The RNA Chaperone Hfq Independently Coordinates Expression of the VirB Type IV Secretion System and the LuxR-Type Regulator BabR in *Brucella abortus* 2308

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The type IV secretion system encoded by the *virB* operon is required for full virulence of *Brucella* sp., and the present study links the RNA chaperone Hfq to wild-type expression of *virB* in *Brucella abortus* 2308. Studies employing *virB-lacZ* fusions, quantitative reverse transcription-PCR, and immunoblot analysis showed that both transcription and translation of *virB* are decreased in an isogenic *hfq* mutant compared to those in the parental strain. These results led to the hypothesis that Hfq regulation of *virB* is mediated through an intermediate transcriptional regulator. Subsequent experiments determined that expression of the gene encoding the putative *Brucella* quorum-sensing regulator BabR (also known as BlxR), a known *virB* regulator, is also controlled by Hfq at the posttranscriptional level, and a *cis*-acting element in the 5′ untranslated region of the *babR* transcript responsible for this regulation was identified. Consistent with its role as a *virB* regulator, recombinant *Brucella* BabR binds to the *virB* promoter region in electrophoretic mobility shift assays. However, experiments employing a *babR* mutant strain determined that BabR is a repressor, not an activator, of *virB* transcription. These findings suggest that Hfq regulates *virB* expression through both BabR-dependent and BabR-independent pathways.

The *Brucella* spp. are part of the $\alpha2$ subclass of proteobacteria (28), and these bacteria infect wild and domestic animals, such as cattle, goats, and sheep, causing abortions in pregnant females and sterility in males. The *Brucella* spp. can also infect humans, and brucellosis is described as the most common zoonosis worldwide, with an estimated 500,000 human brucellosis cases being reported each year (29). The bulk of these infections occur in areas where *Brucella* is endemic in the food animal populations, and humans become infected most often by consuming unpasteurized dairy products or handling animals and products from *Brucella*-infected animals (28). Brucellosis in humans is characterized by nonspecific symptoms, including a recurring high fever, extreme fatigue, and a variety of other "flu-like" symptoms that in aggregate are extremely debilitating (12).

During the course of chronic infection, the brucellae reside within macrophages, where they replicate in a specialized compartment associated with the endoplasmic reticulum, and the ability of the brucellae to survive and replicate within macrophages is essential to their virulence (4). While the brucellae do not produce classical virulence factors, such as toxins or adhesins (39), several genetic determinants of infection have been identified that aid the bacterium in infecting and surviving within the host (36). One of these determinants of *Brucella* virulence is the RNA chaperone Hfq (33).

Hfq is a bacterial protein that mediates RNA-RNA interactions that, in turn, regulate gene expression at the posttranscriptional level (25, 37). Hfq serves as a modulator of gene expression by acting as a scaffold for interactions between small regulatory RNAs (sRNAs) and their target mRNA molecules. These sRNA-mRNA interactions are often formed through short stretches of imperfect base pairing, and therefore, Hfq is required to bridge this limited complementarity between sRNA and mRNA. In many instances, sRNA-mRNA interactions facilitated by Hfq inhibit gene expression, usually by occluding the ribosome-binding site (RBS) and/or decreasing the stability of the mRNA, leading to degradation of the transcript (1). However, Hfq and sRNAs can also activate gene expression by alleviating secondary structure in the RBS region of the mRNA and allowing translation to proceed or by binding to the 3′ end of the mRNA, which stabilizes the transcript (13). Additionally, even though Hfq levels change slightly during the transition from exponential to stationary phase, there is generally an abundance of Hfq in the cell, and thus, the growth phase-dependent and stimulus-driven gene expression mediated by Hfq can usually be attributed to differential expression of sRNAs involved in these regulatory events, rather than changes in Hfq levels (52). Hfq is also required for the normal expression of some target genes in the absence of an sRNA by binding to mRNAs and affecting mRNA stability (10, 53); however, this mechanism of Hfq-mediated gene regulation has not been widely described.

An isogenic *hfq* mutant derived from *Brucella abortus* 2308 displays a pleiotropic phenotype compared to the parental strain. The *B. abortus hfq* mutant exhibits increased sensitivity to oxidative stress, decreased survival at low pH, and increased sensitivity to nutrient deprivation (33). While the full repertoire of Hfq-dependent genes responsible for these deficiencies has not been elucidated, the proper expression of some *Brucella* genes has been linked to Hfq, including those encoding the periplasmic superoxide dismutase SodC (14) and the acid resistance protein HdeA (51). Aberrant production of these proteins may contribute to the inability of a *B. abortus hfq* mutant to adapt to oxidative and pH stresses, respectively, but it is likely that other genes involved in the

adaptive response to stress conditions also require Hfq for their proper expression.

Hfq has been linked to the virulence of several pathogenic bacteria, including *Pseudomonas aeruginosa* (44), *Vibrio cholerae* (7), *Salmonella enterica* serovar Typhimurium (43), *Francisella tularensis* (23), *Neisseria meningitidis* (9), and *Escherichia coli* (42). Likewise, an isogenic *hfq* mutant derived from *B. abortus* 2308 exhibits severe attenuation in experimentally infected mice (33), and a *B. melitensis hfq* mutant derived from strain 16M is attenuated in goats (35) and nonhuman primates (R. Borschel and M. J. Nikolich, personal communication). The present study was undertaken to identify the molecular mechanisms underlying the link(s) between Hfq and wild-type gene expression in *B. abortus* 2308 and to determine how Hfq is linked to virulence in *Brucella*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. abortus* 2308 and derivative strains were routinely grown on Schaedler agar (BD, Franklin Lakes, NJ) containing 5% defibrinated bovine blood (Quad Five, Ryegate, MT) (SBA) or in brucella broth (BD). In some cases, *Brucella* strains were grown on tryptic soy agar (BD) containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 100 μg/ml). In order to induce maximal expression of the *virB* operon, *Brucella* strains were grown in a low-pH (pH 4.5) defined medium (21). *E. coli* strains were grown routinely on tryptic soy agar (BD) or in Luria-Bertani broth. When appropriate, growth media were supplemented with ampicillin (100 μg/ml), kanamycin (45 μl/ml), or chloramphenicol (30 μl/ml; *E. coli* strains only).

Construction of *virB1*- and *babR-lacZ* fusions. All of the transcriptional and translational *lacZ* fusions used in these studies were plasmid borne, and the plasmids used (pMR10 [13] and pMR15 [3]) contain the same vector backbone (e.g., pGL10, an RK2 derivative). These plasmids are maintained at 2 to 4 copies per genome in *Brucella* (34). Additionally, the nucleotide sequences of the gene fusions were verified by DNA sequence analyses.

The promoter regions of the *Brucella virB* and *babR* genes were fused to a *lacZ* reporter as transcriptional fusions. For the *virB* promoter fusion construct (pC³003), approximately 450 bp of the *virB1* upstream region was amplified by PCR using primers *virB*-Tsc-For and *virB*-Tsc-Rev, *B. abortus* 2308 genomic DNA as a template, and *Taq* polymerase. For the *babR* promoter fusion (pC³006), approximately 180 bp of the *babR* upstream region was amplified by PCR using primers *babR*-Tsc-For and *babR*-Tsc-Rev (Table 1) and *B. abortus* 2308 genomic DNA as a template. The amplified DNA fragments were sequentially digested with BamHI and HindIII and subsequently ligated into BamHI/HindIII-digested pMR15, which contains a promoterless *lacZ* gene. pC³003 and pC³006 were introduced into *B. abortus* 2308 and derivatives of this strain by electroporation.

For construction of *virB-lacZ* and *babR-lacZ* translational fusions, the upstream regions of the corresponding *Brucella* genes to the second codon of the coding region were cloned in frame with *lacZ* in pLKC-481 (48) using the HindIII and BamHI restriction sites. The *virB* fragment was amplified by PCR using *B. abortus* 2308 genomic DNA as a template and primers *virB*-Tsl-For and *virB*-Tsl-Rev (Table 1), and the *babR* fragment was amplified with primers *babR*-Tsl-For and *babR*-Tsl-Rev (Table 1). Following successful cloning of the fragments into pLCK-481, the intact gene fusions were liberated from the plasmid by digestion with PstI and SmaI. These fragments were then cloned into the *Brucella*-compatible vector pMR10 (14) for introduction into *B. abortus* 2308 and its derivatives.

In order to truncate the 5′ untranslated region (UTR) of the mRNA of the *babR-lacZ* translational fusion, an approximately 500-bp DNA fragment of the *babR* upstream region to the transcriptional start site of *babR* was amplified by PCR using primers *babR*-Trunc-Up-For and *babR*-Trunc-Up-Rev (Table 1), genomic DNA from *B. abortus* 2308 as a tem-

plate, and *Pfx* polymerase (Invitrogen). Using similar PCR conditions and primers *babR*-Trunc-Down-For and *babR*-Trunc-Down-Rev (Table 1), an ~500-bp DNA fragment beginning with the +21 position of the *babR* 5′ UTR was amplified. The upstream fragment was digested with BamHI, while the downstream fragment was digested with PstI, and both fragments were treated with polynucleotide kinase. Both of the DNA fragments were included in a single ligation mixture with BamHI/PstI-digested pUC19 (50). The resulting plasmid (pC³012) was used as a template for PCR with primers *babR*-Tsl-For and *babR*-Tsl-Rev, and a *babR-lacZ* translational fusion harboring a truncated 5′ UTR was constructed exactly as the *babR-lacZ* translational fusion described in the previous paragraph to generate pC³014.

B. abortus strains harboring the *lacZ* fusion constructs were grown in brucella broth or low-pH (pH 4.5) defined medium, and β-galactosidase assays were performed as described previously (24).

Construction and genetic complementation of a *B. abortus hfq* mutant. The *hfq* gene (BAB1_1134) in *B. abortus* 2308 was mutated using a nonpolar, unmarked gene excision approach. The *hfq* gene, along with approximately 1 kb of each flanking sequence, was amplified by PCR using primers *hfq*-For and *hfq*-Rev (Table 1), *B. abortus* 2308 genomic DNA as a template, and *Taq* polymerase, and the amplified DNA fragment was ligated into pGEM-T Easy (Promega). The resulting plasmid (pJMG001) was used as a template for inverse PCR with primers Inverse-*hfq*-For and Inverse-*hfq*-Rev. Inverse PCR was performed using Herculase Hotstart DNA polymerase (Stratagene). A DNA fragment of approximately 5 kb was generated from inverse PCR, and following gel purification, the ends of the DNA fragment were phosphorylated using polynucleotide kinase. The free ends of the DNA fragment were then ligated together to yield plasmid pJMG002, which contains an in-frame deletion *hfq* allele and approximately 1 kb of flanking DNA sequence on each side. The in-frame nature of the *hfq* mutation was confirmed by DNA sequencing. The mutated *hfq* gene and the flanking DNA were liberated by treatment with NotI, and following gel purification, the liberated DNA fragment was treated with the Klenow fragment of DNA polymerase. This fragment of DNA was then ligated into SmaI-digested pEX100T (38), which contains an ampicillin resistance marker and a *sacB* gene, and the resulting plasmid was named pJMG003. pJMG003 was introduced into *B. abortus* 2308 by electroporation, and merodiploid clones were obtained by selection on SBA plus ampicillin. A single ampicillin-resistant clone was grown overnight in brucella broth and then plated onto SBA containing 10% sucrose. Genomic DNA from sucrose-resistant, ampicillin-sensitive colonies was isolated and screened by PCR for loss of the *hfq* gene. Following confirmation of its genotype by Southern blotting, the isogenic *hfq* mutant derived from *B. abortus* 2308 was named B8.

Genetic complementation of the *hfq* mutation was achieved by in-*trans* complementation using pBBR1MCS-4 (20). An approximately 800-bp DNA fragment containing the *hfq* gene was amplified by PCR using primers *hfq*-comp-For and *hfq*-comp-Rev (Table 1), genomic DNA from *B. abortus* 2308 as a template, and *Pfx* polymerase (Invitrogen). The resulting DNA fragment was digested with StyI and then treated with polynucleotide kinase, and the digested and treated fragment was ligated into XbaI/HincII-digested pBBR1MCS-4 to create pJMG004. pJMG004 was introduced into *B. abortus hfq* mutant strain B8 by electroporation.

Construction and genetic complementation of a *B. abortus babR* mutant. The *babR* gene (BAB1_0190) of *B. abortus* 2308 was mutated using a nonpolar, unmarked gene excision strategy. An approximately 1-kb fragment of the *babR* upstream region to the second codon of the *babR* coding region was amplified by PCR using primers Δ*babR*-Up-For and Δ*babR*-Up-Rev (Table 1), genomic DNA from *B. abortus* 2308 as a template, and *Pfx* polymerase. Similarly, a fragment containing the last two codons of the *babR* coding region to approximately 1 kb downstream of the *babR* open reading frame was amplified with primers Δ*babR*-Down-For and Δ*babR*-Down-Rev (Table 1). The upstream fragment was digested with BamHI, while the downstream fragment was digested with PstI, and both fragments were treated with polynucleotide kinase in the

TABLE 1 Oligonucleotide primers used in this study

Primer	Sequence (5′→3′)[a]
virB-Tsc-For	GCAAGCTTATGACAGGCATATTTCAACGCGAC
virB-Tsc-Rev	GCGGATCCCGATTATTATGATAGCCTTAATTATAG
virB-Tsl-For	GCGGATCCCATGACAGGCATATTTCAACGCGAC
virB-Tsl-Rev	GCAAGCTTCACCATAGGATCGTCTCCTTCTC
babR-Tsc-For	GCAAGCTTATTATCTAATTTAGAATGAAG
babR-Tsc-Rev	GCGGATCCATAAATATGAATTCTTGG
babR-Tsl-For	GCGGATCCCTAATTTAGAATGAAGTTATATTC
babR-Tsl-Rev	GCAAGCTTTTTCATTGCAGACATCCCAG
babR-Trunc-Up-For	GCGGATCCAAAAGACCACCTTGTTCCG
babR-Trunc-Up-Rev	CTTTCATCATAATTAGCGATTT
babR-Trunc-Down-For	TGAAAGATATGCTAGGAATGTTT
babR-Trunc-Down-Rev	GCCTGCAGACAAAGCTCAAGAGGCCGA
hfq-For	CATCAATGGTGATGACTGGCCAAA
hfq-Rev	CGAGCGTGGCGAAAAGCATA
Inverse-*hfq*-For	GCCTGACGCTTTCCGATATTG
Inverse-*hfq*-Rev	AGCCATTGTTTTTATTCCTT
hfq-comp-For	AAAGCATAAGGAAGTCGTGTCG
hfq-comp-Rev	CGCTTTTTTATCCTTGGGTT
16S-RT-For	TCTCACGACACGAGCTGACG
16S-RT-Rev	CGCAGAACCTTACCAGCCCT
virB1-RT-For	TGGCAGCACCTACGCACAAG
virB1-RT-Rev	GCCTTGTTCCTGACCGGGCA
babR-RT-For	AAGAATTATGCGCATGACCT
babR-RT-Rev	GTTCCCACCCATCTGGAAAT
babR-seq-For	TTATGTCCCTGGGGCATCGAAAGAC
babR-seq-Rev	AATAGACATGACATAGGATACATAC
babR-PE	CGTTTCAAAATAAATATGAATTCTTGGAAT
Δ*babR*-Up-For	GCGGATCCGATATCCGCCTCTTTACGGA
Δ*babR*-Up-Rev	TTTCATTGCAGACATCCCAGTAGCGTTTC
Δ*babR*-Down-For	CTCTGACCTTTTCCAGTGTCCCTCGGCGG
Δ*babR*-Down-Rev	GCCTGCAGATCGGCAGATAAGAAGAGGA
babR-comp-For	AACTGCAGGAGGAAACGGCAAACAGCCCT
babR-comp-Rev	AATGTGTAGCCTTTTTCAGGGAAAG
babR-UTR-T7-For	TAATACGACTCACTATAGGGTAATCTTCGTAGAAGAAATATGAA
babR-UTR-T7-Rev	TGTTTCCCATTTCATTGCAGAC
babR-20-UTR-For	TAATACGACTCACTATAGGGTGAAAGATATGCTAGGAATGTTTAG
rHfq-For	GCGGTCTCCGCGCATGGCTGAACGATCGCAAATCT
rHfq-Rev	GCGGTCTCCTATCAGGCTTCCTCGCCTTCAAACAT
rBabR-For	CATGAAATGGGAAACATTTTATG
rBabR-Rev	AGGAGACCAAATGTACGGGCC
P*virB*-For	CTGACTGGGCGTCATTCACAC
P*virB*-Rev	CGTCTCCTTCTCAGAGAATGGACG
babR-RACE-Rev	CAGGTCATGCGCATAATTCTTTACT

[a] Underlined sections indicate restriction endonuclease recognition sequences.

presence of ligase buffer. Both of the DNA fragments were included in a single ligation mixture with BamHI/PstI-digested pUC19 (54). The nucleotide sequence of the ligated *Brucella* DNA fragments in the resulting plasmid (pC³009) was determined to confirm the desired mutant genotype. The mutated *babR* gene with 1-kb flanking regions was then liberated from the vector by digestion with BamHI and PstI, and the liberated fragment was ligated into pNPTS138 (46), which contains a kanamycin resistance marker and the *sacB* gene for counterselection with sucrose. The resulting plasmid (pC³010) was introduced into *B. abortus* 2308 and the *hfq* mutant B8 by electroporation, and merodiploid clones were obtained by selection on SBA plus kanamycin. A single kanamycin-resistant clone was grown overnight in brucella broth and then plated onto SBA containing 10% sucrose. Genomic DNA was isolated from sucrose-resistant, kanamycin-sensitive colonies and screened by PCR for loss of the *babR* gene. The isogenic *babR* mutant derived from *B. abortus* 2308 was named CC058.

Genetic complementation of the *babR* mutation was achieved by in-

trans complementation using pBBR1MCS-4 (20). A DNA fragment containing the *babR* gene and approximately 300 bp of upstream and 100 bp of downstream DNA was amplified by PCR using primers *babR*-comp-For and *babR*-comp-Rev (Table 1) and genomic DNA from *B. abortus* 2308 as a template. The resulting DNA fragment was digested with PstI and then treated with polynucleotide kinase, and the digested and treated fragment was ligated into PstI/HincII-digested pBBR1MCS-4 to create pC³011. pC³011 was introduced into the *B. abortus babR* mutant strain by electroporation.

Expression and purification of recombinant proteins. The *Strep*-tagII system (IBA, Göttingen, Germany) was used to produce recombinant *B. abortus* proteins in *E. coli* strain BL21. For recombinant BabR (rBabR), the coding region of the *babR* gene (BAB1_0190) was amplified using primers rBabR-For and rBabR-Rev (Table 1), *B. abortus* 2308 chromosomal DNA as a template, and *Taq* polymerase. The amplified DNA fragment was treated with polynucleotide kinase and blunt end ligated into PshAI-digested, phosphatase-treated pASK-IBA6, which encodes an

amino-terminal *Strep*-tagII on the protein of interest. The plasmid encoding recombinant Hfq (rHfq) was constructed in a similar fashion; however, the *hfq* coding region (BAB1_1134) was amplified with primers rHfq-For and rHfq-Rev (Table 1). The amplified DNA fragment was digested with BsaI and ligated into BsaI-digested pASK-IBA6. The resulting plasmids, prBabR and prHfq, were transformed into *E. coli* strain BL21, and the strain harboring the recombinant protein expression plasmid was grown to an optical density at 600 nm of approximately 0.6 before protein expression was induced by the addition of anhydrotetracycline (200 μg/ml [final concentration]). Following 2 h of incubation at 37°C, the cells were collected by centrifugation (4,200 $\times$ *g* for 10 min at 4°C) and lysed by treatment with CelLyticB (Sigma, St. Louis, MO) in the presence of the protease inhibitor phenylmethylsulfonyl fluoride. The supernatant from the suspension of lysed cells was collected by centrifugation (14,000 $\times$ *g* for 10 min at 4°C), and the collected supernatant was passed through an affinity column packed with *Strep*-Tactin Sepharose. The column was washed extensively with buffer W (100 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, pH 8.0), and recombinant protein was eluted with 2.5 mM desthiobiotin in buffer W. The degrees of purity of recombinant BabR and Hfq were high, as judged by SDS-PAGE.

Real-time reverse transcription (RT)-PCR. Total RNA was isolated from cultures of *B. abortus* 2308, the *hfq* mutant B8, and the complemented mutant grown to the appropriate phase of growth in brucella broth. An equal volume of 1:1 ethanol-acetone was added to the cultures, and the mixture was stored at −80°C. Cultures were thawed, and the cells were collected by centrifugation at 18,000 $\times$ *g* for 5 min at room temperature. RNA was extracted from the cells using the TRIzol reagent (Invitrogen, Carlsbad, CA), and following ethanol precipitation of the RNA, genomic DNA was removed by treatment with RQ1 DNase I (Promega, Madison, WI) following the manufacturer's instructions. cDNA was generated from the final RNA preparation using the SuperScript III cDNA Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and this cDNA was used for real-time PCR employing SYBR green PCR SuperMix (Roche, Mannheim, Germany). For these experiments, primers for 16S RNA were used as a control, while gene-specific primers were used for evaluating relative mRNA levels (Table 1). Parameters for PCR included a single denaturing step of 5 min at 95°C, followed by 40 cycles of amplification consisting of denaturation for 15 s at 95°C, annealing for 15 s at 50°C, and extension for 15 s at 72°C. Fluorescence from SYBR green incorporation into double-stranded DNA was measured with an iCycler machine (Bio-Rad), and the relative abundance of mRNA was determined using the Pfaffl equation (30).

Immunoblot analysis. Whole-protein lysates were collected from *Brucella* cultures harvested during exponential growth or after entry into stationary phase. Cultures were collected by centrifugation at 15,000 $\times$ *g* for 10 min at 4°C, and the cell pellet was suspended in 1 ml of protein sample buffer (0.3% SDS, 200 mM dithiothreitol, 22 mM Tris base, 28 mM Tris-HCl). The cells were then boiled for 1 h and subjected to 10 cycles (setting 4, 20 s per cycle) of disintegration in Lysing Matrix B (MP Biomedicals, Solon, OH) using a Bio 101 FastPrep FP120 cell disintegrator (Thermo Scientific Savant; Thermo Fisher Scientific Inc., Waltham, MA). Protein concentrations were determined using a Bradford assay with bovine serum albumin (BSA) standards (Bio-Rad, Hercules, CA). Ten micrograms of total protein per strain was separated by 15% SDS-PAGE, and MagicMark and SeeBluePlus2 standards (Invitrogen, Carlsbad, CA) were included on the gel. Proteins were transferred to a nitrocellulose membrane by electroblotting at approximately 400 mA for 45 min. Membranes were blocked at room temperature in 5% skim milk in TBST (Tris-buffered saline [50 mM Tris, 150 mM NaCl, pH 7.4]−0.05% Tween 20), and primary antibodies (anti-VirB1 [1:1,000 dilution]) were incubated with the membranes at room temperature in 5% skim milk. Secondary antibodies (anti-rabbit–horseradish peroxidase [HRP]) were used at a 1:10,000 dilution. All washing steps were performed with TBST. Development of the HRP signal was performed using WestPico (Pierce [Thermo

Scientific], Rockford, IL). The anti-VirB1 antiserum was kindly provided by Christian Baron, Department of Biochemistry, Université de Montréal.

Determination of the *babR* transcriptional start site. Total RNA was extracted from *B. abortus* as described above. The oligonucleotide primer *babR*-PE was end labeled with [γ-^{32}P]ATP (Perkin-Elmer, San Jose, CA) and polynucleotide kinase (Promega, Madison, WI), and the labeled primer was annealed to 50 μg of *Brucella* RNA. Primer extension analysis was carried out using the avian myeloblastosis virus reverse transcriptase primer extension system (Promega, Madison, WI) according to the manufacturer's instructions. DNA sequence analysis was performed using the same radiolabeled primer and the SequiTherm EXCEL II DNA sequencing kit (Epicentre, Madison, WI) following the manufacturer's protocol. Primer extension products and DNA sequencing reaction products were separated on 6% denaturing polyacrylamide gels and visualized by autoradiography.

5′ rapid amplification of cDNA ends (RACE) was carried out using the FirstChoice RLM-RACE kit (Ambion, Austin, TX) according to the manufacturer's instructions. *B. abortus* 2308 was grown in brucella broth to late exponential phase, and RNA was isolated and treated with DNase I as described above. The *babR* gene-specific primer *babR*-RACE-Rev was used in the PCR steps of the 5′ RACE protocol where *Taq* polymerase was also employed. The *babR* 5′ RACE product (see Fig. 3B) was gel purified and cloned into pGEM-T Easy. Plasmid DNA was purified from potential clones, and DNA sequencing analysis was performed to identify the *babR* transcriptional start site.

Electrophoretic mobility shift assays (EMSAs). (i) rBabR EMSAs. All rBabR EMSA experiments were carried out in a 20-μl total reaction volume containing a binding buffer composed of 10 mM Tris-HCl (pH 7.4), 50 mM KCl, 1 mM dithiothreitol, 6% glycerol, 0.5 mM EDTA, 50 μg/ml BSA, and 50 μg/ml salmon sperm DNA. A 150-bp DNA fragment corresponding to the promoter region of the *B. abortus* 2308 *virB* operon was amplified by PCR using primers P$_{virB}$-For and P$_{virB}$-Rev (Table 1) and chromosomal DNA from *B. abortus* 2308 as a template. The amplified DNA fragment was purified by agarose gel electrophoresis, and the fragment was end labeled with [γ-^{32}P]ATP (Perkin-Elmer, San Jose, CA) and polynucleotide kinase (Promega, Madison, WI). Increasing amounts of recombinant BabR were mixed with the radiolabeled P$_{virB}$ DNA in binding buffer, and the reaction mixtures were incubated at room temperature for 20 min. As a control, a 50$\times$ molar concentration of nonradiolabeled P$_{virB}$ DNA (specific competitor) or nonradiolabeled BAB2_0612 DNA (nonspecific competitor) was added to some reaction mixtures. The binding reaction products were subjected to electrophoresis on 6% native polyacrylamide gels in 0.5$\times$ TBE running buffer for approximately 1 h at 4°C. Following electrophoresis, gels were dried onto Whatman 3MM filter paper using a vacuum gel dryer system and visualized by autoradiography.

(ii) rHfq EMSAs. All rHfq EMSA experiments were performed in a 20-μl total reaction volume containing binding buffer composed of 10 mM Tris-HCl (pH 7.4), 50 mM KCl, 1 mM dithiothreitol, 6% glycerol, 0.5 mM EDTA, 50 μg/ml BSA, and 50 μg/ml baker's yeast tRNA. *In vitro* transcription using the MAXIscript system (Ambion, Austin, TX) was employed to generate the single-stranded RNA probe for these experiments. This system relies on the T7 RNA polymerase promoter to drive the transcription of the desired ribonucleotide molecule. PCR utilizing chromosomal DNA from *B. abortus* 2308 as a template and primers *babR*-UTR-T7-For and *babR*-UTR-T7-Rev (Table 1) was used to amplify an approximately 150-bp DNA fragment corresponding to the 5′ UTR and first 5 codons of the *babR* mRNA. A DNA fragment coding for a mutated form of the *babR* 5′ UTR (missing the first 20 nucleotides) was also generated, and for this, a PCR was performed as described above with primers *babR*-20-UTR-For and *babR*-UTR-T7-Rev (Table 1). The single-stranded RNA probes were then generated according to the manufacturer's recommendations. Radiolabeled probe was generated using uniform incorporation with [α-^{32}P]UTP (Perkin-Elmer, San Jose, CA). Nonradiolabeled probe was also generated in order to serve as a specific competitor in the EMSA experiments. For the binding assays, increasing amounts of rHfq

were mixed with labeled *babR*-UTR RNA probe in binding buffer, and the reaction mixtures were incubated at 37°C for 30 min. The binding reaction products were then subjected to electrophoresis on 5% native polyacrylamide gels in 0.5× TBE running buffer for ~2 h at room temperature on ice. Following electrophoresis, gels were dried onto Whatman 3MM filter paper using a vacuum gel dryer system and visualized by autoradiography.

RESULTS

Hfq is required for wild-type production of the VirB1 protein in B. abortus 2308. To better define the Hfq regulon in *B. abortus* 2308, two-dimensional gel electrophoresis was performed on whole-protein lysates from the parental strain and an isogenic *hfq* mutant. One of the components of the VirB type IV secretion system, VirB1, was found to be produced at approximately 6-fold higher levels in parental strain 2308 than in the *hfq* mutant (data not shown). VirB1 is encoded by the first gene in the *virB* operon, *virB1*, and to date, the sole promoter described for this operon is located upstream of *virB1* (5, 27). In order to gain insight into Hfq-mediated regulation of VirB1 protein levels, a pMR10-based *virB-lacZ* translational fusion was constructed, and β-galactosidase activity produced by this fusion was assessed in *B. abortus* 2308, the isogenic *hfq* mutant B8, and a derivative of B8 carrying a plasmid-borne copy of *hfq*.

β-Galactosidase activity from the *virB-lacZ* translational fusion was significantly reduced in the *hfq* mutant compared to that in strain 2308, and genetic complementation of *B. abortus* B8 with plasmid-borne *hfq* restored the β-galactosidase activity of this strain to wild-type levels (Fig. 1A). These data indicate that Hfq has a positive influence on VirB1 production in the parental 2308 strain, and these data also confirm the results of the original two-dimensional gel electrophoresis analyses that first identified the relationship between Hfq and VirB1. Immunoblot analysis of *Brucella* protein lysates with anti-VirB1 antisera also revealed a significant difference between the VirB1 protein levels in *B. abortus* 2308 and the *hfq* mutant (Fig. 1B). VirB1 protein levels were greatly reduced in the *hfq* mutant compared to those in parental strain 2308, and this reduction was observed during exponential growth of the bacteria; however, no significant difference in VirB1 levels was seen during stationary phase. Importantly, the levels of VirB1 protein in the *hfq* mutant were restored to wild-type levels by the expression of *hfq in trans* in the mutant. Altogether, these data demonstrate that Hfq is required for wild-type production of the VirB1 protein in *B. abortus* 2308.

It is possible that Hfq can affect the levels of a specific protein by directly interacting with the mRNA encoding that protein and altering translation and/or transcript stability; however, the possibility also exists that Hfq indirectly alters target protein levels by altering the abundance of a transcriptional regulator that will result in decreased transcription of the target gene. To determine if Hfq affects the transcription of the *virB* promoter, a *virB-lacZ* transcriptional fusion was constructed on the same plasmid backbone as the *virB-lacZ* translational fusion (e.g., pMR10), and for this construct, the region from −450 to +1 relative to the *virB* transcriptional start site was cloned in front of *lacZ*. The β-galactosidase activity of the strains carrying this fusion was reduced in the *B. abortus hfq* mutant compared to that in parental strain 2308, and promoter activity was restored to wild-type levels by in-*trans* complementation of the mutant with a plasmid-borne copy of *hfq* (Fig. 1C). Real-time RT-PCR was employed as an
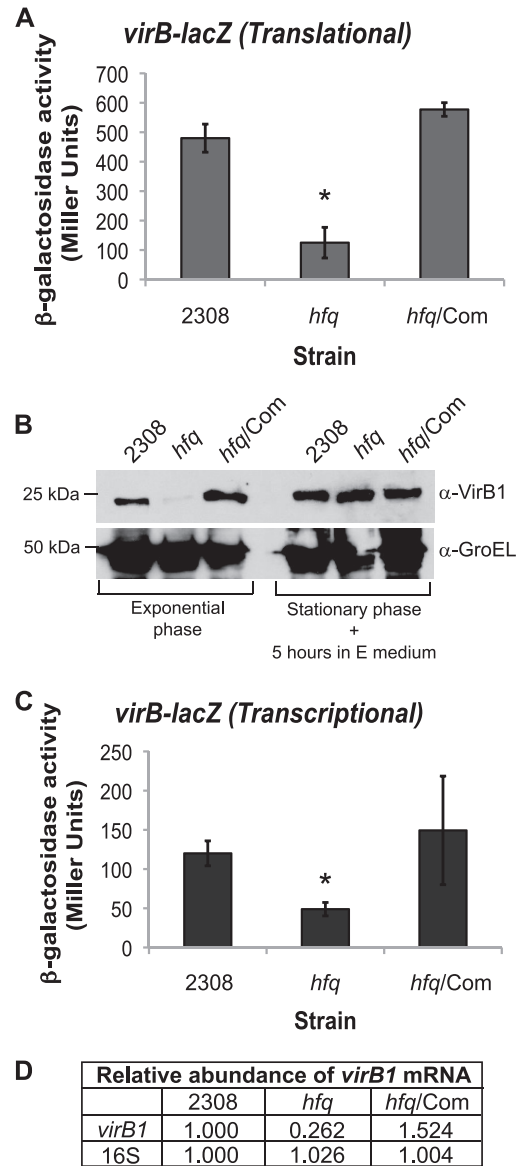
A

virB-lacZ (Translational)

B

α-VirB1

α-GroEL

C

virB-lacZ (Transcriptional)

D

Relative abundance of *virB1* mRNA			
	2308	*hfq*	*hfq*/Com
virB1	1.000	0.262	1.524
16S	1.000	1.026	1.004

FIG 1 Hfq is required for maximal expression of the VirB system. Plasmid constructs pC³005 (A) and pC³003 (C) (Table 2) were transformed into *B. abortus* 2308, the *hfq* isogenic mutant (*hfq*), and the *hfq* mutant strain expressing *hfq* from a plasmid (*hfq*/Com), and following cultivation in low-pH (pH 4.5) defined medium, β-galactosidase activity was assessed. The asterisk denotes a statistically significant difference between the β-galactosidase activities of parental strain 2308 and the *hfq* mutant strain (*P* < 0.01). (B) Immunoblot analysis of VirB1 protein levels. *Brucella* strains were cultivated in low-pH minimal E medium. GroEL levels are shown as a protein loading control, and molecular weight markers are shown to the left. (D) Real-time RT-PCR analysis of *virB1* transcript levels. *Brucella* strains were grown in low-pH (pH 4.5) defined medium, and total RNA was isolated. Oligonucleotide primers specific for *virB1* or 16S rRNA were used to amplify the target genes by PCR, and quantification of the amplified DNA fragments was performed using SYBR green incorporation. The values represent the relative abundances of specific mRNAs, with the level of mRNA from parental strain 2308 designated 1.000.

independent means of assessing the relationship between Hfq and *virB* transcription, and *virB* mRNA was found to be significantly less abundant in the *hfq* mutant than in strain 2308 (Fig. 1D). Genetic complementation of the *hfq* mutant restored *virB* mRNA

TABLE 2 Plasmids used in this study

Plasmid	Description	Reference
pUC19	Cloning vector; Amp^r	54
pGEM-T Easy	Cloning vector; Amp^r	Promega
pEX100T	Cloning vector; contains *sacB* gene; Amp^r	38
pBBR1MCS-4	Broad-host-range cloning vector; Amp^r	20
pNPTS138	Cloning vector; contains *sacB* gene; Kan^r	46
pLKC-481	Cloning vector containing promoterless *lacZ* gene; Amp^r Kan^r	48
pMR10	Broad-host-range cloning vector; Kan^r	14
pMR15	Broad-host-range vector containing promoterless *lacZ* gene; Kan^r	3
pC^{3}003	*virB-lacZ* transcriptional fusion in pMR15	This study
pC^{3}004	*virB-lacZ* translational fusion in pLKC-481	This study
pC^{3}005	*virB-lacZ* translational fusion fragment from pC^{3}004 in pMR10	This study
pC^{3}006	*babR-lacZ* transcriptional fusion in pMR15	This study
pC^{3}007	*babR-lacZ* translational fusion in pLKC-481	This study
pC^{3}008	*babR-lacZ* translational fusion fragment from pC^{3}007 in pMR10	This study
pC^{3}009	In-frame deletion of *babR* plus 1 kb of each flanking region in pUC19	This study
pC^{3}010	Mutated *babR* locus plus flanking regions from pC^{3}009 in pNPTS138	This study
pC^{3}011	*babR* locus including entire promoter region in pBBR1MCS-4	This study
pC^{3}012	*babR* locus with truncated 5′ UTR in pUC19	This study
pC^{3}013	*babR-lacZ* translational fusion with truncated 5′ UTR in pLKC-481	This study
pC^{3}014	Truncated *babR-lacZ* translational fusion fragment from pC^{3}013 in pMR10	This study
pJMG001	Full-length *hfq* gene plus 1 kb of each flanking region in pGEM-T Easy	This study
pJMG002	Religation of pJMG001 following inverse PCR to delete *hfq* coding region	This study
pJMG003	Mutated *hfq* locus plus flanking regions from pJMG002 in pEX100T	This study
pJMG004	*hfq* locus including entire promoter region in pBBR1MCS-4	This study

abundance in this strain to a level above that observed in strain 2308. These data indicate that in addition to affecting the levels of VirB1 protein, Hfq is also involved in the regulation of *virB1* transcript levels. Thus, it was concluded that Hfq likely regulates *virB* expression through an indirect mechanism, and this led to the hypothesis that Hfq is influencing the levels of a regulator of *virB* transcription.

Production of the *virB* regulator BabR is altered by Hfq. Several transcriptional regulators that control the expression of the *virB* operon have been identified (16, 31), and the hypothesis that one of these regulators is the link between Hfq and *virB* expression in *B. abortus* 2308 was tested. In order to test this hypothesis, the promoter regions of the *arsR6* (BAB1_1605), *gntR4* (BAB1_1894), *araC2* (BAB2_0136), *vjbR* (BAB2_0118), and *babR/blxR* (BAB1_0190) genes were cloned in frame with *lacZ* using the same plasmid-based strategy that was employed to construct the *virB-lacZ* translational fusion. β-Galactosidase assays with parental *B. abortus* 2308, the isogenic *hfq* mutant strain, and the in-*trans*-complemented *hfq* mutant strain harboring these plasmids revealed no significant differences in β-galactosidase activity between the strains for the *lacZ* fusions to *arsR6*, *gntR4*, *araC2*, and *vjbR* (data not shown). However, the β-galactosidase activity of the *babR-lacZ* translational fusion was significantly reduced in the *hfq* mutant strain compared to that in parental strain 2308, and genetic complementation of the *hfq* mutant elevated β-galactosidase activity production by this strain to levels above those observed in strain 2308 (Fig. 2A). Moreover, when the *Brucella* strains harboring the *babR-lacZ* translational fusion were grown on agar containing the β-galactosidase indicator X-Gal, strain 2308 and the complemented *hfq* mutant formed blue colonies but the *hfq* mutant formed white colonies, indicating that *babR* expression is severely inhibited in the latter strain (Fig. 2B).

To test if Hfq is modulating the amount BabR protein at the level of transcription or translation, the transcription of *babR* in the *B. abortus hfq* mutant was assessed (Fig. 2C and D). Similar to the *virB-lacZ* transcriptional fusion, a plasmid-borne *babR-lacZ* transcriptional fusion containing the region from −72 to +93 relative to the *babR* transcriptional start site was constructed in pMR15, and the β-galactosidase activity of *Brucella* strains carrying this fusion was measured. There was no significant difference between the β-galactosidase activities produced by parental strain 2308 and the *hfq* mutant strain (Fig. 2C). Unlike strains harboring the *babR-lacZ* translational fusion, strains harboring the *babR-lacZ* transcriptional fusion did not exhibit any color differential when grown on agar containing X-Gal (Fig. 2B). As an independent approach to assessing the link between Hfq and *babR* transcription, real-time RT-PCR was used to assess the abundances of *babR* mRNA in the different *B. abortus* strains, and it was determined that *babR* mRNA levels are not significantly decreased in the *hfq* mutant compared to those in wild-type strain 2308 (Fig. 2D). Taken together, these data suggest that the positive link between Hfq and BabR translation is direct.

Definition of the *babR* promoter and the 5′ UTR of the *babR* mRNA. In order to regulate gene expression at the posttranscriptional level, Hfq must bind to mRNAs, and, in some instances, this binding occurs at the 5′ UTR of the target mRNA (45). Therefore, it was important to define the promoter region of *babR* in order to further characterize the Hfq-mediated regulation of *babR* expression (Fig. 3). The *babR* gene (BAB1_0190) in *B. abortus* 2308 is found on chromosome 1. The genes in this locus include *metH* (B$_{12}$-dependent methionine synthase; BAB1_0188), *hyp* (hypothetical protein; BAB1_0189), *babR* (LuxR-type transcriptional regulator; BAB1_0190), and *atf* (aminotransferase; BAB1_0191) (Fig. 3A). The transcriptional start site for the *babR* mRNA was determined by primer extension analysis and confirmed by 5′ RACE analysis, and these techniques revealed the presence of a
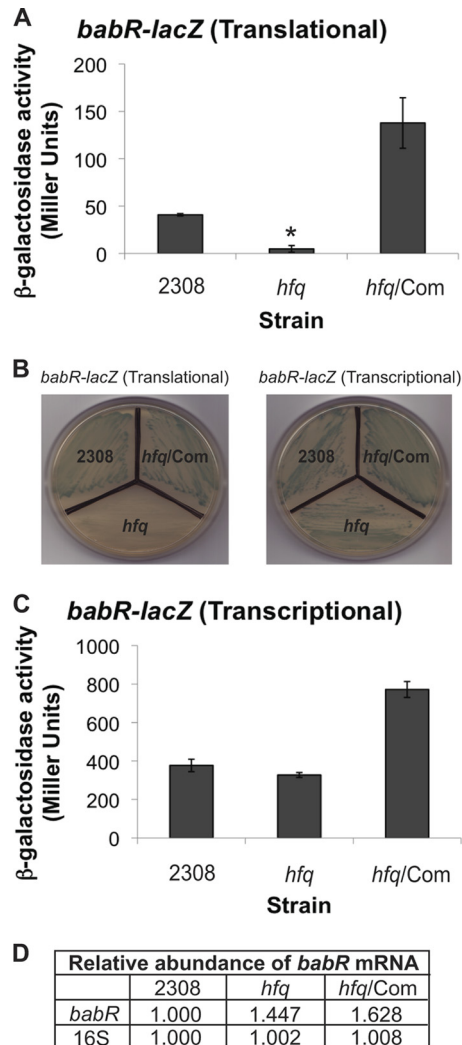
A *babR-lacZ* (Translational)

B

babR-lacZ (Translational)   *babR-lacZ* (Transcriptional)

C *babR-lacZ* (Transcriptional)

D

Relative abundance of *babR* mRNA			
	2308	*hfq*	*hfq*/Com
babR	1.000	1.447	1.628
16S	1.000	1.002	1.008

FIG 2 Identification of an Hfq-regulated transcription regulator, BabR. Plasmid constructs pC³008 (A) and pC³006 (C) (Table 2) were transformed into *B. abortus* 2308, the *hfq* isogenic mutant (*hfq*), and the *hfq* mutant strain expressing *hfq* from a plasmid (*hfq*/Com), and following cultivation in brucella broth, β-galactosidase activity was assessed. The asterisk denotes statistically significant differences between the β-galactosidase activities of parental strain 2308 and the *hfq* mutant strain ($P < 0.01$). (B) Pictures of *Brucella* stains carrying either pC³008 (left) or pC³006 (right) grown on tryptic soy agar containing X-Gal. (D) Real-time RT-PCR analysis of *babR* transcript levels. *Brucella* strains were grown in brucella broth, and total RNA was isolated. Oligonucleotide primers specific for *babR* or 16S rRNA were used to amplify the target genes by PCR, and quantification of the amplified DNA fragments was performed using SYBR green incorporation. The values represent the relative abundances of specific mRNAs, with the level of mRNA in parental strain 2308 designated 1.000.

119-nucleotide 5′ UTR upstream of the *babR* coding region in the *babR* transcript (Fig. 3B). The predicted RNA secondary structure of the *babR* 5′ UTR and the first five codons of the coding sequence was determined using mfold (http://mfold.rna.albany.edu/?q=mfold), and interestingly, this program predicted only two very similar secondary structures (Fig. 3C). The calculated free-energy (ΔG) values for these structures were −22.44 and −24.20 kcal/mol, indicating that the formation of these predicted structures is highly favorable.

Truncation of the *babR* 5′ UTR alleviates the requirement of Hfq for *babR* expression. The direct binding of Hfq to the *babR* 5′ UTR is evidence for the involvement of Hfq in the modulation of *babR* expression at the posttranscriptional level. In order define the region of the *babR* 5′ UTR responsible for the effect of Hfq on expression, the first 20 nucleotides of the *babR* 5′ UTR were deleted, and a *babR-lacZ* translational fusion with this truncated 5′ UTR was constructed (Fig. 4A). The truncated fusion, as well as the full-length UTR fusion, was transformed into parental *B. abortus* strain 2308 and isogenic *hfq* mutant strain B8, and β-galactosidase activity was measured (Fig. 4B). Similar to previous data (Fig. 2A), the β-galactosidase activity produced by the strains harboring the full-length *babR-lacZ* translational fusion was significantly decreased in the *hfq* mutant compared to that in strain 2308; however, there was no significant difference between the strains carrying the truncated *babR* 5′ UTR fusion. As shown in Fig. 2B, parental strain 2308 produces blue colonies when grown on agar containing the β-galactosidase indicator X-Gal, and the *hfq* mutant produced white colonies when grown on the same medium. On the other hand, both parental strain 2308 and the *hfq* mutant strain harboring the truncated *babR* 5′ UTR-*lacZ* fusion produced blue colonies (Fig. 4B). Altogether, these data show that the first 20 nucleotides of the *babR* 5′ UTR are required for the effect of Hfq on the *babR-lacZ* translational fusion.

In vitro, bacterial Hfq proteins will bind to target mRNAs in the absence of a cognate sRNA, and in this study, *Brucella* Hfq was tested for the ability to bind the 5′ UTR of the *babR* mRNA. A recombinant version of *B. abortus* 2308 Hfq was expressed and purified, and EMSAs were used to test for interactions of Hfq with the *in vitro*-transcribed *babR* 5′ UTR and a mutated form of the *babR* 5′ UTR that is missing the first 20 nucleotides (Fig. 5). Hfq bound to the full-length *babR* 5′ UTR in a concentration-dependent manner, and while Hfq also bound the mutated *babR* 5′ UTR, higher concentrations of Hfq were required for binding of the mutated 5′ UTR than for that of the full-length *babR* 5′ UTR These data indicate that Hfq binds directly to the *babR* mRNA and furthermore that the first 20 nucleotides of the *babR* 5′ UTR are required for maximal binding by Hfq. Therefore, these interactions are likely important for the requirement of Hfq in mediating wild-type BabR protein levels. From the *babR-lacZ* fusion data (Fig. 4) and the *babR* 5′ UTR/Hfq EMSA data (Fig. 5), it is concluded that Hfq functions at the posttranscriptional level to modulate the expression of *babR* in *B. abortus* by binding directly to the 5′ UTR of the *babR* mRNA, and moreover, the first 20 nucleotides of the *babR* 5′ UTR are required for this regulatory event.

BabR is a repressor of *virB* expression and binds to the *virB* promoter. It was originally reported that BabR (also called BlxR) has a positive influence on the *virB* promoter (32), but more recently, another study concluded that BabR is a repressor of *virB* transcription (49). Both studies were performed using *B. melitensis* 16M, and the differences in the results obtained could be attributed to differences in experimental and growth conditions. For this study, it was important to clearly define the effect of BabR on *virB* transcription in *B. abortus* 2308 in order to establish whether or not it plays a role in the genetic circuitry between Hfq and VirB1 protein levels. Therefore, an isogenic *babR* mutant of *B. abortus* 2308 was constructed and the *virB1* transcription levels in this strain were assessed (Fig. 6).

The same *virB-lacZ* transcriptional fusion used previously in Fig. 1 was transformed into the *babR* mutant strain and the mu-
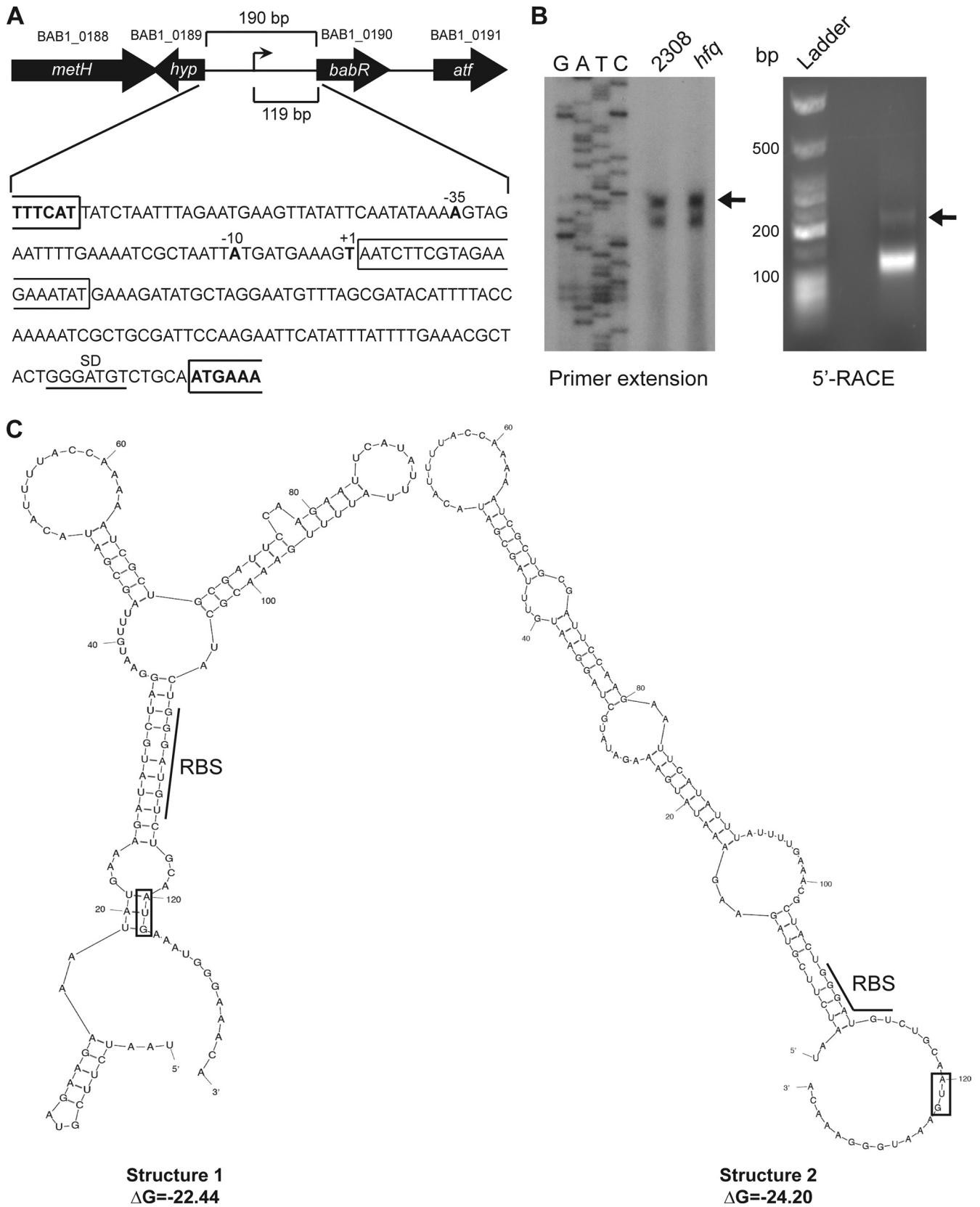
FIG 3 Mapping and characterization of the *babR* promoter region. (A) Schematic of the chromosomal region containing *babR*. The plus strand sequence of DNA corresponding to the region between the *hyp* gene and *babR* is shown below the schematic, and the identified transcriptional start site (+1) is labeled. This transcriptional start site is 119 nucleotides upstream of the *babR* start codon. The first 20 nucleotides of the 5′ UTR are boxed to indicate that this region was
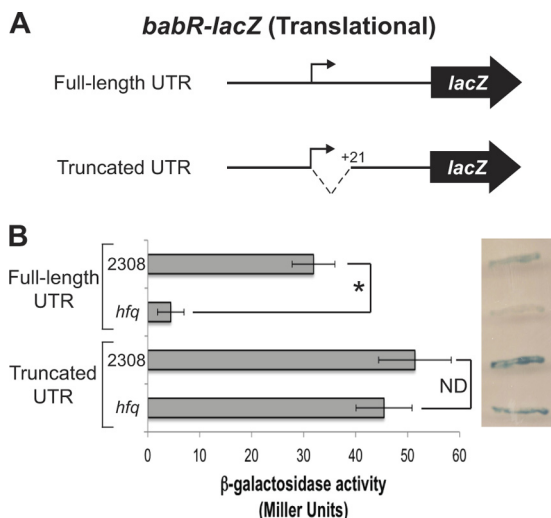
FIG 4 Truncation of the *babR* 5′ UTR alleviates the requirement for Hfq. (A) Schematic of the *babR-lacZ* translational fusions used in this experiment. The full-length fusion construct pC^{3}008 (also used in Fig. 2) contains the *babR* promoter region and the entire *babR* 5′ UTR cloned in frame with *lacZ*. The truncated fusion construct pC^{3}014 (Table 2) contains the *babR* promoter region and a truncated *babR* 5′ UTR cloned in frame with *lacZ*. (B) β-Galactosidase activities of strains carrying the full-length and truncated *babR* 5′ UTR-*lacZ* translational fusions. *B. abortus* 2308 and the isogenic *hfq* mutant harboring pC^{3}008 or pC^{3}014 were growth in brucella broth, and β-galactosidase activity was measured. The asterisk indicates a statistically significant difference between the β-galactosidase activities of parental strain 2308 and the *hfq* mutant strain ($P < 0.01$). To the right of the graph is an image of *Brucella* stains carrying pC^{3}008 or pC^{3}014 grown on tryptic soy agar containing X-Gal. ND, no statistically significant difference.

FIG 5 Hfq binds the 5′ UTR of the *babR* transcript. The full-length 5′ UTR of the *babR* mRNA and a mutated version of the *babR* 5′ UTR (−20 nucleotides from the 5′ end) were transcribed *in vitro* and radiolabeled by [α-^{32}P]UTP incorporation. An EMSA was performed using the labeled *in vitro*-transcribed 5′ UTRs and purified recombinant *B. abortus* Hfq. Increasing concentrations of rHfq were incubated with the labeled RNA probe, and the binding reaction products were resolved in 5% native polyacrylamide gels and visualized by autoradiography.

tant strain expressing *babR* from a plasmid, and β-galactosidase activity was measured (Fig. 6A). The activity of this fusion was significantly increased in the *babR* mutant compared to that in wild-type strain 2308, and β-galactosidase was returned to wild-type levels by complementation of *babR* in the mutant. Real-time RT-PCR analysis of relative *virB1* mRNA levels demonstrated a similar trend (Fig. 6B). The relative abundance of *virB1* mRNA was approximately 1.9-fold higher in the *babR* mutant than in parental strain 2308. Altogether, these results confirm the role of BabR as a regulator of *virB* transcription, and these data also indicate that BabR acts as a repressor of *virB* transcription in *B. abortus* 2308.

The role of BabR as a transcriptional regulator of the *virB* operon was previously determined by other laboratories, and these groups employed targeted mutagenesis of *babR* and subsequent experiments with *virB* promoter fusions and/or microarray analyses (32, 49). However, no studies have addressed the ability of BabR to bind directly to the *virB* promoter region, and there-
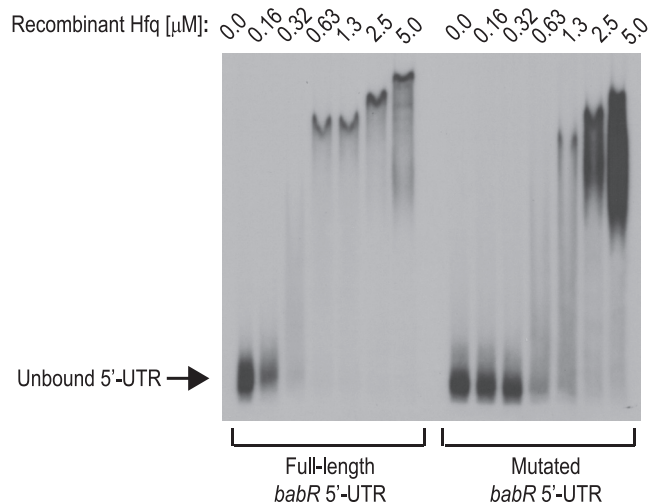
fore, it was not known if BabR directly or indirectly regulates *virB* transcription. In this study, a recombinant version of BabR was expressed and purified, and binding of BabR to the *virB* promoter region was tested by EMSA (Fig. 7). A DNA fragment corresponding to the first 150 bp directly upstream of the *virB1* start codon was used in these experiments, and BabR bound this fragment of DNA in a concentration-dependent manner. Importantly, the binding of BabR to the P$_{virB}$ DNA was specific, as indicated by the ability of excess unlabeled P$_{virB}$ DNA to abolish binding, while a nonspecific fragment of DNA (the coding region of BAB2_0612) was not able to compete with P$_{virB}$ DNA for binding to BabR. From the EMSA analyses, it was concluded that BabR directly binds to the *virB* promoter in order to repress transcription.

DISCUSSION

The *Brucella virB* locus has been the subject of numerous studies, and several transcriptional regulators have been identified that influence *virB* expression (31). These regulators include the histidine utilization regulator HutC (41) and the two-component regulator BvrRS (22). The stringent response (p)ppGpp synthetase/hydrolase protein Rsh has also been linked to *virB* expression in *Brucella* sp. (8). Additionally, mutational analyses of 88 transcriptional regulators in *B. melitensis* 16M revealed five additional tran-

removed for the experiments shown in Fig. 5. The −10 and −35 elements of the *babR* promoter are also labeled. The genes in this region are *metH* (B12-dependent methionine synthase; BAB1_0188), *hyp* (hypothetical protein; BAB1_0189), *babR* (LuxR-type transcriptional regulator; BAB1_0190), and *atf* (aminotransferase; BAB1_0191). (B) Identification of the transcriptional start site of the *babR* mRNA. Primer extension (left panel) was performed using total RNA isolated from *Brucella* strains (wild-type 2308 and the *hfq* mutant) grown in brucella broth. The primer extension reaction products, along with DNA sequencing reaction products produced with the same radiolabeled primer, were separated on 6% denaturing polyacrylamide gels and visualized by autoradiography. 5′ RACE was also employed (right panel) using RNA isolated from *B. abortus* 2308 grown in brucella broth. Molecular weight standards and the 5′ RACE product were separated by 2.0% agarose gel electrophoresis. The indicated DNA band was extracted from the gel, cloned, and sequenced to determine the transcriptional start site. (C) Secondary structure predictions of the RNA containing the *babR* 5′ UTR and the first five codons of the *babR* coding region. The RBS of each predicted structure is underlined, while a box is placed around the start codon (AUG) of each predicted structure.
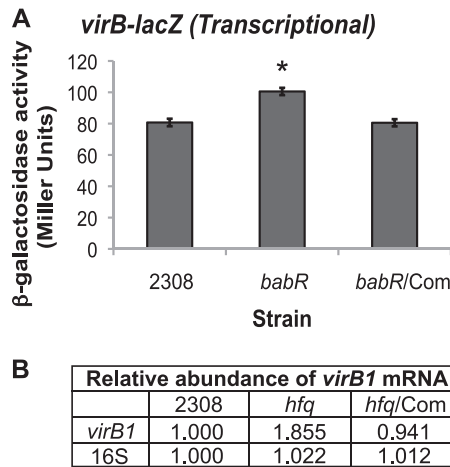
A

virB-lacZ (Transcriptional)

(Bar chart: β-galactosidase activity (Miller Units) vs Strain. Y-axis 0–120. Strains: 2308 (~80), babR (~101, marked with *), babR/Com (~80).)

B

Relative abundance of *virB1* mRNA			
	2308	*hfq*	*hfq*/Com
virB1	1.000	1.855	0.941
16S	1.000	1.022	1.012

FIG 6 BabR is a repressor of *virB* expression. (A) The *virB-lacZ* transcriptional fusion construct pC³003 (Table 2) was transformed into *B. abortus* 2308, the *babR* isogenic mutant (*babR*), and the *babR* mutant strain expressing *babR* from a plasmid (*babR*/Com), and following cultivation in low-pH (pH 4.5) defined medium, β-galactosidase activity was assessed. The asterisk denotes a statistically significant difference between the β-galactosidase activities of parental strain 2308 and the *babR* mutant strain ($P < 0.01$). (B) Real-time RT-PCR analysis of *virB1* transcript levels. *B. abortus* 2308 strains were grown in low-pH (pH 4.5) defined medium, and total RNA was isolated. Oligonucleotide primers specific for *virB1* or 16S rRNA were used to amplify the target genes by PCR, and quantification of the amplified DNA fragments was performed using SYBR green incorporation. The values represent the relative abundances of specific mRNAs, with the level of mRNA in parental strain 2308 designated 1.000.

Non-specific competitor: - - - - - - - +
Specific competitor: - - - - - - + -
Recombinant BabR [nM]: 0 67.2 134 269 538 1080 1080 1080

BabR-P*virB* complex →

Free P*virB* DNA →

FIG 7 BabR binds the *virB1* promoter. An EMSA was employed to test the binding of purified rBabR to the *virB* promoter region. A DNA probe containing the first 150 bp of the *virB* upstream region was amplified by PCR, and the fragment was radiolabeled with [γ-³²P]ATP. Increasing concentrations of rBabR were incubated with the labeled DNA, and in some binding reaction mixtures, unlabeled specific (P*virB* DNA) and nonspecific (BAB2_0612 coding sequence DNA) competitor DNA fragments were included as controls. The binding reaction products were resolved in 6% native polyacrylamide gels and visualized by autoradiography.

scriptional activators (ArsR6, AraC2, AraC8, DeoR1, and GntR4) of *virB* expression (16). The LuxR-type regulators VjbR (6, 50) and BabR (also called BlxR) (32, 50) have also been reported to influence *virB* expression. In the present study, we have shown that Hfq, most likely in conjunction with an sRNA, is also involved in the regulation of *virB* expression. From the data collected (Fig. 1 and 2), it was hypothesized that Hfq functions via an intermediate transcriptional regulator to modulate *virB* expression. This hypothesis was based on the fact that in other bacteria, Hfq has been shown to alter the levels of transcriptional regulators, which, in turn, leads to the altered transcription of genes targeted by these regulators (17, 18). Subsequent experiments determined that BabR, a known transcriptional regulator of *virB*, requires Hfq for normal expression. Therefore, a working hypothesis was developed for this regulatory circuit in which Hfq and a small RNA are needed for the efficient translation of BabR, which, in turn, leads to regulation of *virB* expression.

The present work confirmed a regulatory link between BabR and *virB*, and it was established that BabR is a *virB* repressor in *B. abortus* 2308 (Fig. 6). Previously, the proper expression of *virB* had been linked to BabR, as mutation of *babR* was reported to lead to changes in *virB* expression (32, 49), and these studies utilized promoter fusions to reporter genes and quantitative RT-PCR to draw these conclusions. The ability of BabR to directly bind to DNA to exert its regulatory effects was not previously reported, but here it is shown that BabR binds to the promoter region of *virB* (Fig. 7). Thus, we demonstrated a direct and specific interaction between BabR and the *virB* promoter. BabR is a member of the LuxR superfamily, which consists of transcriptional regulators that are involved in the regulation of quorum-sensing systems, and these
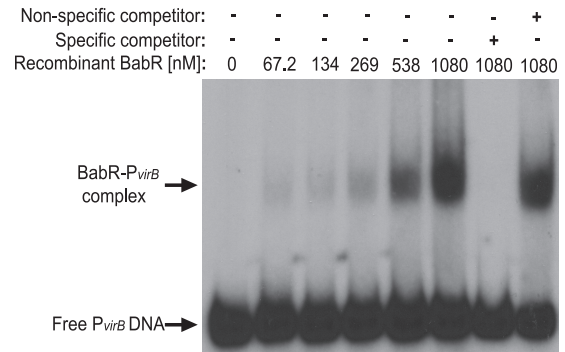
regulators often respond to small-molecule signals, such as homoserine lactones (HSL), that alter the capacity of the proteins to bind target promoters in DNA (26). Interestingly, addition of the *Brucella* quorum-sensing molecule C12-HSL (47) to the EMSA reaction mixtures did not affect the binding of BabR to the *virB* promoter (data not shown). This supports results reported by Uzureau et al. showing that BabR-mediated regulation of the *virB* system is independent of the C12-HSL quorum-sensing molecule (49). By DNase I footprinting analyses, the specific DNA sequence in the *virB* promoter that is bound by another *Brucella* LuxR-type regulator, VjbR, has been identified (2). In order to compare the binding sites of the two *Brucella* LuxR-type regulators, the BabR binding box in the *virB* promoter was sought. Unfortunately, repeated attempts using DNase I footprint analysis failed to identify the specific sequence bound by BabR. Nonetheless, BabR binds directly to the *virB* promoter, but how and under what conditions VjbR and BabR cooperate to regulate *virB* expression remain to be determined.

Having determined that Hfq is required for the proper translation of BabR, we next sought to better define the link between Hfq and BabR. Using EMSAs, it was shown that *Brucella* Hfq binds the *babR* 5′ UTR with a high degree of affinity (Fig. 5). Additionally, the first 20 nucleotides of the *babR* 5′ UTR are required in order for Hfq to mediate its effect on *babR* expression (Fig. 4). The 5′ UTR of the *babR* mRNA is predicted to form a highly stable secondary structure with hairpins that occlude the RBS (Fig. 3), and *Brucella* Hfq binds this 5′ UTR with high affinity (Fig. 5). Even though the ability of Hfq to serve as a modulator of gene expression is usually through its capacity to bring together target mRNAs with sRNAs (15), it is possible that binding of Hfq alone (without an sRNA) to the *babR* 5′ UTR can alleviate the secondary structure of the 5′ UTR and allow the proper translation of BabR. Indeed, a *babR-lacZ* translational fusion is completely unable to be activated in the *B. abortus hfq* mutant, but expression of *hfq* from a plasmid restores the production of β-galactosidase in this mutant (Fig. 2A and B). There is evidence from work with *E. coli* that Hfq can alter gene expression in the absence of an sRNA (10, 53), and therefore, the possibility that Hfq functions without an sRNA to regulate
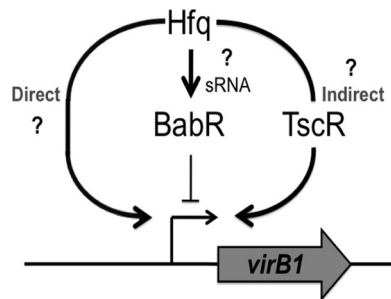
FIG 8 Model of Hfq-mediated regulation of *babR* and *virB1*. There is a clear relationship between Hfq and wild-type levels of VirB1 in *B. abortus* 2308. It was shown in this study that Hfq positively regulates the production of BabR, a known *virB1* transcriptional regulator; however, BabR is a repressor of *virB1* transcription. Therefore, BabR is not the sought-after genetic link between Hfq and *virB1* expression. Two alternative means of *virB1* regulation by Hfq may be taking place. Hfq may be directly altering the levels of VirB1 protein by binding to the *virB1* mRNA and altering translation (Direct). Alternatively, Hfq may be affecting the levels of a transcriptional regulator protein (TscR), and altered levels of this regulator would lead to increased levels of *virB1* mRNA, resulting in increased levels of VirB1 protein (Indirect).

babR expression cannot be excluded. While the present study identified BabR as a target for Hfq in *B. abortus* 2308, the identity of, or requirement for, an sRNA in this regulatory event was not described. It is likely that Hfq works in concert with an sRNA in order to exert its regulatory effects on the *babR* mRNA, and our laboratory is currently employing several approaches to identify such an sRNA molecule. While Hfq is essential for *babR* expression, these experiments do not confirm or exclude the requirement of an sRNA for proper *babR* expression. Rather, it can only be concluded that Hfq is absolutely required for the expression of *babR*.

The role of Hfq in the virulence of *Brucella* is clear and well documented (33, 35), but this requirement of Hfq for wild-type virulence has remained undefined mechanistically. The present study sheds some light on this subject, as we have shown that Hfq aids in the proper expression of the *virB* locus, which is an important genetic determinant of *Brucella* virulence (11, 19, 27, 40, 55). It was determined that both the transcription and the translation of *virB* are affected by Hfq (Fig. 1), and these findings led to the hypothesis that Hfq indirectly alters VirB1 protein levels by controlling the expression of a *virB* transcriptional regulator. However, from these data, the possibility cannot be excluded that Hfq also acts directly on the *virB* mRNA to affect the expression of the *virB* system, and furthermore, it is possible that Hfq is regulating *virB* expression both directly and indirectly (Fig. 8). Additionally, the present study was carried out using *B. abortus* 2308, where *virB* is constitutively expressed at a low level, and can be further induced in a low-pH environment (40). Other *Brucella* spp., such as *B. melitensis* and *B. suis*, do not exhibit constitutive *virB* expression, and thus, how Hfq is involved in *virB* expression in other *Brucella* spp. is not known. Nonetheless, studies are ongoing in our laboratory to determine if Hfq directly regulates VirB1 protein levels by acting at the level of the *virB* mRNA, as well as to clearly define the indirect regulatory relationship between Hfq and *virB* expression.

It is important to note that the data presented here indicate that BabR is not the sought-after genetic link between Hfq and *virB* expression. It was initially hypothesized that BabR served as this

link; however, it was determined that BabR is a repressor of *virB* transcription in *B. abortus* 2308 (Fig. 6). Hfq positively influences BabR production (Fig. 2), and therefore, Hfq-mediated increases in BabR levels should result in decreased *virB* expression. Given that Hfq also elicits a positive effect on *virB* expression (Fig. 1), BabR cannot serve as the direct connection between Hfq and *virB* expression. Thus, it is concluded that Hfq coordinates the expression of *virB* in *B. abortus* 2308 through both BabR-dependent and BabR-independent mechanisms. This seems likely, given that mutations in either *hfq* or *virB* result in the attenuation of *Brucella* strains, but mutation of *babR* is not linked to *Brucella* virulence (32). As discussed earlier, the possibility exists that Hfq is functioning at multiple levels, both directly and indirectly, to maximally modulate the expression of the *virB* locus (Fig. 8). In conclusion, Hfq independently mediates the proper expression of the LuxR-type transcriptional regulator BabR and the VirB type IV secretion system in *B. abortus*, but the sRNA(s) involved in these regulatory events remains to be elucidated.

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