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The AraC-Like Transcriptional Regulator DhbR Is Required for Maximum Expression of the 2,3-Dihydroxybenzoic Acid Biosynthesis Genes in *Brucella abortus* 2308 in Response to Iron Deprivation▽

Eric S. Anderson, James T. Paulley, and R. Martin Roop II*

Department of Microbiology and Immunology, East Carolina University School of Medicine, Greenville, North Carolina 27834

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Phenotypic evaluation of isogenic mutants derived from *Brucella abortus* 2308 indicates that the AlcR homolog DhbR (2,3-*dih*ydroxy*b*enzoic acid [2,3-DHBA] biosynthesis *r*egulator) modulates the expression of the genes involved in 2,3-DHBA production, employing 2,3-DHBA or brucebactin as a coinducer.

Brucella abortus is a gram-negative intracellular pathogen and the causative agent of brucellosis, a disease that produces abortion and infertility in its natural bovine host and which is known as undulant fever in humans (8). Like most prokaryotes, *Brucella* spp. require iron as an essential micronutrient (13, 35). Two siderophores have been identified in *B. abortus*, 2,3-dihydroxybenzoic acid (2,3-DHBA) (21) and the more complex 2,3-DHBA-based siderophore brucebactin (16). The production of both siderophores requires proteins encoded by the *dhbCEBA* operon (6, 16). Although studies indicate that neither siderophore is required for the maintenance of chronic spleen infection by *B. abortus* 2308 in the mouse model (5, 16, 25), a *dhbC* mutant derived from this strain exhibits profound attenuation in pregnant ruminants (6). These experimental findings indicate that the biosynthesis of 2,3-DHBA and/or brucebactin plays an important role in the virulence of this bacterium in the natural ruminant host.

While insufficient iron can restrict bacterial growth, excess intracellular iron can result in the formation of damaging hydroxyl radicals via Fenton chemistry and thus be toxic (28). For this reason, the uptake of iron into both prokaryotic and eukaryotic cells is tightly regulated to maintain an ideal balance between meeting the cells' physiological needs and avoiding excess (2, 10). Most bacteria rely on the *f*erric iron *u*ptake *r*egulator (Fur) or a transcriptional repressor with Fur-like properties to regulate siderophore production and uptake (9, 12).

In some bacteria, a second level of regulation of siderophore biosynthesis is mediated by AraC-like transcriptional activators that use the end product siderophore as a coactivator for induction of the siderophore biosynthesis genes as well as the genes required for siderophore uptake. Members of this regulator family include the AlcR, PchR, YbtA, and RhrA proteins of *Bordetella bronchiseptica*, *Pseudomonas aeruginosa*, *Yersinia pestis*, and *Sinorhizobium meliloti*, respectively (4, 14, 18, 22, 29). It has been postulated that these transcriptional regulators allow bacteria to fine-tune the expression of siderophore biosynthesis and transport genes in such a manner that maximum expression of these genes occurs only in environments where the corresponding ferrisiderophore complex can serve as an efficient iron source (7). The gene annotated as BAB2_1152 in the *B. abortus* 2308 genome sequence is predicted to encode a 259-amino-acid protein that exhibits significant amino acid sequence homology with AlcR (18.8% identity, 30.6% similarity), RhrA (17.1% identity, 28.6% similarity), PchR (14.8% identity, 31.6% similarity), and YbtA (12.5% identity, 23.7% similarity). Unlike the genes encoding these other AraC-like transcriptional activators, however, BAB2_1152 is found at a chromosomal location distal to the genes involved in 2,3-DHBA and brucebactin biosynthesis in *B. abortus* 2308 (BAB2_0011–BAB2_0016).

To determine if the product of BAB2_1152 plays a role in regulating siderophore production by *B. abortus* 2308, the corresponding genetic locus was disrupted in this strain by using a previously described strategy (11). Two separate regions of 685 and 929 bp flanking the BAB2_1152 open reading frame in the *B. abortus* 2308 genome were amplified by PCR (upstream forward primer, 5′-AACAACTCGGAAACGTTGGCAGGT TGCAGCTT-3′; upstream reverse primer, 5′-CCATCGATA ATATCATCAGCATCAGCTTCGGAAA-3′; downstream forward primer, 5′-CCATCGATCGTTCCGCAAGCTCTATGGA TTTTCA-3′; downstream reverse primer, 5′-CCGCATCGAC GTTCTTGCTTTTGATCTGT-3′). A ClaI restriction site was added to the 3′ end of the upstream 685-bp fragment and the 5′ end of the 929-bp downstream fragment as shown in Fig. 1. Each PCR product was individually ligated into pGEM-T Easy (Promega), resulting in the construction of plasmids pdhbR1 and pdhbR2, respectively. The upstream fragment was subsequently excised from pdhbR1 as an NcoI/ClaI fragment and ligated into ClaI/NcoI-digested pdhbR2, producing plasmid pdhbR1-2. Following the linearization of pdhbR1-2 with ClaI, the linearized plasmid was treated with the Klenow fragment of DNA polymerase I and ligated with the chloramphenicol acetyltransferase (*cat*) gene from pBlue-Cm2 (30). This plasmid was introduced into *B. abortus* 2308 by electroporation, and transformants were selected on Schaedler agar supple-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, East Carolina University School of Medicine, 600 Moye Boulevard, Greenville, NC 27834. Phone: (252) 744-1357. Fax: (252) 744-3535. E-mail: roopr@ecu.edu.

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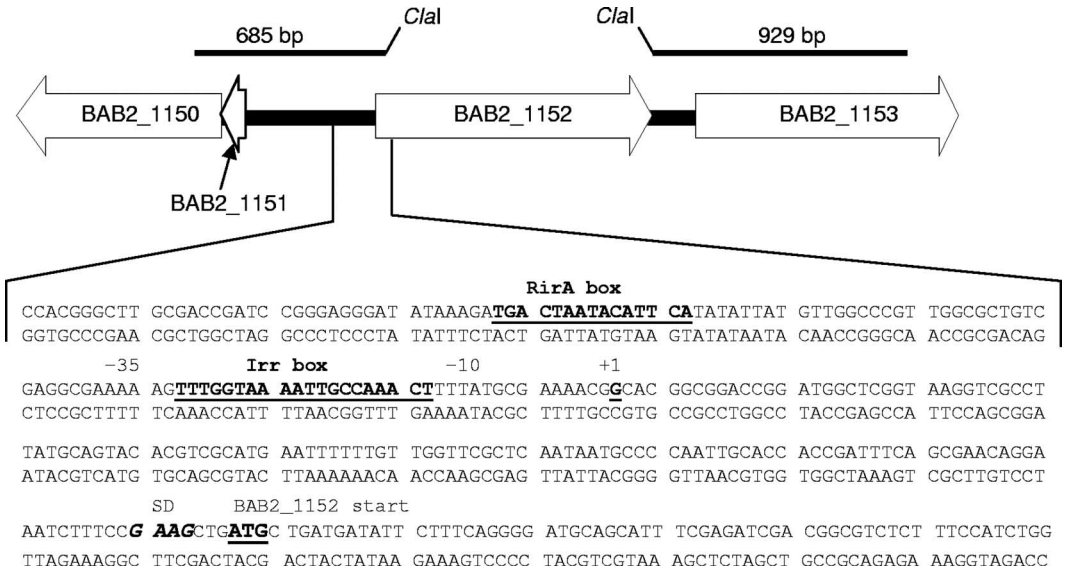

FIG. 1. Genetic organization of the BAB2_1150–BAB2_1153 locus in *B. abortus* 2308. BAB2_1150 encodes a TonB-dependent outer membrane protein (BhuA) that is involved in heme transport (26), BAB2_1151 is predicted to encode a hypothetical protein, and BAB2_1153 is predicted to encode a homolog of LivK, a periplasmic protein involved in branched-chain amino acid transport in *Escherichia coli* and other bacteria. The black bars above the locus represent the upstream and downstream regions flanking the *cat* gene in the mutated BAB2_1152 locus in *B. abortus* BEA6. The transcriptional start for the BAB2_1152 gene (*dhbR*) is noted by +1, the corresponding G is shown in boldface and is underlined, and the −10 and −35 regions relative to the transcriptional site are also shown. A putative ribosome binding site and the assigned ATG start codon for the *dhbR* coding region (BAB2_1152) are shown in boldface and italics and in boldface and underlined, respectively.

mented with 5% defibrinated bovine blood containing 5 μg/μl chloramphenicol. The genotype of the resulting *B. abortus* mutant designated BEA6 was confirmed by PCR analysis of genomic DNA from this strain using BAB2_1152-, *cat*-, and pGEM-specific primer sets. Crystal violet exclusion was used to verify that BEA6 retained its smooth lipopolysaccharide phenotype (1).

The *B. abortus* BAB2_1152 null mutant exhibited approximately 40% less siderophore production as measured by the catechol-specific Arnow assay (3) (Fig. 2A) or the liquid chrome azurol S assay (33) (which detects all classes of siderophores) (data not shown) than the parental 2308 strain when these strains were grown under iron-deprived conditions. The *B. abortus* BAB2_1152 null mutant also produced about half as much β-galactosidase activity from the *dhbC-lacZ* fusion in pP_{dhbC}::*lacZ* (6) as the parental strain when these two strains were cultivated under iron-deprived conditions (Fig. 2B), indicating that the reduced levels of siderophore production exhibited by the *B. abortus dhbR* mutant in response to iron deprivation are the result of decreased levels of *dhbCEBA* transcription.

To confirm the link between the BAB2_1152 null mutation in BEA6 and the reduced siderophore production and *dhbCEBA* expression in this mutant compared to that of the parent strain, a 1,285-bp region encompassing the region from bp 413 upstream to bp 73 downstream of the BAB2_1152 open reading frame was amplified from *B. abortus* 2308 genomic DNA by PCR (primer F, 5′-AGCGAACCGCTTTGCTTTAAATGCT-3′; primer R, 5′-AAAAACAGGTCTGCATCGTGATGGA-3′) by using *Pfu* polymerase (Stratagene) and ligated into SmaI-digested pMR10 (15), producing plasmid pEA17. Introduction of pEA17 into the *B. abortus* BAB2_1152 null mutant restored siderophore production levels by this strain in response to iron deprivation to the same levels as those produced by the parental strain (Fig. 2A). Plasmid incompatibility prevented the use of pEA17 in conjunction with the pP_{dhbC}::*lacZ* reporter plasmid as both are RK2 derivatives, so the same *dhbR*-containing fragment employed to create pEA17 was ligated into SmaI-digested pBBR1MCS4 (20). Introduction of the resulting plasmid, pEAD4, into the *dhbR* mutant containing the *dhbC-lacZ* transcriptional fusion restored β-galactosidase production by this strain in response to iron deprivation to levels equivalent to those displayed by the parental 2308 strain (Fig. 2B). These results demonstrate that the product of the gene designated BAB2_1152 is required for wild-type expression of the *dhbCEBA* operon in *B. abortus* 2308 in response to iron limitation. Based on this relationship, we have given this gene the designation DhbR (2,3-*DHB*A biosynthesis *r*egulator).

AlcR, PchR, YbtA, and RhrA require the end products of the siderophore biosynthesis genes they regulate as coinducers in order to function efficiently as transcriptional activators (7, 19, 22, 27). To determine if this same relationship holds true for the *Brucella* DhbR, the levels of *dhbC* expression in *B. abortus* 2308 and the *dhbR* mutant BEA6 in response to iron deprivation were compared to the levels of *dhbC* expression in response to iron limitation in *B. abortus* BHB2 (2308 *dhbC*), an isogenic mutant that cannot produce 2,3-DHBA or brucebactin (6). As shown in Fig. 2B, the *B. abortus dhbR* and *dhbC* mutants displayed levels of reduction in *dhbC* expression in response to iron limitation equivalent to those of the parental 2308 strain exposed to the same conditions, which suggests that DhbR requires 2,3-DHBA or brucebactin as a coinducer in

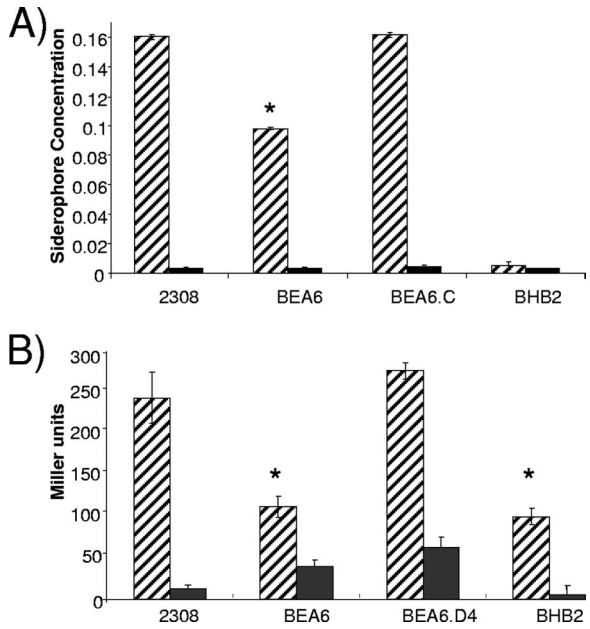

FIG. 2. DhbR is required for maximal siderophore production (A) and *dhbCEBA* transcription (B) by *B. abortus* 2308 in response to iron limitation. *B. abortus* strains 2308, BEA6 (2308 *dhbR*), BEA6.C [BEA6(pEA17)], and BHB2 (2308 *dhbC*) were grown in 100 ml low-iron minimal medium (hatched bars) or low-iron minimal medium supplemented with 50 μM $FeCl_3$ (black bars) in 500-ml flasks at 37°C with shaking. (A) Following 48 h of cultivation in these media, samples of the supernatants of these cultures were evaluated for the presence of siderophores using the Arnow assay (3). (B) β-Galactosidase production in Miller units (24) by derivatives of *B. abortus* 2308, BEA6, BEA6.D4 [BEA6(pEAD4)], and BHB2 carrying plasmid pP_{dhbC}::*lacZ* (6) following 48 h of growth under the same cultural conditions is also shown. The results presented are from single experiments that are representative of multiple (three or more) experiments performed for each analysis from which equivalent results were obtained. *, significance at a P of <0.05 for comparisons of BEA6 or BHB2 versus 2308, BEA6.C, or BEA6.D4 in a two-tailed Student t test.

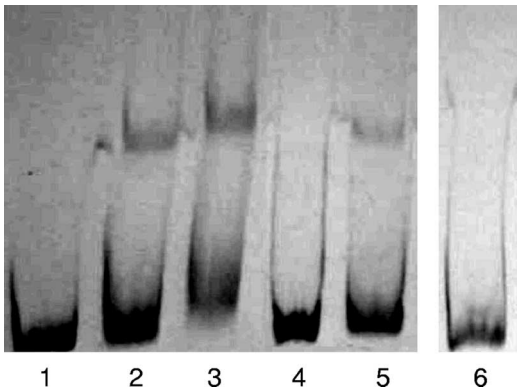

FIG. 3. DhbR binds to the *dhbCEBA* promoter region in *B. abortus* 2308 in an EMSA. Lane 1, 0.125 μg ^{33}P-labeled *dhbC* promoter-specific DNA fragment; lane 2, 0.125 μg ^{33}P-labeled *dhbC* promoter-specific DNA fragment plus 2 μg MBP-DhbR fusion protein; lane 3, 0.125 μg ^{33}P-labeled *dhbC* promoter-specific DNA fragment plus 10 μg MBP-DhbR fusion protein; lane 4, 0.125 μg ^{33}P-labeled *dhbC*-promoter-specific DNA fragment plus 2 μg MBP-DhbR fusion protein plus 6.25 μg unlabeled *dhbC* promoter-specific DNA fragment; lane 5, 0.125 μg ^{33}P-labeled *dhbC* promoter-specific DNA fragment plus 2 μg MBP-DhbR fusion protein plus 6.25 μg unlabeled nonspecific competitor DNA; lane 6, 0.125 μg ^{33}P-labeled *dhbC* promoter-specific DNA fragment plus 2 μg unfused MBP. The results presented are from a single experiment that is representative of multiple (three or more) experiments performed from which equivalent results were obtained.

order to serve as an efficient transcriptional activator for the *dhbCEBA* operon in *B. abortus* 2308.

To determine if DhbR binds directly to the *dhbC* promoter in *B. abortus* 2308, a DhbR–maltose-binding protein (MBP) fusion protein was generated, and the interactions of the fusion protein with a DNA fragment corresponding to the *dhbC* promoter region were examined in an electrophoretic mobility shift assay (EMSA). The *dhbR* coding region was amplified from *B. abortus* 2308 genomic DNA by PCR (primer Fwd, 5′-ATGCTGATGATATTCTTTCA-3′; primer Rev, 5′-ACCG GAACCGCTCCGGCAGAGGATCC-3′) using high-fidelity *Pfu* polymerase (Stratagene) and cloned into XmnI/BamHI-digested pMal-c2X. The resulting plasmid encodes a fusion protein comprised of the first 411 amino acids of MalE fused to the N terminus of DhbR. Confirmation of the *malE-dhbR* fusion in this plasmid was accomplished by PCR amplification of the *dhbR* gene using plasmid DNA as a template. Purification of the MalE-DhbR fusion peptide was performed on an amylose resin column following the manufacturer's instructions. For the EMSAs, a 298-bp DNA fragment encompassing the *dhb* promoter region (6) was amplified from *B. abortus* 2308 genomic DNA (forward primer, 5′-CGGCATCCAGCC ATCTGCAAAA-3′; reverse primer, 5′-ATCACCTCACGCA TGCCGCT-3′) and radiolabeled with [^{33}P]dATP using the following procedures. Briefly, 21 μl of purified PCR fragment (100 ng/μl) was added to 3 μl of *Taq* polymerase 10× reaction buffer, 10 μCi [^{33}P]dATP, 10 units of *Taq* DNA polymerase, and 3 μl of double-distilled water. This mixture was incubated at 70°C for 30 min, unincorporated radiolabeled nucleotide was removed from the fragment using the QIAquick nucleotide removal kit (Qiagen), and the fragment was suspended in 30 μl double-distilled water. Binding of the MalE-DhbR fusion protein to the radiolabeled DNA fragment was measured by mixing 2, 5, or 10 μg purified MBP-DhbR protein with 0.125 μg radiolabeled *dhbR* promoter fragment, 2 μg sheared herring sperm DNA, 10 μg bovine serum albumin, and 12 μl of 5× binding buffer (20 mM Tris-HCl [pH 8.0], 200 mM NaCl, 20 mM $MgCl_2$, 20% glycerol). This mixture was incubated for 20 min at room temperature and then loaded onto an 8% nondenaturing acrylamide gel and subjected to electrophoresis at 200 V. The contents of the gel were transferred to a piece of Whatman 1 M filter paper, dried, and visualized by autoradiography. To evaluate the specificity of the interactions between the MalE-DhbR fusion protein and the radiolabeled DNA fragment, an unlabeled version of this fragment was used as a specific inhibitor and a 278-bp SacI/SapI fragment from pBlueCM2 was used as a nonspecific inhibitor.

Numerous attempts to cleave DhbR from the MBP resulted in denaturation of the DhbR protein, which led to its precipitation out of solution. Because of this, the fusion protein was evaluated for binding in its uncleaved form in the EMSA. Increasing concentrations of the DhbR-MBP fusion protein retarded the migration of the labeled *dhb* promoter fragment into the gel (Fig. 3, lanes 2 and 3) in this assay, and this

mobility shift was abolished by the addition of a 50-fold excess of unlabeled *dhbC* promoter DNA (Fig. 3, lane 4) but not by the addition of a 50-fold excess of nonspecific competitor DNA (Fig. 3, lane 5). In contrast, no evidence for specific interactions between the unfused MBP and the DNA fragment corresponding to the *dhbC* promoter region was obtained in this assay (Fig. 3, lane 6). These results demonstrate a direct and specific interaction between the DhbR component of the DhbR-MBP fusion protein and the *dhbC* promoter region of *B. abortus* 2308, which is consistent with the proposed function of DhbR as a transcriptional activator. Preliminary studies indicate that the presence of 2,3-DHBA does not alter the binding affinity of DhbR for the *dhbC* promoter region in the EMSA (data not shown). This suggests that brucebactin may serve as the coactivator for DhbR, but the instability of this siderophore (16) has to date prevented a definitive evaluation of the interactions between brucebactin and DhbR in the EMSA.

Like the siderophore biosynthesis genes that they regulate, the genes encoding PchR, YbtA, and AlcR are tightly repressed in their respective bacteria when intracellular iron reaches a threshold level (7, 23, 27). To determine whether or not *dhbR* exhibits iron-responsive expression in *B. abortus* 2308, total RNA was isolated from cultures of this strain grown in low-iron minimal medium and low-iron minimal medium supplemented with 50 μM $FeCl_3$, and real-time reverse transcriptase PCR analysis was used to compare the relative levels of *dhbR* transcripts in these RNA preparations by using the procedures described by Paulley et al. (26). A 26-fold-greater level of *dhbR* transcripts was detected in the RNA preparations obtained from *B. abortus* 2308 cultures grown under iron-deprived conditions than was detected in the RNA preparations obtained from cultures grown under iron-replete conditions (data not shown), indicating that *dhbR* expression is iron repressible. Primer extension analysis was performed on RNA preparations obtained from iron-deprived cultures by employing the methods described by Robertson et al. (30), and a G residue 130 nucleotides upstream of the predicted ATG start codon for this gene was identified as the transcriptional start site for *dhbR* (Fig. 1). Putative binding sites for RirA (34) and Irr (17), two iron-responsive regulators that play important roles in controlling the expression of iron metabolism genes in other alphaproteobacteria (31), are located upstream of the transcriptional start site for *dhbR* in *B. abortus* 2308 (Fig. 1). Genome sequence analysis indicates that both of these regulators are present in *B. abortus* 2308 (32), but whether or not RirA or Irr plays a role in the iron-responsive nature of *dhbR* expression in this strain has yet to be determined experimentally.

The studies presented in this report demonstrate that DhbR is required for maximal expression of the 2,3-DHBA biosynthetic genes in *B. abortus* 2308 in response to iron limitation and suggest that 2,3-DHBA and/or brucebactin serves as a coinducer(s) for this transcriptional activator. Based on the regulatory activity of DhbR homologs in other bacteria, it will also be important to determine if DhbR regulates the expression of the genes involved in the conversion of 2,3-DHBA to brucebactin (6, 16) and/or the transport systems for these siderophores in *Brucella* spp. (32). The genomic location of the *dhbR* gene in *B. abortus* 2308, *B. abortus* 9-941, *Brucella melitensis* 16 M, and *Brucella suis* 1330 is unique compared to that of its counterparts in *Bordetella*, *Yersinia*, *Pseudomonas*, and *Sinorhizobium* spp. in that *dhbR* is not located contiguous with genes involved in siderophore biosynthesis or transport. In fact, in all four of the *Brucella* strains for which the genome sequence is available, *dhbR* resides next to a gene that has recently been linked to the utilization of heme as an iron source (26). Consequently, in order for us to gain a clearer picture of the role of DhbR in iron metabolism in *Brucella* spp., it will be important to determine if this regulator controls the expression of genes other than those responsible for siderophore biosynthesis and transport. An evaluation of the virulence properties of *Brucella dhbR* mutants will also tell us whether DhbR-mediated regulation of siderophore biosynthesis and transport and possibly other iron metabolism genes are important for the prioritization of iron source utilization by these bacteria in their mammalian hosts.

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