The Iron-Responsive Regulator Irr Is Required for Wild-Type Expression of the Gene Encoding the Heme Transporter BhuA in Brucella abortus 2308

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Brucella abortus is a Gram-negative intracellular pathogen that causes abortion and infertility in its natural bovine host. Incidental infection in humans results in a prolonged illness known as undulant fever. As with most bacteria, iron is an essential micronutrient for Brucella strains (5, 32). This requirement for iron presents a particular challenge for the brucellae, as in nature these bacteria are found exclusively in association with mammalian hosts (13, 23). Iron not incorporated into host tissues is sequestered by host iron-binding proteins such as transferrin and lactoferrin in extracellular spaces and by ferritin within host cells (6). This tight sequestration of iron in mammals serves to prevent iron toxicity in the host, as well as limiting the availability of this nutrient to invading microbes. Indeed, iron restriction plays an important role in both the innate and acquired immune responses of the host (3, 14, 33, 34).

Previous studies have shown that B. abortus 2308 can use heme as an iron source (17). The TonB-dependent outer membrane heme transporter BhuA is required for heme utilization by this strain in vitro, and an isogenic bhuA mutant cannot maintain a chronic spleen infection in experimentally infected mice. Like most other bacterial genes that encode heme transporters, bhuA expression in B. abortus 2308 is maximal during growth under iron-deprived conditions (28). In contrast, Irr generally represses genes in these bacteria (28). Recent studies have linked Irr to the regulation of iron acquisition (31), and Bartonella quintana (16).

Experimental evidence indicates that Irr can serve as either a repressor or an activator (25, 35). Since Irr is functional when cellular iron levels are low, Irr often serves as an activator of genes involved in iron acquisition in the alphaproteobacteria (28). In contrast, Irr generally represses genes in these bacteria that encode proteins that require iron for their activity, proteins that participate in cellular processes that require iron (e.g., heme biosynthesis), or iron storage proteins (28). Recent studies have shown that Irr regulates siderophore and heme biosynthesis genes in B. abortus 2308 (10, 11), and the studies described in this report were designed to investigate the role of
this regulator in the iron-responsive expression of the gene encoding the heme transporter BhuA in this strain.

MATERIALS AND METHODS

Culture media and growth conditions. Routine cultivation of Escherichia coli strains was carried out in Luria-Bertani (LB) broth or on tryptic soy agar (TSA) plates with appropriate antibiotic supplementation as necessary. Brucella strains were routinely grown in brucella broth (Difco) at 37°C or on Schaedler agar (Difco) supplemented with 5% defibrinated bovine blood (SBA) incubated at 37°C under 5% CO₂. Ampicillin, chloramphenicol, and kanamycin were added to these culture media as needed at final concentrations of 100, 5, and 45 µg/ml, respectively. Low-ion minimal medium was prepared as previously described (9), and 50 µM FeCl₃ was added to this medium as a control for growth under iron-replete conditions.

Construction and genetic complementation of B. abortus irr mutants. A 2,051-bp fragment containing the irr open reading frame (ORF) (BAB1_2175) and flanking sequences was amplified from B. abortus 2308 genomic DNA using Taq DNA polymerase (primers: Fwd, 5'GGCCTGGTATTCACTAGG-3'; Rev, 5'GCGCCGCACTGAAAACTC-3') and ligated into the Smal site of pBluescript Ks+ (Stratagene). The resulting plasmid was linearized with NruI and ligated to a 508-bp Smal-HindIII fragment containing the chloramphenicol resistance gene (cat) gene from pBluescript (2) or to a 1.4-kb Smal fragment containing the kanamycin resistance gene (aph3a) from pKS-kan (8). These plasmids were then used to transform isogenic irr mutants from B. abortus 2308 via gene replacement using previously described procedures (4). The genotypes of the resulting B. abortus irr mutants (designated BEA2 [irr:cat] and KHS2 [irr:aph3a]) were confirmed by PCR amplification of the mutated irr loci and restriction enzyme digestion and DNA sequence analysis of the amplified DNA fragments. To facilitate genetic complementation studies, the same 2,051-bp irr-containing PCR fragment described above was also cloned into pGEM-T Easy (Promega), excised from the resulting plasmid by EcoRI digestion, and ligated with EcoRI-digested pBBR1MCS-4 (8). This plasmid, pKHS2, was introduced into B. abortus BEA2 by electroporation (4).

Determination of the transcriptional start site for bhuA by primer extension. The transcriptional start site for the bhuA gene was determined by primer extension analysis performed on total RNA preparations obtained from B. abortus 2308 cultures grown for 120 h in low-ion minimal medium using the primer 5'TGATTT GATITCCAAATGGTTCC-3' and the methods described by Robertson et al. (22).

Construction of bhuA-lacZ transcriptional fusions and β-galactosidase assays. The construction of the bhuA-lacZ transcriptional fusion was described previously (17). The primer 5'GGGACGGGTTCGTCTTTGCTCATAAA-3' and 5'GGGACGGGTTCGTCTTTGCTCATAAA-3' used to construct the bhuA-lacZ fusion with 29-bp and 120-bp 5' deletions were designed to amplify B. abortus 2308 genomic DNA with the forward primers 6a.SaF 5'GGCGTGCCGCGGATTTTCGATAA-3' and 5aF 5'TATTGCTCTTATATCATCCCTTC-3' in conjunction with the same reverse primer (5'GACGTCGCAAGGGACAATA-3') used to construct the bhuA-lacZ fusion with 29-bp deletion. The resulting PCR fragments were ligated into pUC19 and transformed into E. coli DH5α. The resulting pUC19 plasmids were then used to transform isogenic irr mutants in order to determine the number of viable brucellae present. These experiments were performed prior to receiving notification from the Centers for Disease Control and Prevention's Select Agent Program that the introduction of Brucella melitensis, Brucella suis, and B. abortus strains carrying chloramphenicol resistance genes into experimentally infected animals is no longer permitted.

Immunoblot analysis. Whole-cell protein lysates were collected from Brucella cultures grown to the desired time points (i.e., 72, 96, or 120 h) in low-ion minimal medium and low-ion minimal medium supplemented with 50 µM FeCl₃. Cells were harvested by centrifugation (6,800 × g, 10 min, room temperature), and the pellet was suspended in 1 ml of protein sample buffer (0.5% SDS, 200 mM DTT, 22 mM Tris base, and 2 mM Tris-HC). The cells were then boiled for 1 h, followed by 10 cycles of 20 s per cycle of digestion in lysing matrix B (Pierce). These protein homogenates were serially diluted and plated on SBA to determine the number of viable brucellae present.

RESULTS AND DISCUSSION

Irr regulates the expression of the genes encoding the BhuA homologs, HmuR and HutA, in two close phylogenetic relatives of the brucellae, Bradyrhizobium japonicum and Bartonella quintana, respectively (16, 25). In Bradyrhizobium japoni-
cum, Irr is required for the induction of hmuR in response to iron deprivation, and genetic and biochemical evidence suggest that Irr serves as a transcriptional activator of hmuR (25). In contrast, in Bartonella quintana, overproduction of Irr has a negative impact on hutA expression, and Irr has been shown to bind upstream of the hutA promoter in an electrophoretic mobility shift assay (16). As shown in Fig. 1, transcription of hutA is induced during stationary phase following growth in low-iron minimal medium in B. abortus 2308 but not in the isogenic irr mutant BEA2. Moreover, introduction of a plasmid-borne copy of irr into BEA2 restores low-iron-responsive hutA expression in this strain. These data indicate that Irr is required for the low-iron-responsive induction of hutA transcription in B. abortus 2308. Such a role is reminiscent of the one that Irr plays in the iron-responsive expression of hmuR and several other genes encoding TonB-dependent outer membrane iron transporters in Bradyrhizobium japonicum, where Irr serves as a transcriptional activator (25, 27). Consistent with its inability to induce hutA expression in response to iron deprivation, the B. abortus irr mutant BEA2 is also unable to use heme as an iron source in an in vitro assay (Fig. 2A), while the parental 2308 strain and the complemented mutant (BEA2.C) grow equally well around disks containing heme on plates containing the iron-specific chelator EDDHA. In contrast to the results reported by Martínez et al. (11), who reported that a B. abortus irr mutant is not attenuated at 1 and 3 weeks postinfection in BALB/c mice. However, it should be noted that no attenuation was observed for the irr mutant at 1 or 2 weeks postinfection in mice in the present study (Fig. 3), with attenuation being observed starting at week 4 postinfection. In addition, C57BL/6 mice were used in the study reported here, while BALB/c mice were employed in the study contrast to the results reported by Martínez et al. (11), who reported that a B. abortus irr mutant is not attenuated at 1 and 3 weeks postinfection in BALB/c mice. However, it should be noted that no attenuation was observed for the irr mutant at 1 or 2 weeks postinfection in mice in the present study (Fig. 3), with attenuation being observed starting at week 4 postinfection. In addition, C57BL/6 mice were used in the study reported here, while BALB/c mice were employed in the study
described by Martínez et al. (11). From a regulatory standpoint, it is important to point out that the mouse infections described in this report were performed prior to notification from the Centers for Disease Control and Prevention’s Select Agent Program that the introduction of *B. melitensis*, *B. suis*, and *B. abortus* strains carrying chloramphenicol resistance genes into experimentally infected animals is no longer permitted.

To begin to define the *cis*-acting regulatory elements that control the iron-responsive expression of *bhuA* in *B. abortus* 2308, primer extension was employed to determine the transcriptional start site for this gene. A guanine residue (G) 141 nucleotides (nt) upstream of the predicted *bhuA* start codon was identified as the start site for the *bhuA* transcript (Fig. 4). This transcript would include the open reading frame (ORF) annotated as BAB2_1151 in the *B. abortus* 2308 genome sequence which is predicted to encode a hypothetical protein. Whether or not the BAB2_1151 ORF is translated is unknown and is currently under investigation. Notably, 156 nucleotides upstream of the transcriptional site for *bhuA* is a consensus Irr binding site (also known as an ICE box) (Fig. 4).

The spacing of this putative Irr binding site relative to the *bhuA* transcriptional start site is similar to that found in the brl4504 gene (121 nt) in *Bradyrhizobium japonicum*, which requires Irr for its induction in response to iron deprivation (27).

To evaluate the importance of the ICE box for iron-responsive expression of *bhuA* in *B. abortus* 2308, β-galactosidase production by derivatives of this strain carrying pbhuA-lacZ (a pMR15-based plasmid carrying a *bhuA-lacZ* transcriptional fusion [17]) and truncated versions of this plasmid was evaluated. Plasmid pbhuA6.5a-lacZ contains a lacZ fusion containing 179 bp upstream of the *bhuA* transcriptional start site that includes the putative Irr binding site (Fig. 4 and 5). Plasmid

FIG. 4. Genetic organization of the *dhbR-bhuA* locus in *B. abortus* 2308. The transcriptional start site for the *bhuA* gene (which would also include the hypothetical gene designated BAB2_1151) is denoted by +1, and the corresponding guanine residue (G) is shown in boldface and highlighted. The nucleotides comprising the putative Irr binding site upstream of *bhuA* are enclosed in a box (ICE box). The oligonucleotide primers used to construct the truncated versions of the plasmid-borne *bhuA-lacZ* fusion are indicated by the arrows labeled 7AF, 6.5AF, and 5AF.

FIG. 5. Identification of the iron-responsive *bhuA* promoter in *B. abortus* 2308 by deletion analysis. β-Galactosidase production by *B. abortus* 2308 carrying the plasmid-borne *bhuA-lacZ* transcriptional fusions following 120 h of growth in low-iron minimal medium (low Fe) or low-iron minimal medium supplemented with 50 μM FeCl3 (high Fe) is shown. The results presented are from a single experiment that is representative of multiple (≥3) experiments performed from which equivalent results were obtained. The regions of the *dhbR-bhuA* locus included in the *bhuA-lacZ* fusions are shown as dashed arrows, and the location of the putative Irr binding motif is denoted as the ICE box.
contrast, the growth in low-iron minimal medium but basal expression of the Irr protein; lane 6, 75 ng 32P-labeled bhuA promoter-specific DNA fragment plus increasing concentrations (0.25, 0.5, 0.75, and 1 ng) of Irr protein; lane 7, 75 ng 32P-labeled bhuA promoter-specific DNA fragment plus 1 μg Irr plus 750 ng unlabeled pBlue-Cm2 specific DNA fragment (nonspecific inhibitor). The results presented are from a single experiment that is representative of multiple (≥3) experiments performed from which equivalent results were obtained.

pbhu5a-lacZ, on the other hand, contains a lacZ fusion containing 88 bp upstream of the bhuA transcriptional start site lacking an Irr binding site. As shown in Fig. 4, both of the bhuA-lacZ transcriptional fusions (pbhuA5a-lacZ and pbhuA6.5a-lacZ) containing the putative Irr binding site exhibit enhanced expression in B. abortus 2308 following growth in low-iron minimal medium but basal expression when this strain is grown under iron-replete conditions. In contrast, the bhuA-lacZ transcriptional fusion lacking the Irr binding site (pbhuA5a-lacZ) displays basal levels of expression in both low-iron medium and low-iron medium supplemented with 50 μM FeCl₃. These experimental findings verify that the putative Irr-binding site upstream of bhuA is required for the low-iron-responsive induction of this gene in B. abortus 2308 and, moreover, suggest that Irr is serving as a transcriptional activator. This proposition is further supported by the observation that Irr directly binds to the bhuA promoter region in a specific manner in an EMSA (Fig. 6).

Considering the essential role that Irr plays in regulating the iron-responsive expression of bhuA in B. abortus 2308, an important question to address is how Irr activity is modulated in this strain. As noted previously, the stability of the Irr protein in Bradyrhizobium japonicum is controlled by cellular iron levels and Irr stability may also exist in B. abortus 2308. Indeed, the presence of Irr appears to be essential for the wild-type expression of bhuA, which encodes a major virulence determinant (17). Because the brucellae must carefully balance cellular iron levels to avoid the enhancement of endogenous oxidative stress arising from their respiratory metabolism and exogenous oxidative stress derived from the NADPH and inducible nitric oxide synthase (iNOS) activity of host macrophages (23), it will be important to obtain further insight into the nature of the Irr regulon in Brucella. In addition to Irr, Brucella strains also possess a homolog of the rhizobial iron regulator RirA, another important regulator of iron metabolism genes in the alphaproteobacteria (30), and preliminary studies indicate that RirA also plays an important role in controlling the expression of iron metabolism genes in B. abortus 2308, including bhuA (18). Thus, it will also be important to determine how Irr and RirA work together to regulate iron metabolism in this strain.

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REFERENCES

