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Function, Regulation, and Transcriptional Organization of the Hemin Utilization Locus of *Bartonella quintana*\(^{\dagger}\)

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*Bartonella quintana* is a gram-negative agent of trench fever, chronic bacteremia, endocarditis, and bacillary angiomatosis in humans. *B. quintana* has the highest known hemin requirement among bacteria, but the mechanisms of hemin acquisition are poorly defined. Genomic analyses revealed a potential locus dedicated to *hemin utilization* (*hut*) encoding a putative hemin receptor, HutA; a TonB-like energy transducer; an ABC transport system comprised of three proteins, HutB, HutC, and HmuV; and a hemin degradation/storage enzyme, HemS. Complementation analyses with *Escherichia coli hemA* show that HutA functions as a hemin receptor, and complementation analyses with *E. coli hemA tonB* indicate that HutA is TonB dependent. Quantitative reverse transcriptase PCR analyses show that *hut* locus transcription is subject to hemin-responsive regulation, which is mediated primarily by the iron response regulator (Irr). Irr functions as a transcriptional repressor of the *hut* locus at all hemin concentrations tested. Overexpression of the ferric uptake regulator (*fur*) represses transcription of *tonB* in the presence of excess hemin, whereas overexpression of the rhizobial iron regulator (*rirA*) has no effect on *hut* locus transcription. Reverse transcriptase PCR analyses show that *hutA* and *tonB* are divergently transcribed and that the remaining *hut* genes are expressed as a polycistronic mRNA. Examination of the promoter regions of *hutA*, *tonB*, and *hemS* reveals consensus sequence promoters that encompass an H-box element previously shown to interact with *B. quintana* Irr.

*Bartonella quintana*, the gram-negative bacterial agent of epidemic trench fever during World Wars I and II, is one of several *Bartonella* species of current medical relevance (27). *B. quintana* is transmitted to humans via the human body louse (*Pediculus humanus corporis*) and is reemerging in large metropolitan areas among destitute individuals as the cause of “urban trench fever.” Risk factors for urban trench fever include alcoholism, homelessness, and exposure to body lice (33). Unlike classical trench fever, a self-limiting flulike disease, the reemerging disease is associated with chronic bacteremia and endocarditis regardless of immune status (33). *B. quintana* infection can also result in bacillary angiomatosis, which is the development of proliferative vascularized lesions of the skin (43). Although bacillary angiomatosis primarily affects patients infected with human immunodeficiency virus (HIV) or other immunodeficiencies, a limited number of cases have been reported in immunocompetent individuals (46).

HIV infects approximately 0.47% of the general U.S. adult population, and there are an estimated 800,000 homeless people in the United States on any given day (26, 38). Some studies suggest that HIV prevalence is up to five times higher in homeless populations than in the general population (1). Despite the relatively large population at risk for *B. quintana* infection, trench fever is recognized as a “neglected infection of poverty” (19). Accordingly, insufficient data exist for a general estimate of prevalence in the United States and very little is known about the pathogenesis of this bacterium.

Utilization of host heme-containing proteins as a source of iron is a common strategy for bacterial pathogens (10). In addition to using these heme or hemin (the Fe\(^{3+}\) oxidation product of heme) sources, *Bartonella* species are unique in their ability to parasitize human erythrocytes (27, 37). In the absence of erythrocyte lysates or hemoglobin, in vitro growth of *B. quintana* requires media supplemented with the highest known concentrations of hemin among bacterial species (31). Free heme is toxic in humans due to its lipophilic nature and ability to participate in the generation of reactive oxygen species via Fenton chemistry. Therefore, it is either rapidly catalyzed by a heme oxygenase system or neutralized by one of several host heme-binding proteins, maintaining a very low concentration (22). However, complexed heme, primarily hemoglobin, is abundant (16). Acquisition of heme in the limiting environment of the human host is pivotal to the survival and pathogenesis of *B. quintana*. In contrast to the human host, in the gut of blood-sucking arthropods free heme is thought to exceed toxic levels during the initial digestion of a blood meal (34). The ability of *B. quintana* to withstand the heme-limiting environment of the human host and the heme-replete gut of the body louse suggests that its heme acquisition systems are tightly regulated.

Little is known about molecular mechanisms or regulation of heme acquisition by *Bartonella*. Previous studies by our lab focused on the heme-binding proteins (HbP\(\text{A}\) to HbP\(\text{E}\)), a five-member family of outer membrane porin-like proteins (28). In addition to binding hemin, HbP proteins are transcriptionally regulated in response to variations in ambient temperature, oxygen level, and hemin concentration (5). This regulation is mediated in part by Irr (iron response regulator), a
member of the ferric uptake regulator (Fur) superfamily first described for Bradyrhizobium japonicum, which responds directly to hemin (17). Irr acts as either a transcriptional activator or a repressor by binding the iron control element (ICE) of \textit{B. japonicum}, and the effect of Irr on target genes is believed to be a consequence of the ICE’s location relative to the transcriptional start site (TSS) (39). In \textit{B. quintana}, Irr operates by binding a unique DNA motif found in the promoter region of all \textit{hbp} genes, termed the “H box” (6). \textit{B. quintana} has at least two additional iron- and/or hemin-responsive regulators, namely, Fur and RirA (rhizobial iron regulator \textit{A}) (2). In gammaproteobacteria, Fur functions as a transcriptional regulator of iron and hemin uptake systems, with activation dependent on intracellular iron concentrations (18). In contrast, Fur has been shown to play a diminished or nonexistent role in alphaproteobacteria (20).

\textbf{In silico analyses.} Genomic sequences for \textit{B. quintana} strain Toulouse were accessioned at the RhizoDB website (http://xbase.bham.ac.uk/rhizodb/) (11) or the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). BLAST was employed for all database searches (3), and ClustalW version 2.0 (23) was used for multiple amino acid sequence alignments. The Protein DataBank (www.protein.dataбанк.org) was used as the standard antibiotic concentrations when required. For induction of gene expression, IPTG (isopropyl- \textit{p}-thiogalactopyranoside) was added to mid-log cultures at a final concentration of 2 mM and cultures were grown for an additional 4 to 5 h. For growth of \textit{E. coli} strain E353, overnight cultures were centrifuged and the supernatant fraction was stored at 20°C until needed. Samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 12.5% (wt/vol) acrylamide gels (4). Gels were rinsed three times for 5 min in deionized water and then stained with 0.05% (wt/vol) Coomassie blue in deionized water for 30 min. Following destaining in water, the purified HutA band was excised and used to generate rabbit anti-HutA antiserum as previously described (42).

\section*{Materials and Methods}

\textbf{Bacterial strains and growth conditions.} \textit{E. coli} was routinely grown overnight at 37°C in Luria-Bertani (LB) or tryptone-yeast extract (TY) medium, with standard antibiotic concentrations when required. For induction of gene expression, IPTG (isopropyl- \textit{p}-thiogalactopyranoside) was added to mid-log cultures at a final concentration of 2 mM and cultures were grown for an additional 4 to 5 h. For growth of \textit{E. coli} strain E353, overnight cultures were centrifuged at 100,000 \textit{g} at 4°C in an SW60Ti rotor (Beckman Coulter, Fullerton, CA). The Sarkosyl-insoluble pellet was resuspended in 0.2 M NaOH and filtered sterilized for a stock solution. In order to maintain pBBR1MCS and derivatives in \textit{B. quintana}, medium was supplemented with 1 \textmu M chloramphenicol.

\textbf{Preparation and manipulation of nucleic acids.} \textit{B. quintana} genomic DNA was purified with a DNAeasy blood and tissue kit (Qiagen, Valencia, CA). Plasmids were purified with a QiAprep spin miniprep kit (Qiagen), a Perfectprep plasmid minikit (Eppendorf, Hamburg, Germany), or a Wizard Plus midiprep DNA purification system (Promega, Madison, WI). Routine procedures were employed for PCR amplification, ligation, cloning, and restriction endonuclease digestion (4). PCR and sequencing primers were synthesized by Operon Biotechnologies (Huntsville, AL).

\begin{table}[h]
\centering
\caption{Strains and plasmids used in this study.}
\begin{tabular}{lll}
\hline
Strains & Description & Source or reference \\
\hline
\textit{B. quintana} & & \\
JK31 & Low-passage human isolate & J. Kochler \\
JK31+pBR & harboring pBBR1MCS & 6 \\
JK31+pBR-FUR & harboring pBBR-FUR & 6 \\
JK31+pBR-RJA & harboring pBBR-RJA & 6 \\
JK31+pBR-IRR & harboring pBBR-IRR & 6 \\
E. coli & & \\
JM109 & Host strain for cloning & Promega \\
TOP10F & Host strain for cloning & Invitrogen \\
EB53 & harboring pBBR1MCS & 50 \\
IR754 & harboring pBBR1MCS & 6 \\
EB53+pWSK29 & harboring pBBR1MCS & 6 \\
EB53+pnP1 & harboring pnP1 & This study \\
IR754+pWSK29 & harboring pBBR1MCS & This study \\
IR754+pnP1 & harboring pnP1 & This study \\
Plasmids & & \\
pCR2.1-TOPO & TA cloning vector & This study \\
pNP2 & pCR2.1TOPO with \textit{B. quintana} hutA & This study \\
pWSK29 & Low-copy-number expression vector & 50 \\
pNP1 & pWSK29 with \textit{B. quintana} hutA & This study \\
pOE0 & pQE30 vector & Qiagen \\
pNP3 & pQE30 with \textit{B. quintana} hutA & This study \\
pBBR-FUC & shuttle vector for \textit{Bartonella} & 21 \\
pBBR-RJA & pBBR1MCS with \textit{B. quintana} hutA & This study \\
pBBR-IRR & pBBR1MCS with \textit{B. quintana} hutA & This study \\
\end{tabular}
\end{table}

\textbf{Complementation assays.} The ability of \textit{E. coli} \textit{hutA} strain E353 or IR754 (containing pNP1 or vector alone) to use hemin was examined as previously described, with modifications (47). Briefly, overnight cultures were centrifuged at 3,900 \times g for 5 min at 4°C and pellets were resuspended in 5 ml TY without ALA. Cultures were incubated for 2 h at 37°C with shaking to deplete intracellular ALA and hemin and then used to inoculate 8-ml cultures of TY alone or supplemented with either ALA (50 \mu M) or hemin chloride (10 \mu M or 50 \mu M) to an initial optical density at 600 nm of OD600 of 0.02. Cultures were incubated at 37°C with agitation, and OD600 was measured every 4 h for 24 h.

\section*{Generation of anti-HutA antisera.} A His\textsubscript{6}-tagged mature \textit{B. quintana} HutA protein was generated and purified under denaturing conditions using a QIAexpress kit (Qiagen). Purified protein fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 12.5% (wt/vol) acrylamide gels (4). Gels were rinsed three times for 5 min in deionized water and then stained with 0.05% (wt/vol) Coomasie blue in deionized water for \textasciitilde 30 min. Following destaining in water, the purified HUtA band was excised and used to generate rabbit anti-HutA antisera as previously described (42).

\section*{Sarkosyl fractionation and immunoblotting.} Proteins were quantified by a bicinchoninic acid protein kit (Pierce, Rockford, IL). Sarkosyl fractionation was performed essentially as previously described (52). Briefly, overnight cultures of \textit{E. coli} were harvested, washed in phosphate-buffered saline (pH 7.4), and resuspended in sterile distilled H\textsubscript{2}O. Cell lysis was done with a Fastprep bead homogenizer as described above. Cells were incubated for 30 min in 2% (vol/vol) N-lauroyl sarcosinate (Sigma, St. Louis, MO) at room temperature and then centrifuged for 1 h at 100,000 \textit{g} at 4°C in an SW60Ti rotor (Beckman Coulter, Fullerton, CA). The Sarkosyl-insoluble pellet was resuspended in 0.2 M phenylmethylsulfonyl fluoride in deionized water (Sigma). Both the resuspended pellet and the Sarkosyl-soluble supernatant fraction were stored at \textasciitilde 20°C until needed. Samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to supported nitrocellulose (GE Water & Process Technologies, Tewksbury, MA) for immunoblotting (49). The resulting blots
were cloned into pCR2.1-TOPO and used to transform 
"E. coli"
was added, and tailed cDNA was PCR amplified with the abridged anchor primer
version 2.0 (Invitrogen, Carlsbad, CA). Briefly, RNA was reverse transcribed
and
icant.
ning ready reaction kit 3.1 (ABI, Foster City, CA). Sequence data were analyzed
hutB
control for contaminating DNA.
A reaction mixture lacking reverse transcriptase was used as a PCR template to
hacute
protocol. The resulting cDNA was used as a PCR template with primer sets for
described using SuperScript III first-strand synthesis for RT-PCR (Invitrogen) per

FRAP and NPNL domains (10) (Fig. 1B). A conserved histidine
pathogenic bacteria shows conservation of characteristic
alignment of HutA with hemin/hemoglobin receptors of other
of TonB-dependent proteins (data not shown) (30). A ClustalW

HemR has been replaced by a tyrosine (Tyr 505) in

and

TY media supplemented with 0.05 mM ALA and neither strain
despite hemin/ALA starvation. Regardless, these data indicate

However, expression of
HutA could not restore growth of IR754 in media

RESULTS

In silico analyses of B. quintana hut locus. The hut locus consists of six genes encoding a potential receptor, HutA; an
ABC transport system, HutBC and HmuV; a TonB ortho-

developmental polymerase-conjugated goat anti-rabbit antibodies (Sigma), 4-chlo-
ronaphthol, and hydrogen peroxide, as previously described (42).

qRT-PCR and RT-PCR. Differences in hut locus expression were quantified
for B. quintana grown on BA supplemented with low (0.05 mM) or high (2.5
mM) hemin relative to an optimal hemin concentration (0.15 mM) or for JK31
overexpressing fur, irr, or irr relative to JK31 with pBRR1MCs vector alone (6).
For each condition, 500 ng RNA was reverse transcribed per the manufacturer's
instructions with an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Tem-
plate cDNA (0.67 ng) and 500 nM of each primer were used per 25 μl reaction
mixture with iQ Sybr green supermix (Bio-Rad) as recommended. qRT-PCR
mixtures were incubated for 5 min at 95°C and then 40 cycles at 95°C for 30 s
followed by 55°C for 30 s. Data were obtained with a MyQ real-time PCR
detection system and Optical System software, version 1.0 (Bio-Rad). Mean
values from each triplicate reaction were used to determine individual differ-
ences in gene expression by the 2−ΔΔCT method using 16S rRNA as the internal
control (24).

Transcriptional organization of the hut locus genes was examined by reverse
transcribing the hmuV transcript and using it for PCR amplification of individual
hut genes as previously described (29). Briefly, 500 to 1,100 ng DNase-treated
RNA from JK31 grown on BA containing 0.05 mM hemin was reverse trans-
scribed using SuperScript III first-strand synthesis for RT-PCR (Invitrogen) per
protocol. The resulting cDNA was used as a PCR template with primer sets for
hems, hutA, hutB, hutC, and hmuV (see Table S1 in the supplemental material).
A reaction mixture lacking reverse transcriptase was used as a PCR template to
control for contaminating DNA.

TSS mapping. RNA was isolated from B. quintana grown on BA supple-
mented with 0.05 or 0.15 mM hemin and used for TSS mapping of tonB, hutA,
and hmuV with a system for 5′ rapid amplification of cDNA ends (5′ RACE),
version 2.0 (Invitrogen, Carlsbad, CA). Briefly, RNA was reverse transcribed
with SuperScript II and the resulting cDNA was RNA treated. Following pu-
rification of cDNA with a QIAquick PCR purification kit (Qiagen), a 3′ dCT
was added, and labeled cDNA was PCR amplified with the abridged anchor primer
supplied in the 5′ RACE kit and a nested, gene-specific primer. PCR products
were cloned into pCR2.1-TOPO and used to transform E. coli TOP10F−
per the TOPO TA cloning (Invitrogen) protocol. Plasmids were screened for appropri-
ately sized inserts and sequences.

DNA sequencing. Sequence data were obtained with an automated DNA
sequencer (AB3130xl genetic analyzer) and a BigDye Terminator cycle sequenc-

Statistical analyses. Three independent determinations were used to calculate
the means and standard deviations for all numerical data. Statistical significance
was determined using Student’s t test, with P values of <0.05 considered signif-

B. quintana HutA (10) (Fig. 1B). This substitution is also seen in BhuR, the Bordetella avium heme/hemoprotein receptor (30). Like BhuR, HutA shares more homology with the “heme scavenger” subclass of receptors than with the hemoglobin subclass (e.g., HmbR of Neisseria meningitidis) (45). Three-
dimensional modeling of B. quintana HutA showed structural similarity to the ferric citrate receptor (FecA) of E. coli (14),
where threading revealed the expected 22 antiparallel β-strands and 11 extracellular loops characteristic of TonB-
dependent receptors (data not shown) (13). Tyr 505 was cen-
trally positioned in one of the extracellular loops of the prot.

 coat, as were four additional tyrosines (i.e., residues 278, 451,
511, and 512) and histidine 389 (9). In silico data strongly
suggest that the hut locus is a system dedicated to hemin
acquisition and that HutA functions as the receptor.

Complementation of E. coli hemA strains. The outer membrane of E. coli K-12 is impermeable to hemin, and growth
defects from mutations in porphyrin biosynthesis genes cannot be
overcome with hemin supplements (41). E. coli hemA aroB
strain EB53 is a K-12 derivative with a mutation in glutamyl-
tRNA reductase, required for biosynthesis of ALA and ulti-
ately protoporphyrin IX (7). Exogenously supplied ALA can
restore growth of EB53; however, utilization of hemin requires a
functional hemin receptor (44). To test the hypothesis that B.
quintana HutA functions as a hemin receptor, hutA was cloned
into pWSK29 to produce pNP1 and used to transform EB53.
When grown in the absence of ALA and hemin, EB53/
pWSK29 and EB53/pNP1 exhibited the characteristic “leaky”
growth (maximum OD600 of ~0.185 in 24 h [data not shown])
previously noted for these strains (41). Normal growth curves
were obtained for both EB53/pWSK29 and EB53/pNP1 when
TY broth was supplemented with 0.05 mM ALA (data not shown).
However, when media were supplemented with 10 μg/ml hemin,
EB53/pNP1 grew to a significantly higher OD600 by 24 h (P < 0.029) than EB53/pWSK29 (Fig. 2A). This
difference was more pronounced when strains were grown in
media supplemented with 50 μg/ml hemin (P < 0.0002) (Fig.
2B). Interestingly, rescue of the hemA mutation in E. coli by B.
quintana HutA is not apparent until ~16 h postinoculation
despite hemin/ALA starvation. Regardless, these data indicate
that B. quintana HutA functions as a hemin receptor and that
its expression in EB53 is sufficient to allow utilization of hemin
as a sole porphyrin source.

In order to examine HutA’s potential dependence on TonB
for energization, pNP1 was also used to transform E. coli
strain IR754 (47). As observed for the EB53 strains, both IR754/
pWSK29 and IR754/pNP1 exhibited normal growth curves in
TY media supplemented with 0.05 mM ALA and neither strain
surpassed the basal “leaky” growth level in media lacking both
ALA and hemin (data not shown). However, expression of B.
quintana HutA could not restore growth of IR754 in media
supplemented with either 10 μg/ml or 50 μg/ml hemin (Fig. 2A
and B, respectively), in direct contrast to EB53. These data
clearly indicate that HutA-mediated hemin uptake is depen-
dent on E. coli TonB.

Synthesis of HutA in B. quintana and E. coli. We were able
to identify native HutA in B. quintana and recombinant His-
tagged HutA in E. coli strain JM109/pNP3 (data not shown)
but were unable to detect HutA in E. coli strain EB53/pNP1 by
Coomassie blue staining or Western blotting. However, expres-
transcription and induction of hutA mRNA in *E. coli* strain EB53/pNP1 were detectable by qRT-PCR. As expected, cDNA from EB53/pWSK29 gave results similar to those obtained from a no-template control (data not shown). Recombinant HutA protein is undoubtedly localized to the outer membrane of EB53/pNP1, as deduced from its ability to rescue the *hemA* mutation and restore growth in the presence of hemin. Failure to detect HutA in EB53/pNP1 is possibly due to a combination of low-level expression and antiserum cross-reactivity. A similar situation was reported for detection of the recombinant *Bartonella henselae* orthologue (Pap 31) in *E. coli* strain M15 until a monoclonal antibody was employed (52).

Transcription of *hut* locus genes is hemin responsive. *hut* locus genes were expected to be tightly regulated in response to available hemin. To investigate this hypothesis, RNA was isolated from *B. quintana* grown on media supplemented with low (0.05 mM), optimal (0.15 mM), and high (2.5 mM) concentrations of hemin and used to examine differences in *hut* transcript levels by qRT-PCR. Data show that expression of *hut* locus genes from JK31 grown on BA-0.05 mM hemin is only ~1.5-fold higher than that obtained from JK31 grown on BA-0.15 mM hemin, suggesting that this range of hemin concentrations does not substantially alter expression of the *hut* locus. The most pronounced change was observed when transcript levels from JK31 grown on BA-2.5 mM hemin were compared to those from JK31 grown on BA-0.15 mM, where results show a 2.2-fold decrease in transcription of *hut* locus genes in response to excess hemin (Fig. 3). Furthermore, the *hut* locus genes are coordinately regulated, as evidenced by the fact that differences for each *hut* locus gene are repressed to approximately the same magnitude. These results show that *hut* locus genes are transcriptionally downregulated in response to excess hemin.

**Hemin-responsive control is mediated by *B. quintana* Irr.** To elucidate regulation of the *hut* locus genes, JK31 strains that overexpress one of three iron/hemin-responsive regulator
genes were used for qRT-PCR experiments to investigate the effects on transcription. RNA was isolated from overexpression and vector-only strains grown in parallel on BA-0.05 mM hemin, BA-0.15 mM hemin, and BA-2.5 mM hemin to control for cofactor availability. Average differences between JK31+pBBR-RIRA and JK31+pBBR indicate a 7- to 16-fold increase in \( rirA \) transcription but less than a 1.8-fold increase in transcription of any \( hut \) locus gene under optimal or hemin-limiting growth conditions. Likewise, overexpression of \( rirA \) results in less than a 1.8-fold decrease in transcription of any \( hut \) locus gene when RNA is isolated from strains grown in the presence of excess hemin (Fig. 4A). These data suggest that \( rirA \) overexpression does not appreciably affect transcription of \( hut \) locus genes regardless of ambient hemin concentration.

Similar results were obtained when \( hut \) locus transcript levels were compared for JK31+pBBR-FUR relative to JK31+pBBR after growth on BA-0.05 mM hemin. These conditions resulted in less than a 1.7-fold increase in any \( hut \) locus gene, while \( fur \) overexpression was evident by a 4.4-fold increase (Fig. 4B). On BA-0.15 mM hemin, \( fur \) transcription was increased ~8-fold in JK31+pBBR-FUR relative to JK31+pBBR, \( hemS \) levels were almost identical, and the remainder of the \( hut \) locus genes showed a minor decrease in transcription. \( fur \) overexpression results in a 2.5-fold increase in \( fur \) and a 4-fold decrease in \( tonB \), when comparing levels of \( hut \) locus expression from strains grown in the presence of excess hemin. The remainder of the \( hut \) locus shows only a minor decrease in expression, as seen in strains grown with optimal hemin concentrations. These data suggest that \( fur \) overexpression in the presence of excess hemin exerts a repressive effect on \( tonB \) that is not imposed on other members of the \( hut \) locus.

\( irr \) overexpression results in decreased transcription of the entire \( hut \) locus (Fig. 4C). Average differences in transcription of \( hut \) locus genes from JK31+pBBR-IRR relative to JK31+pBBR showed a 7- to 12-fold increase in \( irr \) mRNA and an
2.5-fold decrease in transcription of all hut genes regardless of hemin concentration. Interestingly, the decrease in transcription during irr overexpression is similar in magnitude to the decrease in hut locus expression in the presence of excess hemin (Fig. 3). These data suggest that hemin-responsive regulation of hut genes is mediated, at least in part, by Irr.

Transcriptional organization of the hut locus. The genomic arrangement of the hut locus suggested that hutA and tonB might be divergently transcribed, while hemS, hutBC, and hmuV could be polycistronic (Fig. 1A). To test these hypotheses, RNA from JK31 was reverse transcribed with a hmuV primer. PCR analyses were performed on the resulting cDNA using primers specific to each member of the hut locus (except tonB), and a separate PCR with genomic DNA as a template was used as a positive control for each gene. Data indicate that hemS, hutB, hutC, and hmuV are all present in the hmuV transcript, as evidenced by the PCR amplicons generated with primers specific to each of these genes from the hmuV cDNA. In contrast, no hutA amplicon is generated from the cDNA (Fig. 5). Furthermore, no PCR amplicons were generated from the reactions using the sample that was not reverse transcribed, which confirms the absence of contaminating DNA. These data show that hemS, hutB, hutC, and hmuV are cotranscribed as part of a polycistronic transcript from the hemS promoter, while hutA is transcribed as a separate mRNA.

TSS mapping and identification of H-box elements. To further elucidate regulation of the hut locus, TSSs were mapped for tonB, hutA, and hemS by 5’ RACE. The hutA TSS was found 121 bp upstream of its start codon, and the tonB TSS was mapped 40 bp upstream of its start codon. Putative –10 and

FIG. 4. qRT-PCR analyses of B. quintana hut locus in response to overexpression of various iron response regulators. Average differences in hut locus transcription from JK31+pBBR-RIRA (A), JK31+pBBR-FUR (B), and JK31+pBBR-IRR (C) relative to JK31+pBBR. Strains were grown in parallel on BA-0.05 mM hemin, BA-0.15 mM hemin, and BA-2.5 mM hemin. Data represent the means ± standard deviations from three independent determinations.
-35 sites were identified in the 78-bp divergent promoter region (Fig. 6A). Examination of the promoter region between tonB and hutA showed ~69% identity with a 40-bp consensus sequence previously identified in Bartonella hbp promoter regions (6). The H box completely encompasses the -10 and -35 sites of hutA and is located 1 bp before the potential -35 site of tonB. In contrast, no obvious similarity to the Fur-binding motifs of E. coli or B. japonicum Fur proteins was found upstream of tonB (15).

The hemS TSS was mapped 70 bp upstream of the start codon, and potential -10 and -35 sites were identified relative to the TSS (Fig. 6B). The hemS promoter region also contains a site with ~57% identity to the H-box consensus sequence. This region overlaps the predicted -35 site of hemS but does not extend to the -10 site. Identification of motifs similar to the H box and surrounding consensus sequence in the promoter regions of hutA, tonB, and hemS is consistent with qRT-PCR data showing repressive effects of irr overexpression on hut locus expression (Fig. 4C). Together, these results suggest that Irr represses transcription of hut locus genes by binding at or near RNA polymerase recognition sites.

DISCUSSION

Genomic analyses of the B. quintana genome show multiple systems possibly involved in hemin and/or iron acquisition. One of these, the hut locus, appeared to be the most likely and complete candidate for a hemin uptake system. Based on multiple lines of in silico evidence, including amino acid similarity, domain conservation, and structural similarity, we hypothesized that HutA was functioning as a hemin receptor (Fig. 1).

We tested the hypothesis by functional expression of B. quintana hutA in E. coli hemA strain EB53 (12, 41). Expression of hutA trans-complemented EB53 was tested in the presence of hemin at two concentrations (Fig. 2). The E. coli hemA strains have a leaky phenotype, which accounts for the low-level increase in optical density over time (41). The results required an ~12- to 16-h lag before a difference in growth rate between complemented and control strains was discernible. This observation may be due to limited homology between TonB and TonB boxes of B. quintana and E. coli (30). Nevertheless, sufficient homology in TonB allowed B. quintana HutA to function as a hemin receptor in EB53, whereas HutA could not complement an otherwise isogenic E. coli strain (IR754), where both tonB and hemA are mutagenized (Fig. 2).

The B. quintana hut locus is similar to other bacterial hemin acquisition systems in that it is transcriptionally regulated in a hemin-responsive manner (Fig. 3). Growth on low hemin results in a slight increase in hut locus mRNAs relative to the quantity obtained from growth on optimal hemin. Although the difference in hemin concentrations between BA-2.5 mM hemin and BA-0.15 mM is much greater, the decrease in hut gene transcription is fairly modest. Based on these data, it is tempting to speculate that changes in extracellular hemin are buffered in B. quintana, possibly by an accessory hemin-binding system, such as the Hbp proteins. Such a system could enhance the ability of B. quintana to withstand hemin fluctuations in the divergent environments of the body louse and human bloodstream.

Effects of overexpression of hemin/iron-responsive regulators (irr, rirA, and fur) indicate that hemin-responsive changes...
in *hut* locus transcription are mediated primarily by Irr. Although Irr homologs have been reported to act as transcriptional activators of hemin/iron genes when hemin is limiting (25, 39), *B. quintana* Irr represses *hut* locus genes. *B. quintana* Irr may also transcriptionally activate *hut* locus genes in the absence of hemin, but the absolute requirement for hemin by *B. quintana* prohibits investigating this possibility (31). Our data suggest that *irr* overexpression results in repression of *hut* locus genes regardless of ambient hemin concentration, despite previous reports suggesting that *B. japonicum* Irr is degraded upon binding hemin (51). Interestingly, the N-terminal heme response motif (amino acids 28 to 33) of *B. japonicum* Irr is not conserved in *B. quintana* Irr (51). Likewise, only two of three histidine residues implicated in a second hemin-binding...
site (51) are present in B. quintana Irr. Of note, both B. quintana and E. coli Fur have two histidines in this domain but are not degraded by hemin (data not shown). B. japonicum Irr represses protoporphyrin biosynthesis genes when heme is present, but the majority of these genes are not present in the B. quintana genome, suggesting that B. quintana Irr plays a distinct role (6, 36). Of specific interest, heme-mediated degradation of Irr in B. japonicum requires ferrochelatase (36), but an orthologue is absent in B. quintana (2).

RT-PCR analyses show that the hut locus is expressed as three transcripts, originating from a divergent promoter region between hutA and tonB and a polycistronic mRNA transcribed from the region upstream of hemS (Fig. 5). Consistent with qRT-PCR data from the irr overexpression strain, both promoters possess regions with considerable identity to a consensus sequence containing the H box (Fig. 6) (6). Unlike Hbp proteins, the majority of which were activated by Irr, the consensus sequence encompassing the H box in the hut locus promoters either overlaps the −10 and −35 regions (hutA) or is located nearby (tonB and hemS). A similar location for the ICE motif was reported for B. quintana genes repressed by Irr (39, 40). These data strongly suggest that Irr directly represses the hut locus.

In contrast to irr, rirA overexpression showed only minor changes in transcription of hut locus genes, regardless of hemin concentration (Fig. 4C). In the presence of excess hemin, fur overexpression resulted in decreased transcription of tonB (Fig. 4B). However, no obvious consensus Fur box sequence is evident in the promoter region of tonB (15). The effect of fur overexpression on tonB may be indirect in B. quintana, but this seems unlikely as tonB is known to be repressed by Fur in other bacteria (32). Most likely, Bartonella Fur recognizes a unique consensus sequence, as described for B. japonicum (15).

To our knowledge, this is the first study to characterize a complete system of hemin acquisition in Bartonella. Our data indicate that the hut locus is surprisingly similar to heme uptake systems described for other gram-negative bacterial pathogens and is controlled primarily by Irr. However, given the importance of heme to the survival and pathogenesis of Bartonella, B. quintana provides a unique model for studying its acquisition and utilization. Interesting areas of future study include the interplay between the Hut proteins and potential accessory systems including proteins able to bind hemin (e.g., Hbp proteins), proteins able to remove heme from hemoglobin, and proteins able to function as hemoglobin receptors. Any of these systems would contribute to the success of Bartonella pathogenesis by buffering fluctuations in available heme and by allowing Bartonella to use the most abundant source of heme in the human host (16).

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