

# The *bhuQ* Gene Encodes a Heme Oxygenase That Contributes to the Ability of *Brucella abortus* 2308 To Use Heme as an Iron Source and Is Regulated by Irr

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The *Brucella* BhuQ protein is a homolog of the *Bradyrhizobium japonicum* heme oxygenases HmuD and HmuQ. To determine if this protein plays a role in the ability of *Brucella abortus* 2308 to use heme as an iron source, an isogenic *bhuQ* mutant was constructed and its phenotype evaluated. Although the *Brucella abortus* *bhuQ* mutant DCO1 did not exhibit a defect in its capacity to use heme as an iron source or evidence of increased heme toxicity *in vitro*, this mutant produced increased levels of siderophore in response to iron deprivation compared to 2308. Introduction of a *bhuQ* mutation into the *B. abortus* *dhbC* mutant BHB2 (which cannot produce siderophores) resulted in a severe growth defect in the *dhbC bhuQ* double mutant JFO1 during cultivation under iron-restricted conditions, which could be rescued by the addition of FeCl<sub>3</sub>, but not heme, to the growth medium. The *bhuQ* gene is cotranscribed with the gene encoding the iron-responsive regulator RirA, and both of these genes are repressed by the other major iron-responsive regulator in the alphaproteobacteria, Irr. The results of these studies suggest that *B. abortus* 2308 has at least one other heme oxygenase that works in concert with BhuQ to allow this strain to efficiently use heme as an iron source. The genetic organization of the *rirA-bhuQ* operon also provides the basis for the proposition that BhuQ may perform a previously unrecognized function by allowing the transcriptional regulator RirA to recognize heme as an iron source.

Iron represents an essential micronutrient for *Brucella* strains (27). Acquiring sufficient iron to meet their physiological needs is particularly challenging for the brucellae because these bacteria are found in nature almost exclusively in mammalian hosts, an environment where the iron restriction faced by pathogenic microbes is well documented (29). *Brucella* strains can use heme as an iron source *in vitro*, and studies with an isogenic mutant have shown that the presence of the TonB-dependent outer membrane heme transporter BhuA is required for the wild-type virulence of *B. abortus* 2308 in experimentally infected mice (21), suggesting that heme is a biologically relevant source of iron for the brucellae during infection.

Heme oxygenases catalyze the release of iron from heme, and these enzymes contribute to the ability of a variety of bacteria to utilize heme as an iron source (25, 30, 37). The product of the gene designated BMEII0706 in the *Brucella melitensis* 16M genome sequence shares 58 and 50% amino acid identity with the heme oxygenases HmuD and HmuQ, respectively, from *Bradyrhizobium japonicum*, and this *Brucella* protein exhibits heme oxygenase activity in an *in vitro* assay (23). Based on its documented biochemical activity and its homology to the IsdG/HmuQ family of heme oxygenases, we have given this protein the designation BhuQ (*Brucella* heme utilization oxygenase Q). The purpose of the experiments described in this report was to determine if the homologous protein in *Brucella abortus* 2308 (which is encoded by BAB2\_0677) plays a role in the capacity of this strain to use heme as an iron source.

## MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** *Brucella abortus* strains (Table 1) were routinely grown in brucella broth at 37°C with aeration or on Schaedler agar supplemented with 5% bovine blood (SBA) at 37°C under 5% CO<sub>2</sub>. Kanamycin (45 µg/ml) (Sigma) and/or ampicillin (25 µg/ml) (Sigma) was added to these media as appropriate for the selection of strains with antibiotic resistance markers. *Escherichia coli* strain DH5α

was used for the propagation of plasmids for procedures involving recombinant DNA, and this strain was cultivated at 37°C in LB broth or on LB agar plates containing either 100 µg/ml ampicillin or 45 µg/ml kanamycin when appropriate. Gerhardt's minimal medium (GMM) (11) and low-iron minimal medium (15) were prepared as previously described.

**Construction of a *B. abortus* *bhuQ* mutant and a *dhbC bhuQ* double mutant.** PCR utilizing *Taq* polymerase (Invitrogen) with the oligonucleotide primers *bhuQ*-1F and *bhuQ*-1R (see Table S1 in the supplemental material) was used to amplify a 1,605-bp fragment encompassing the *bhuQ* gene (BAB2\_0677) from *B. abortus* 2308 genomic DNA. This fragment was then cloned into pGEM-T Easy (Promega). Inverse PCR employing AccuPrime Pfx supermix (Invitrogen) with this plasmid as a template and the primers *bhuQ*-2F and *bhuQ*-2R (see Table S1) was then employed to generate a blunt-ended linear fragment from which 70 bp internal to the *bhuQ* coding region had been removed. This fragment was ligated with a 1,345-bp fragment containing the *aph3a* gene from pKS-Kn (14). The resulting construct, pGEMΔ*bhuQ*, was introduced into *B. abortus* strain 2308 by electroporation and transformants were selected on SBA supplemented with 45 µg/ml kanamycin. Putative *B. abortus* *bhuQ* deletion mutants were identified based on their resistance to kanamycin and sensitivity to ampicillin, and their genotypes were confirmed by PCR analysis and DNA sequence analysis. Chromosomal DNA preparations from putative deletion mutants and strain 2308 were harvested, and oligonucleotides *bhuQ* F1 and Kan R (see Table S1) were used to determine the presence of the *aph3a*-based gene in the proper orientation, the absence of the ampicillin resistance gene from pGEM-T Easy (Promega) (*amp* F and *amp* R), and the absence of the 70 bp in the middle of *bhuQ*

Received 7 March 2012 Accepted 18 May 2012

Published ahead of print 25 May 2012

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Supplemental material for this article may be found at <http://jb.asm.org/>.

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doi:10.1128/JB.00367-12

TABLE 1 Bacterial strains used in this study

Strain or plasmid	Genotype or description	Reference or source
<b>Strains</b>		
<i>Escherichia coli</i> DH5 $\alpha$	F <sup>-</sup> $\phi$ 80lacZ $\Delta$ M15 $\Delta$ (lacZYA-argF)U169 recA1 endA1 hsdR17(r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>+</sup> ) phoA supE44 thi-1 gyrA96 relA1 $\lambda$ <sup>-</sup>	Invitrogen
<i>Brucella abortus</i>		
2308	Virulent challenge strain	Laboratory stock
DCO1	70-bp gene deletion of <i>bhuQ</i> (BAB2_0677) with an <i>ahpA3</i> kanamycin resistance gene insertion	This study
DCO1RC	DCO1 with <i>bhuQ</i> (BAB2_0677) reconstructed on the chromosome using pNPTS138	This study
BHB2	Nonpolar, in-frame deletion of <i>dhbC</i>	5, this study
JFO1	$\Delta$ <i>dhbC</i> $\Delta$ <i>bhuQ</i>	This study
JFO1RC	$\Delta$ <i>dhbC</i> $\Delta$ <i>bhuQ</i> with <i>bhuQ</i> (BAB2_0677) reconstructed on the chromosome using pNPTS138	This study
BEA2	<i>Dirr</i>	1, this study
<b>Plasmids</b>		
pGEM-T Easy	ColE1-based cloning vector; Ap <sup>r</sup>	Promega
pKS + Kan	794-bp <i>aph3A</i> gene from Tnp <i>hoA</i> cloned into Sall-HindIII-digested pBluescript II KS+	14
pNPTS138	<i>sacB</i> -containing counterselection vector; Amp <sup>r</sup>	32

(*bhuQ* F1 and *bhuQ* R1). One confirmed *B. abortus* *bhuQ* mutant was selected for further phenotypic evaluation and given the designation DCO1.

The approach described in the previous paragraph was also used to introduce a *bhuQ* mutation into *B. abortus* BHB2 (6). BHB2 has an unmarked, in-frame deletion in its *dhbC* gene, which renders it unable to produce the two siderophores produced by *Brucella* strains, 2,3-dihydroxybenzoic acid (15) and brucebactin (12). The *B. abortus* *dhbC bhuQ* double mutant constructed in this fashion was given the designation JFO1.

**Reconstruction of the *bhuQ* loci in the *B. abortus* *bhuQ* and *dhbC bhuQ* mutants.** Because the *bhuQ* gene is the terminal gene in an operon and lies downstream of a transcriptional regulator (see Fig. 4), reconstruction of the mutated *bhuQ* genes in DCO1 and JFO1 was chosen as a strategy for verifying the link between genotype and the phenotypes exhibited by these strains, rather than genetic complementation with a plasmid-borne *bhuQ* gene. A 920-bp fragment encompassing the *bhuQ* gene from *B. abortus* strain 2308 genomic DNA was amplified by PCR using the primers *bhuQ*-3F and *bhuQ*-3R (see Table S1) and cloned into the BamHI and Sall sites of pNPTS138Ap (Table 1), an ampicillin-resistant derivative of the *sacB*-containing ColE1-based vector pNPTS138 (32). The resulting plasmid, designated pNPTS138*bhuQ*, was introduced into *B. abortus* DCO1 and JFO1 by electroporation, and a previously described *sacB*-based counterselection strategy (6) was used to select for derivatives of these mutants in which the mutated *bhuQ* genes had been replaced by the parental *bhuQ* gene. The genotypes of the resulting *B. abortus* strains, designated DCO1RC and JFO1RC were confirmed by PCR amplification and DNA sequence analysis.

**Measurement of siderophore production by *Brucella* strains.** Following growth of the *B. abortus* strains in low-iron minimal medium (15) at 37°C with shaking (165 rpm), bacterial cells from 1.5-ml portions of the cultures were pelleted by centrifugation (15,550  $\times$  g, 1 min, room temperature), 1 ml of the resulting supernatant was removed to a fresh tube (10 ml), and the level of catechol siderophore present was measured using the Arnow assay (4).

**Determination of the growth characteristics of *B. abortus* strains in an iron-limited culture medium.** *B. abortus* strains were grown on SBA plates at 37°C with 5% CO<sub>2</sub> for 48 h and harvested into phosphate-buffered saline (PBS). The resulting cell suspensions were used to inoculate 20 ml low-iron minimal medium in 125-ml Erlenmeyer flasks at a final concentration of approximately 10<sup>6</sup> CFU/ml. When applica-

ble, the medium was supplemented with either 50  $\mu$ M FeCl<sub>3</sub> or 25  $\mu$ M deferrated hemin (33). Cultures were incubated at 37°C with shaking at 165 rpm, and at 24-h time points postinoculation, these cultures were serially diluted in PBS and plated on SBA, followed by incubation at 37°C under 5% CO<sub>2</sub>.

**Relative quantification of *bhuQ* transcript levels using real-time RT-PCR.** *B. abortus* 2308 and BEA2 (2308 *irr*) (1) were grown in low-iron minimal medium and low-iron minimal medium supplemented with 50  $\mu$ M FeCl<sub>3</sub> or in Gerhardt's minimal medium with or without 25  $\mu$ M deferrated hemin. Total cellular RNA was isolated from these cultures using a previously described procedure (7). The RNA was treated with RQ1 DNase (Ambion) following the manufacturer's instructions to remove residual contaminating DNA. The absence of DNA from the RNA preparations was confirmed via PCR analysis and lack of an amplified product as visualized on an agarose gel confirmed that the RNA sample was free of DNA contamination. Concentrations of RNA in the samples were determined by measuring the absorbance at 260 nm using a Nano-Drop ND-1000 spectrophotometer.

A SuperScript III first-strand synthesis system for RT-PCR kit (Invitrogen) was used to convert 1  $\mu$ g of RNA from these preparations into cDNA for each preparation following the manufacturer's instructions and using the random hexamers supplied with the kit. The cDNA preparations were then used as the templates in real-time RT-PCR analysis along with FastStart SYBR green master mix 2 $\times$  (Roche) to evaluate the relative levels of gene-specific mRNA transcripts in the total cellular RNA preparations. Gene-specific oligonucleotide primers were utilized for the 16S gene, *dhbC*, *bhuA*, and *bhuQ* (see Table S1 in the supplemental material). The differences in the levels of the *bhuQ*-, *bhuA*-, and *dhbC*-specific transcripts present were calculated using methods described by Pfaffl (22), using the 16S gene as an internal standard. This gene encodes the ribosomal 16S protein, and its expression is constitutive in *B. abortus* 2308 under the experimental conditions used here.

**Determination of the operonic organization of the *ybaK*, *rirA*, *bhuQ*, and *bfr* genes in *B. abortus* 2308.** Reverse transcriptase PCR was performed using cDNA prepared from clean RNA harvested from *B. abortus* 2308 grown for 72 h in low-iron minimal medium. Primers that span the intragenic regions between *ybaK* (BAB2\_0679), *rirA* (BAB2\_0678), *bhuQ* (BAB2\_0677), and *bfr* (BAB2\_0676) (see Table S1 in the supplemental material) were used in order to verify the presence or absence of a continuous transcript containing these genes. The resulting PCR products

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Sa IsdI (1) -MFMAENRRLQLQKGSAAETIERFYNRQ-GIETIEGFQQMFVTKTL--NTEDTDEVKIL
Sa IsdG (1) MKFMAENRRLTLTKGTAKDIERFYTRH-GIETLEGFDGMFVTQTL--EQEDFDEVKIL
Bj HmuD (1) -MYIAMNRFRVAKGSEAAFEQVWLSRDTHLDKVPGFVEFHLLRGP--ELEDHTLYASH
Bj HmuQ (1) -MFIAMNRFQVKKGAETAFAFETVWATRESYLGSMGPFVEFHLLKGP--EAEDHTLYSSH
Ba BhuQ (1) -MFIAMNRFKVRIGSETDFETVWKNRDSQLSDVPGFESFHLLRGATNEDEGYTYLASH

Sa IsdI (55) TIWESEDSFNNWLNSDVFKEAHKNVR-LKSDDDQQSPILSNKVFYDYGHYQK
Sa IsdG (56) TVWKSQAFTDWLKSDVFKAAHKHVR-SKNEDES--SPIINNKVITYDIGYSYMK
Bj HmuD (56) TVWANHAAFEWTKSEAFRAAHHKAG--DNKPLYLGHPPQFEGFEVMQTVGRGAK-
Bj HmuQ (56) TTWVDKAAFEWTRSEEFRAAHARADNRTGESLYLGHPPQFEGFEVIQSERKAAAA
Ba BhuQ (58) TVWRSQEDFIGWTRSEQFRAAHRNAG--ENKPLYLGPQFEGFTAVLGG-----

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**FIG 1** The *Brucella abortus* (Ba) BhuQ protein shares amino acid homology with the heme oxygenases HmuD and HmuQ from *Bradyrhizobium japonicum* (Bj) and IsdI and IsdG from *Staphylococcus aureus* (Sa) and contains the conserved Asn 7, Trp 67 and His 77 residues (large bold font), which have been shown experimentally to be important for the heme oxygenase activity of IsdG (31).

were separated by electrophoresis in a 0.7% agarose gel and visualized by staining the gel with ethidium bromide.

**Determination of the transcriptional start site for the *rirA*-*bhuQ* operon.** The *bhuQ* gene is cotranscribed with the upstream gene, *rirA*. In order to determine a transcriptional start site for this operon, 5' RNA ligase-mediated rapid amplification of the cDNA end (5' RLM-RACE) was performed using a primer (Rev [see Table S1 in the supplemental material]) anchored in the reverse orientation within the *rirA* ORF following the manufacturer's instructions (FirstChoice RLM-RACE kit, no. AM1700; Ambion). The PCR product generated from this reaction was cloned into pCR2.1 (Invitrogen), and the authenticity of the PCR fragment verified by DNA sequence analysis.

**Identification of the Irr binding site in the *rirA* promoter region.** A recombinant version of the *Brucella* Irr was purified and used in a DNase I footprint analysis with the *rirA* promoter region from *B. abortus* 2308 using previously described methods (1, 17). Briefly, the oligonucleotide primers *rirA* F and *rirA* R (see Table S1 in the supplemental material) were individually labeled with [ $\gamma$ - $^{32}$ P]ATP (Perkin Elmer) using the T<sub>4</sub> polynucleotide kinase reaction (Promega, Madison, WI) prior to their use in PCRs with Pfx polymerase to generate 300-bp DNA fragments representing the *rirA* promoter and transcriptional start site. The resulting PCR products were subjected to agarose gel electrophoresis and purified by gel extraction (Fermentas, Glen Burnie, MD). DNA probes corresponding to 8,000 cpm of the forward-labeled and reverse-labeled templates were incubated separately in EMSA binding buffer (10 mM Tris-HCl [pH 8], 40 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM dithiothreitol [DTT], 5% glycerol) supplemented with 100 ng/ml bovine serum albumin (BSA) and 50 ng/ml salmon sperm DNA (nonspecific competitor) in the presence of 100  $\mu$ M MnCl<sub>2</sub> and increasing concentrations of the recombinant *Brucella* Irr protein. The reaction mixtures were incubated at room temperature for 30 min prior to treatment with 0.08 U of DNase I freshly diluted in 10 $\times$  DNase I buffer (400 mM Tris-HCl [pH 8.0], 100 mM MgSO<sub>4</sub>, 10 mM CaCl<sub>2</sub>) for 4 min. Reactions were stopped by the addition of 5 mM EDTA and heating at 65°C for 10 min. Reaction mixtures were ethanol precipitated and resuspended in 4  $\mu$ l of formamide loading buffer (98% formamide, 10 mM EDTA [pH 8.0], 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue). Digested DNA fragments were separated on a denaturing 6% (wt/vol) acrylamide and 7 M urea sequencing gel in glycerol-tolerant buffer (17). Gels were dried under vacuum and subjected to autoradiography. The sequence protected by Irr was determined by comparing the nucleotide sequences generated for a 100-bp region of the *rirA* promoter region using the SequiTherm Excel II DNA sequencing kit (Epicentre, Madison, WI) and *B. abortus* 2308 DNA preparations exposed to DNase I treatment with and without recombinant Irr as templates.

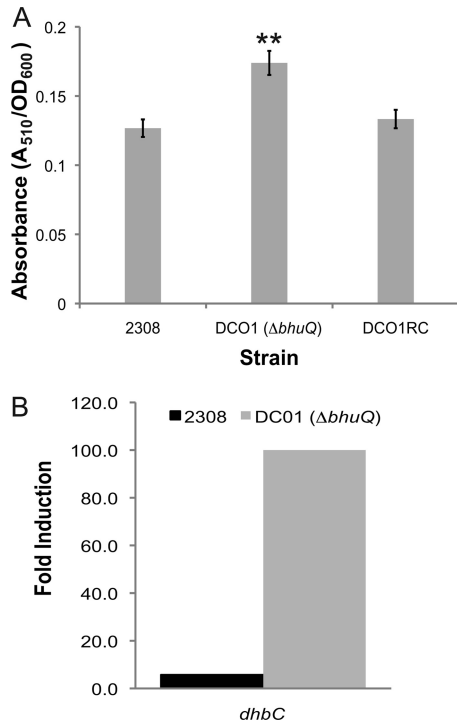
**Statistical analysis.** All statistical analysis was performed using the Student two-tailed *t* test. *P* values of less than 0.05 were considered significant (28).

## RESULTS AND DISCUSSION

Pfam analysis of the *Brucella* BhuQ protein indicates that it belongs to the antibiotic monooxygenase (ABM) family of heme oxygenases along with the HmuD and HmuQ proteins from *B. japonicum* (23) and the IsdI and IsdG proteins from *Staphylococcus aureus* (31). Moreover, BhuQ contains the conserved Asn 7, Trp 67, and His 77 residues, which have been shown experimentally to be important for the heme oxygenase activity of IsdG (36) (Fig. 1), and BhuQ has been shown to bind and degrade heme *in vitro* (23). Despite the documented heme oxygenase activity of BhuQ, however, an isogenic *bhuQ* mutant (DCO1) constructed from *B. abortus* 2308 exhibited a comparable growth pattern in low-iron minimal medium and an equivalent resistance to the iron-specific chelator ethylenediamine-*N,N'*-bis(2-hydroxyphenylacetic acid) (EDDHA) to that displayed by the parental strain. More importantly, both strains were also able to use heme as an iron source with equivalent efficiency in a chelator-based disk assay on a solid growth medium (data not shown).

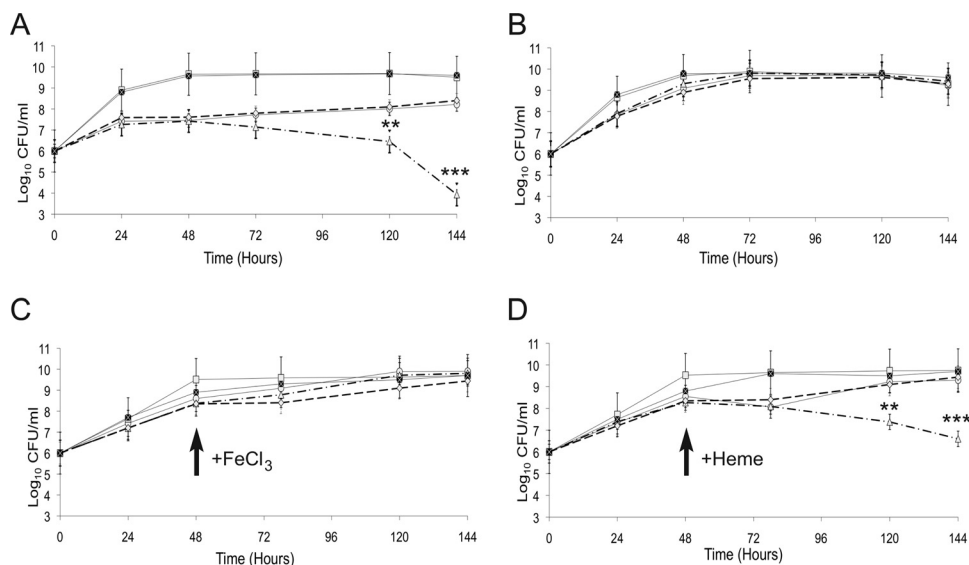
A distinctive characteristic of the *B. abortus bhuQ* mutant that was noticed during its phenotypic characterization, however, was that this mutant consistently and reproducibly produces more siderophore during growth in low-iron minimal medium than the parental 2308 strain (Fig. 2A). This increase in siderophore production is accompanied by a corresponding increase in transcription of *dhbC* in the *bhuQ* mutant in comparison to the parental strain (2308) following growth under iron-limiting conditions (Fig. 2B). This phenotype suggests that the *B. abortus bhuQ* mutant is experiencing a greater degree of iron deprivation in the low-iron minimal medium than 2308 and in turn increasing its siderophore production to compensate. Enhanced siderophore production could explain why the *B. abortus bhuQ* mutant does not exhibit a readily detectable iron acquisition defect in *in vitro* assays such as growth in low-iron minimal medium or sensitivity to EDDHA. The ability of this mutant to use heme as an iron source in the disk diffusion assays, however, indicates that *B. abortus* 2308 possesses one or more additional heme oxygenases that can compensate for the loss of BhuQ and allow the *bhuQ* mutant to use heme as an iron source. The identity of these other heme oxygenases is currently unknown.

*Brucella* strains have the capacity to use multiple iron sources during *in vitro* growth (27), and it is not usual for bacterial strains with mutations affecting single iron transporters to exhibit little or

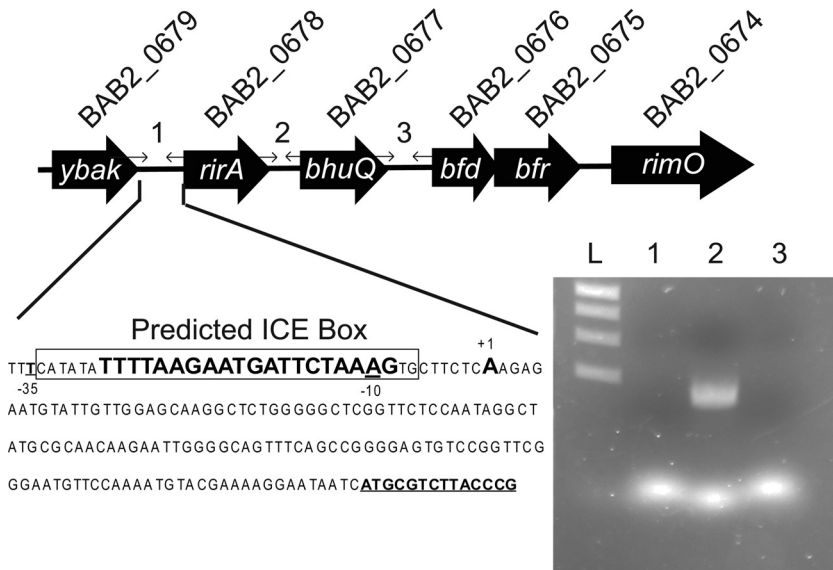


**FIG 2** (A) Siderophore production by *B. abortus* 2308, DCO1 ( $\Delta bhuQ$ ), and DCO1RC (DCO1  $bhuQ^+$ ) following 72 h growth in low-iron minimal medium. The values on the y axis are the levels of catechol siderophore detected by the Arnow assay (4). \*\*,  $P < 0.01$  for comparisons of the data obtained for these strains in the Student two-tailed  $t$  test (28). (B) Iron-responsive expression of *dhbC* in *B. abortus* 2308 and DCO1 (2308  $bhuQ$ ). Values on the y axis are the difference between the levels of *dhbC* transcripts detected in RNA preparations from *B. abortus* 2308 and DCO1 cultures after 72 h of growth in low-iron minimal medium compared to RNA preparations from these cultures after growth in low-iron minimal medium supplemented with 50  $\mu\text{M}$   $\text{FeCl}_3$ .

no defect in iron utilization assays due to compensation by other iron transport systems. Indeed, the fact that the *B. abortus bhuQ* mutant exhibits increased siderophore production in response to iron deprivation compared to the parental strain provided an experimental avenue for assessing the role of BhuQ in iron metabolism. Specifically, if the loss of BhuQ from *B. abortus* DCO1 is leading to an increased demand for iron, and siderophore production is being increased in this mutant to meet this demand, then a derivative of this strain that cannot produce siderophore (e.g., a *B. abortus dhbC bhuQ* double mutant) would be expected to show an enhanced iron deprivation phenotype compared to *B. abortus* 2308 or the *bhuQ* mutant when grown under iron-limiting conditions. This is in fact the relationship that was observed. As shown in Fig. 3A, the *dhbC bhuQ* double mutant JFO1 showed a greatly enhanced growth defect compared to the parental BHB2 (*dhbC* mutant) strain when they were cultivated in low-iron minimal medium, a phenotype that was not observed when these strains were grown in iron-replete medium (Fig. 3B). More importantly, the enhanced growth defect exhibited by the *B. abortus dhbC bhuQ* double mutant during cultivation in low-iron minimal medium could be rescued by the addition of  $\text{FeCl}_3$  (Fig. 3C), but not heme (Fig. 3D), to the growth medium 48 h after inoculation of the bacterial cultures. In contrast, either  $\text{FeCl}_3$  (Fig. 3C) or heme (Fig. 3D) was able to rescue the growth defect exhibited by the *dhbC* mutant BHB2 during growth under low-iron conditions, although in both cases the alleviation of the growth restriction occurred more rapidly when  $\text{FeCl}_3$  was added. To verify the link between the *bhuQ* mutations in *B. abortus* DCO1 and JFO1 and the phenotypes exhibited by these strains, a *sacB*-based counterselection strategy (6) was used to reconstruct the *bhuQ* genes in these mutants. The resulting strains, designated DCO1RC and JFO1RC, exhibited the expected parental phenotype with regard to their production of siderophore in response to iron deprivation (Fig. 2), their sensitivities to iron deprivation (Fig. 3A and B), and



**FIG 3** Growth of *B. abortus* 2308 (filled squares), *B. abortus* DCO1 (2308  $\Delta bhuQ$ ) (squares), BHB2 (2308  $\Delta dhbC$ ) (circles), JFO1 (2308  $\Delta dhbC \Delta bhuQ$ ) (triangles), and JFO1RC (JFO1  $bhuQ^+$ ) (diamonds) in (A) low-iron minimal medium, (B) low-iron minimal medium containing 50  $\mu\text{M}$   $\text{FeCl}_3$ , (C) low-iron minimal medium supplemented with 50  $\mu\text{M}$   $\text{FeCl}_3$  at 48 h postinoculation, and (D) low-iron minimal medium supplemented with deferrated 25  $\mu\text{M}$  heme at 48 h postinoculation. The data presented are from one experiment but representative of multiple experiments ( $>3$ ) in which similar trends were observed. The data were compared with the Student two-tailed  $t$  test (28). \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

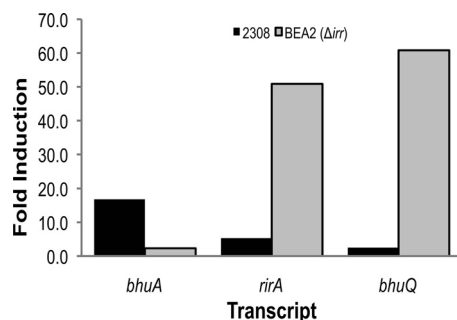


**FIG 4** *rirA* and *bhuQ* are cotranscribed as an operon in *B. abortus* 2308. The sequence shows the start site (+1) for the *rir-bhuQ* transcript, located 134 bp upstream of the *rirA* ORF (bold underlined), and the location of an Irr binding motif predicted by Rodionov et al., labeled as an ICE box and shown in large boldface type (26), while the nucleotide sequence protected by Irr is boxed. At the top, primer sets 1, 2, and 3 denote the intragenic regions used to define the transcript, and the corresponding lanes are marked on the agarose gel below.

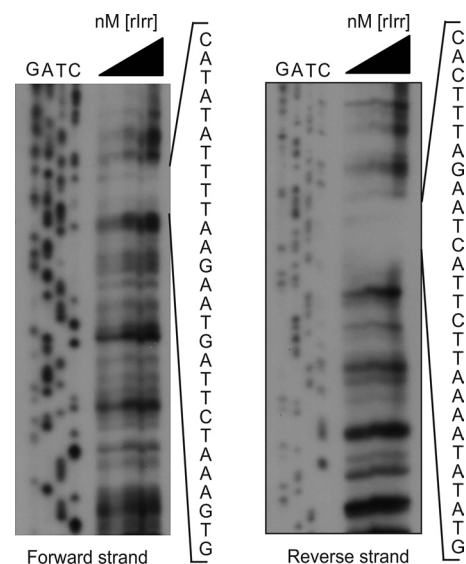
their abilities to use  $\text{FeCl}_3$  and heme as iron sources (Fig. 3C and D). Although these experimental findings establish a role for *BhuQ* in the capacity of the *B. abortus dhbC* mutant to use heme as an iron source, this activity appears to be masked in *B. abortus* 2308 by the activity of other heme oxygenases when alternative iron sources are readily available in the growth medium. The capacity of the *B. abortus bhuQ* mutant to compensate for the loss of one heme oxygenase was also observed in experimental models of infection, as this strain exhibited wild-type virulence in cultured murine macrophages and experimentally infected BALB/c mice (data not shown).

RT-PCR analysis indicates that *bhuQ* is the second gene in an operon transcribed as *rirA* (BAB2\_0678)-*bhuQ* (BAB2\_0677) in *B. abortus* 2308 (Fig. 4). *RirA* is a well-characterized regulator of

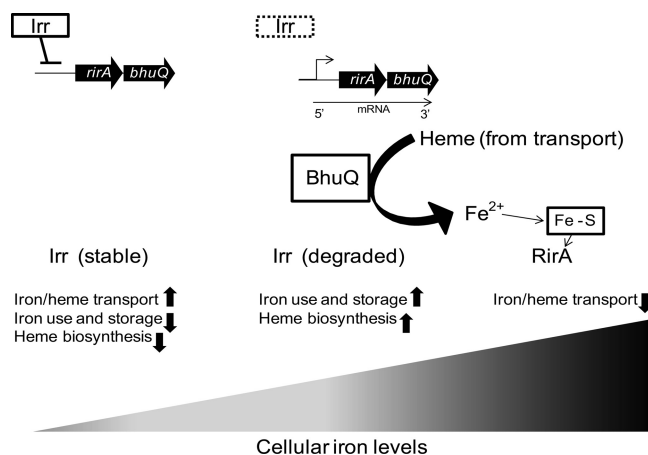
iron metabolism genes in several other alphaproteobacteria (8, 13, 19, 35). A predicted iron control element (ICE) motif is located in the  $-10$  region of the promoter of the *rirA-bhuQ* operon (Fig. 4), suggesting that the iron response regulator Irr regulates the expression of these genes in response to cellular iron levels (16). In fact, when the expression patterns of the *rirA* and *bhuQ* genes in *B. abortus* 2308 were independently evaluated by real-time PCR,



**FIG 5** *rirA* and *bhuQ* expression in response to iron deprivation in *B. abortus* 2308 and the isogenic *irr* mutant BEA2. Values on the y axis are the difference between the levels of *rirA*, *bhuQ*, and *bhuA* transcripts detected in RNA preparations from *B. abortus* 2308 and BEA2 after 72 h of growth in low-iron minimal medium compared to RNA preparations from these cultures after growth in low-iron minimal medium supplemented with  $50 \mu\text{M}$   $\text{FeCl}_3$ . The pattern of *bhuA* transcription was included because this gene exhibits elevated expression in response to iron deprivation in *B. abortus* 2308, and this low-iron-responsive induction is dependent upon the presence of Irr (1).



**FIG 6** Irr binds directly to the *rirA* promoter in *B. abortus* 2308 and protects a 28-nucleotide sequence in a DNase I footprint analysis. The triangle above the lanes indicates that the corresponding reaction mixtures contain increasing concentrations (700 ng, 1.4  $\mu\text{g}$ , 2.1  $\mu\text{g}$ , and 3.5  $\mu\text{g}$ ) of recombinant *Brucella* Irr, and the nucleotide sequences shown to the right of the gels show the nucleotides protected from DNase I digestion in the forward and reverse strands of the *rirA* promoter sequence.



**FIG 7** Model showing the proposed role of BhuQ in allowing the transcriptional regulator RirA to recognize heme as an iron source in *Brucella*. Under iron-deprived conditions, Irr is stable and drives the expression of genes involved in iron and heme transport. As intracellular iron levels increase, Irr is degraded, which allows expression of the *rirA-bhuQ* operon. BhuQ degrades the heme being transported into the cell, releasing  $\text{Fe}^{2+}$ . When cellular iron levels reach a threshold, RirA is postulated to repress the expression of the iron and heme acquisition genes.

both genes exhibited a modest induction in response to iron deprivation (Fig. 5). In contrast, the expression of both of these genes was elevated >50-fold in the *B. abortus irr* mutant BEA2 when this strain was grown under iron-limiting conditions. Thus, it appears that Irr represses the expression of *rirA* in *Brucella* strains during periods of iron deprivation in much the same manner as it does in the related alphaproteobacterium *Agrobacterium tumefaciens* (13). DNase I footprint analysis indicated that Irr directly binds to the *rirA* promoter (Fig. 6), protecting a 28-nucleotide sequence, 5'-CATATATTTTAAGAATGATTCTAAAGTG-3'. Notably, this sequence includes the conserved ICE motif in the promoter region of *rirA* (underlined) predicted by Rodionov et al. (26).

Genetic studies suggest that RirA functions as an iron-responsive repressor of iron acquisition genes in the alphaproteobacteria (35), in much the same fashion as Fur does in other bacteria. The potential benefit of Irr's repressing the expression of an iron-responsive repressor when the bacterial cell is experiencing iron deprivation is not difficult to envision. But such a regulatory link would appear to be counterproductive for the *bhuQ* gene if its product is solely dedicated to the utilization of heme as an iron source. One scenario that might explain a possible benefit of co-regulation of *bhuQ* and *rirA* in *B. abortus* 2308 is that BhuQ production leads to an increased level of heme oxygenase activity which enhances the capacity of RirA to recognize exogenous heme being utilized as an iron source and represses the cell's iron acquisition systems accordingly (Fig. 7).

In addition to their ability to provide iron from heme, some bacterial heme oxygenases also function to protect the cell against heme toxicity (2). Heme has a high redox potential, and too much heme inside a bacterial cell can be toxic (20). Neither *B. abortus* 2308 nor the isogenic *bhuQ* mutant display a growth defect in low-iron medium supplemented with up to 200  $\mu\text{M}$  deferrated hemin. It is possible, however, that any role that BhuQ might be playing in the detoxification of heme is masked by the activity of

another heme oxygenase. Another factor that may affect this observed lack of heme toxicity is that *Brucella* strains possess multiple homologs of the outer membrane heme-binding proteins (Hbps) that have been proposed to play a role in capturing heme and preventing its toxicity in *Bartonella* (18). In *Brucella*, these proteins are known as the Omp25/31 family of proteins (9). Heme export systems have also been proposed as a means by which bacteria protect themselves from heme toxicity (2). Although a heme exporter has not been identified in *Brucella* strains, genes that potentially encode orthologs of proteins linked to porphyrin (34) and heme (24) export in other bacteria can be found in the genome sequence of *B. abortus* 2308.

In order to fully understand the role of BhuQ in iron and heme metabolism in *Brucella* strains, it will be imperative to identify the other heme oxygenase(s) present in these bacteria. Phenotypic evaluation of mutants lacking combinations of these enzymes can then be used to assess the relative contributions of the heme oxygenases to iron and heme metabolism, as well as their potential role in modulating the regulatory capacity of RirA. Such studies may also provide an added practical benefit, as prokaryotic heme oxygenases have been proposed to be targets for the development of antimicrobial agents (10). Brucellosis in humans is notoriously difficult to treat, requiring a combination of antibiotics for a prolonged period (3). Hence, the development of improved chemotherapeutic regimens for treating this disease would be of great benefit to the medical community.

#### ACKNOWLEDGMENT

This work was funded by a grant (AI 63516) from the National Institute of Allergy and Infectious Diseases to R.M.R.

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