination.
two subgroups exist within the hbp family: subgroup I (hbpC and hbpB) and subgroup II (hbpA, hbpD, and hbpE).

The subgroup hypothesis is further supported by analyzing the fold differences at high hemin levels (1.0, 2.5, 5.0 mM) relative to the control (0.15 mM) and is illustrated in Fig. 4. As the hemin concentration is elevated to 1.0 mM, a decrease is evident in all hbp transcript quantities and implies that hemin is saturating but not yet toxic, suggesting that this may be the ideal hemin concentration for B. quintana strain JK31. Increasing concentrations further (2.5 to 5 mM) results in an elevation of subgroup I (hbpC and hbpB), whereas subgroup II (hbpA, hbpD, and hbpE) remains relatively unchanged. This demonstrates that hbpC and hbpB respond to concentrations of free hemin that are approaching toxicity, an environment that would most likely be encountered in the louse gut. These results, combined with the >100-fold increase seen in hbpC transcript at louse-like temperature (Fig. 1), strongly suggest that HbpC function is critical in the louse.

Finally, hbp transcript quantity was not significantly affected by cultivation at several different free iron concentrations or media pH backgrounds, indicating that these environmental cues do not influence expression (data not shown). Due to the inherent peroxidase activity of hemin, it was impossible to investigate the oxidative stress response by cultivation in an H$_2$O$_2$-rich environment.

**Synthesis of subgroup II proteins correlates with expression data.** To determine if hbp transcript profiles correspond to protein profiles, we analyzed whole-cell lysates by two-dimensional electrophoresis, immunoblotting, and matrix-assisted laser desorption ionization—time of flight (MALDI-TOF) mass spectrometry (MS). B. quintana strain JK31 was grown at 37°C on BA-H supplemented with low (0.05 mM) and control (0.15 mM) hemin concentrations. The image in Fig. 5A shows a silver-stained two-dimensional gel of a lysate prepared from B. quintana organisms grown on control (0.15 mM) hemin plates with spots identified by MALDI-TOF MS corresponding to group II proteins (HbpA, HbpD, and HbpE). Immunoblots were prepared and probed with anti-HbpA antibody. It is obvious, from the intensity and size of the immunolabeled spots, that group II proteins are more abundant when bacteria are grown at low hemin (Fig. 5C) relative to control hemin (Fig. 5B). The arrow indicates the size of the HbpD spot, which was confirmed by MALDI-TOF MS to extend into a lower pI range. Previously, we described our rabbit anti-HbpA antibody as monospecific, based on single-dimension immunoblots and knowledge of only a single member of the Hbp family (10).
This experiment clearly demonstrates that anti-HbpA antibody cross-reacts with HbpD and HbpE, the other members of subgroup II. We were unable to locate HpbC or HbpB by MALDI-TOF MS or by cross-reactivity with anti-HbpA from cultures grown at low hemin concentration.

**Expression of lacZ by the hbpA promoter parallels hbpA transcription.** Data presented thus far demonstrate that hbp transcription is affected by physiologically relevant environmental conditions. We were curious to determine if transcription factors were involved in regulation of transcript quantity. To this end, we constructed a reporter plasmid, pHPRO, consisting of the hbpA promoter region, HPRO, fused to a truncated lacZ gene (lacZ′). A diagram of this reporter plasmid is shown in Fig. 6A, along with two control constructs lacking either HPRO, pHPRO′LACZ′ (Fig. 6B), or lacZ, pHPRO′LACZ′− (Fig. 6C). These three constructs were electroporated into low-passage B. quintana strain JK31, and transformation was confirmed by restriction fragment length polymorphism analysis and sequencing. The transformation efficiency was approximately 0.8 transformants per µg DNA.

The resulting strains (JK31-pHPRO+LACZ′+, JK31-pHPRO−LACZ′+, and JK31-pHPRO′LACZ′−) were then analyzed by qRT-PCR in the two environments where hbpA expression is most affected: low hemin (0.05 to 0.15 mM) and low oxygen (5% relative to 21%). Fold differences were calculated for lacZ transcripts and are shown in Fig. 7. Although lacZ appears to be transcribed from a cryptic promoter in the control plasmid lacking HPRO (black bars), where relative amount of transcript (more than 3.5 to 4.0) is not influenced by the environment, data in Fig. 7B clearly show that without HPRO, repression is lost under low oxygen. lacZ is not detectable in strains lacking this truncated open reading frame (ORF), showing that B. quintana does not have a genomic copy of lacZ. hbpA expression in these strains and under the two conditions is similar to previous results in Fig. 2 and 3 (data not shown). These data suggest that a transcriptional regulator(s) is influencing the expression of hbpA, at least under conditions of low O2, and that the hbpA promoter region contains a cis-acting regulatory element.

**DISCUSSION**

*Bartonella* species require extraordinarily high hemin concentrations for growth, yet an explanation for this remains unknown. Several researchers have hypothesized that heme is essential as a source of porphyrin (34) and/or iron (10, 42) or as a hydrogen peroxide-detoxifying agent (33). Recently published genomic sequences (1) provide data that address these hypotheses: (i) neither *B. quintana* nor *B. henselae* is capable of de novo heme synthesis, as genes for nearly all porphyrin biosynthetic enzymes are missing; (ii) ORFs encoding uptake systems for heme (HutABC/HmuV), iron-siderophore (FatBCD/CeuD), and free iron (YfABC) are found in both species; (iii) genes coding for catalase, peroxidase, bacterioferritin, or ferritin-like heme or iron storage molecules and iron detoxification proteins (Dps) are absent in both genomes.

A survey of over 100 genomes using the KEGG database (http://www.genome.ad.jp/kegg/pathway.html) demonstrates that bacteria lacking the enzymes to perform de novo heme synthesis are quite rare. Although the absence of heme biosynthesis in *B. quintana* and *B. henselae* describes why this molecule is essential, it does not explain the extraordinary quantity that is necessary for routine culture. Compared to other pathogens that also lack this capability, the heme supplement for *Bartonella* is approximately 100-fold greater than that of *Porphyromonas gingivalis* and 1,000-fold greater than that of *Haemophilus influenzae* in aerobic iron-replete conditions (23, 25, 34, 49). One explanation assumes that more heme and/or iron is required as a nutrient by *Bartonella* to synthesize a relatively larger number of enzymes that utilize heme prosthetic groups and/or iron. However, while genomic sequence annotations for *B. quintana* and *B. henselae* (1) describe a number of enzymes...
that use iron as a cofactor, only two utilize heme prosthetic groups, succinate dehydrogenase and cytochrome O oxidase.

An alternative explanation for the high requirement is that heme molecules are not just metabolic nutrients per se but are also mediators of metabolic homeostasis, where heme may (i) function as a defense mechanism against ROS (ii) or exogenously generate a decreased oxygen environment for the bacterium. Although there is one report suggesting that *B. quintana* can respire anaerobically (17), it is generally accepted that the *Bartonella* species are aerobic (8). During aerobic respiration, superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) are naturally generated, and superoxide dismutase and catalase/peroxidase are normally employed, respectively, for intracellular detoxification (30). Although genes encoding superoxide...
dismutase can be found in both *B. quintana* and *B. henselae*, there are no genes encoding catalase or peroxidase (1). The apparent absence of a method for endogenous H₂O₂ detoxification suggests that either these bacteria possess an uncharacterized mechanism for its degradation, or they do not respire aerobically. First, since almost all catalases and peroxidases utilize a heme prosthetic group, it is possible that one or more of the hypothetical genes encode a novel heme enzyme for H₂O₂ detoxification and the concurrent requirement for high-level heme uptake. Second, although in vitro growth of *B. quintana* can be accomplished under atmospheric oxygen, the results of this study demonstrate that a microaerophilic environment of 5% O₂ is sufficient for replication. It has been demonstrated biochemically that *B. quintana* does not produce H₂O₂ and is catalase negative, two metabolic traits shared with clostridia and lactobacteria that respire anaerobically, even in the presence of oxygen (34). This suggests that *B. quintana* is not an aerobe and may not require much oxygen for metabolism. Determination of the heme requirements of *B. quintana* at very low O₂ concentrations is currently being examined to help address this hypothesis.

The second nonnutritional method whereby heme can mediate metabolic homeostasis is by creating a decreased oxygen microenvironment. First, *Bartonella* are members of the order *Rhizobiales*, along with several human (*Brucella* spp.) and plant (*Agrobacterium* spp.) pathogens. Many rhizobia form a symbiotic relationship with their legume host plant by fixing atmospheric nitrogen in root nodules. For nitrogen fixation to occur, a microaerophilic environment must be established for the bacteria. This is accomplished by plant-generated leghemoglobin (a molecule similar to hemoglobin) binding to the rhizobial surface, effectively shielding the bacteria, and O₂-labile nitrogenase, from oxygen (3). Considering the close relationship of *Bartonella* and rhizobia, it is tempting to speculate that heme binding is a common strategy used by members of this order to decrease oxygen in the environment. In addition, orthologues of the Hbps can be found in *Brucella* and *Agrobacterium* (13, 32). Second, *P. gingivalis* stores heme dimers on its surface to both exclude oxygen from the cell (44) and function as an antioxidant by the intrinsic peroxidase activity of heme (43). Interestingly, the hemin blotting technique that we first used to identify the Hbp family proteins (10) relies on this intrinsic peroxidase activity and shows that the heme bound to the Hbps might act as H₂O₂ detoxifiers.

The human body louse has been implicated as the insect vector for three major human diseases: epidemic typhus (*Rickettsia prowazekii*), relapsing fever (*Borrelia recurrentis*), and trench fever (*B. quintana*) (15). Body lice live between the skin and clothing of humans, where the temperature is approximately 30°C (27, 29). Lice imbibe human blood several times per day and hemolyze erythrocytes almost immediately (9, 47), resulting in waves of potentially toxic heme, iron, and ROS with each meal. It has been well established that *B. quintana* multiplies extracellularly in the gut of the louse (14, 19) and is
thus repeatedly exposed to these toxic molecules and digestive enzymes.

Our data show that *hbpC* transcript increases >100-fold at louse-like temperature (Fig. 1) and 10-fold at high hemin concentration (Fig. 4). Together, these results strongly suggest that *HbpC* would be preferentially synthesized in the louse gut. Considering the nutritive and nonnutritive requirements for the high heme requirement given above, the most straightforward hypothesis is that *HbpC* functions to bind heme on the surface, creating an antioxidant barrier. Heme and iron detoxification and storage are also challenges for hematophagous arthropod vectors, as hemoglobin is a major protein source (35, 37). This implies that *B. quintana* must compete with the louse for heme and iron molecules and suggests that *HbpC* could also function as a heme storage site. An intriguing parallel occurs in the flea-borne agent of bubonic plague, *Yersinia pestis*, where (i) the outer membrane is the primary site of exogenous heme storage (39), (ii) the storage phenotype is most evident at the temperature of the flea (40), and (iii) heme storage is required for colonization of the flea proventriculus and subsequent transmission (16). Finally, louse fecal matter is the most common vehicle for *B. quintana* transmission to humans, where *B. quintana* can survive up to a year (21). Each environmental cue of fecal matter (21% O₂, low temperature, and high heme) resulted in an increased expression of *hbpC*, suggesting that *HbpC* may contribute to survival in fecal matter. In nature, these conditions are experienced simultaneously and may have a different effect on the *hbp* transcript profile than the individual in vitro simulations presented in this study. We propose that *HbpC* is required for survival of *B. quintana* in the louse and/or fecal matter and are presently addressing this hypothesis with an *hbpC* mutant and our established body louse colony.

*B. quintana* is exposed to a very different environment in the human. Available heme is scavenged by hemopexin, hemoglobin, and serum albumin, and free iron is chelated by a number of molecules depending on whether the bacterium is intracellular or extracellular. As growth of *B. quintana* is dependent on heme availability, mechanisms must be employed for its binding and transport into the cell. Genes encoding uptake systems for heme, iron-siderophore, and free iron are found in both *B. quintana* and *B. henselae* (1), yet their functions have not been studied. To date, all *Bartonella* species examined also contain genes encoding the Hbp family proteins (originally termed “Pap” in *B. henselae*) (50). Our data demonstrate that the relative quantity of subgroup II (*hbpA, hbpD*, and *hbpE*) transcript is significantly upregulated at low hemin concentrations (Fig. 3), suggesting that subgroup II proteins are utilized in the human for acquisition of heme. This notion is supported by qRT-PCR analysis of the *hbp* transcript profile in a Rhesus macaque where the hierarchy (*hbpD > hbpA > hbpE > hbpC > hbpB*) is consistent with this in vitro low-heme condition (data not shown).

Oxygen, heme, and iron are molecules that are required by almost all living organisms to maintain metabolic homeostasis, yet each of these nutrients can become toxic if the concentration is too high (6, 18). Furthermore, heme and iron are capable of transforming oxygen into highly toxic ROS (O₂⁻, H₂O₂, and OH⁻). Humans maintain their bloodstream O₂ between 3 and 5%, much lower than the atmospheric 21% used for routine cultivation of *B. quintana*. Nothing is known about the O₂ concentration in a louse gut, but we hypothesize that large amounts of ROS are present during blood meal digestion. Our data show that all of the *hbp* transcripts decrease significantly when *B. quintana* is grown at an O₂ concentration that simulates the human bloodstream (Fig. 2). This finding strongly suggests that the Hbps respond to O₂ and/or ROS. Again, the simplest explanation is that surface-bound heme functions as an antioxidant barrier, where bacteria grown at 5% oxygen encounter relatively less ROS and, thus, less Hbp is necessary to maintain metabolic homeostasis. Assuming that the Hbps also function in transport of hemin for nutritional purposes (50), it is also possible that enzymes which utilize porphyrin and/or iron are not required to the extent they are at 21% O₂. In nature, one would expect relative *hbp* expression to be highest in louse fecal matter and lowest in the human bloodstream.

*B. quintana* is also unusual in that it apparently lacks mechanisms for iron storage (ferritin), heme storage (bacterioferritin), iron detoxification, and antioxidant defense (catalase and peroxidase). To prevent intracellular oxidative damage and iron toxicity, the regulation of genes involved in heme uptake and subsequent nutritive utilization must be tightly regulated (2, 45). It is obvious that *hbpC* transcription is temperature regulated and that oxygen and hemin also influence transcription of the *hbp* genes. We generated a *lacZ* reporter construct (Fig. 6) to determine if the *hbpA* promoter region could control transcription of an exogenous locus. Although the presence of the cryptic promoter may be affecting *lacZ* expression at low hemin concentrations (Fig. 7A), it is evident that repression of *lacZ* is occurring under low oxygen concentrations (Fig. 7B). This strongly suggests that one or more regulators are affecting transcription and that the *hbpA* promoter region contains a cis-acting regulatory element. Experiments are under way to identify the specific trans-acting regulators and the cognate promoter elements of the *hbp* genes.

In conclusion, the roles that the Hbp family play in essential heme acquisition and maintenance of metabolic homeostasis are unknown. We report here the differential and coordinated expression of *hbp* genes in response to environmental conditions that simulate the human host and louse vector. Based on differential expression patterns, we propose that there are two subgroups of *hbp* genes, subgroup I (*hbpC* and *hbpB*) and subgroup II (*hbpA, hbpD*, and *hbpE*). We are downplaying the role of *hbpB* for several reasons: (i) *hbpB* contains a ~510-bp insert in the center of its ORF and recombinant HbpB does not bind hemin like other Hbps (J. A. Carroll and M. F. Minnick, unpublished data); and (ii) in all qRT-PCR studies, the relatively high average C_T value suggests that very little *hbpB* transcript is produced. Continued work on this heme receptor gene family is expected to yield valuable clues regarding the extraordinary need for heme as well as the roles that these virulence factors play in the survival and pathogenesis of *Bartonella*.

ACKNOWLEDGMENTS

We are grateful to Jane Koehler (UC Davis, San Francisco, CA) for the generous contribution of *B. quintana* strain JK31. We thank Jesse Hay, Beverly Parker, and Holly Cox (University of Montana) as well as Matthew Chenoweth (National Cancer Institute) for their valuable technical assistance with cell disruption, MALDI-TOF, MASCOT fingerprinting analysis, and two-dimensional electrophoresis. Finally, we
thank Patty McIntire (Murdoch Sequencing Facility, University of Montana) for providing sequence data and James Gannon (University of Montana) and James Carroll (University of Pittsburgh) for helpful discussions on bacterial physiology and qRT-PCR, respectively. This work was supported by Public Health Service grant R01 AI50111 from the National Institutes of Health.

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