

# A MarR-Type Regulator Directly Activates Transcription from the *Brucella abortus* *virB* Promoter by Sharing a Redundant Role with HutC

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Type IV secretion systems (T4SS) are multiprotein structures that direct the translocation of specific molecules across the bacterial cell envelope. As in other bacteria, pathogenicity of the genus *Brucella* essentially depends on the integrity of the T4SS-encoding *virB* operon, whose expression is regulated by multiple transcription factors belonging to different families. Previously, we identified IHF and HutC, two direct regulators of the *virB* genes that were isolated from total protein extracts of *Brucella*. Here, we report the identification of MdrA, a third regulatory element that was isolated using the same screening procedure. This transcription factor, which belongs to the MarR-family of transcriptional regulators, binds at two different sites of the *virB* promoter and regulates expression in a growth phase-dependent manner. Like other members of the MarR family, specific ligands were able to dissociate MdrA from DNA *in vitro*. Determination of the MdrA-binding sites by DNase I footprinting and analyses of protein-DNA complexes by electrophoresis mobility shift assays (EMSAs) showed that MdrA competes with IHF and HutC for the binding to the promoter because their target DNA sequences overlap. Unlike IHF, both MdrA and HutC bound to the promoter without inducing bending of DNA. Moreover, the two latter transcription factors activated *virB* expression to similar extents, and in doing so, they are functionally redundant. Taken together, our results show that MdrA is a regulatory element that directly modulates the activity of the *virB* promoter and is probably involved in coordinating gene expression in response to specific environmental signals.

Type IV secretion systems (T4SSs) are multiprotein structures that direct the translocation of specific molecules across the bacterial cell envelope (3). During evolution, these machineries have specialized to secrete different kinds of substrates, which led to the emergence of the different biological functions that T4SSs can exert. Both the *Escherichia coli* Tra and the *Agrobacterium tumefaciens* VirB T4SSs are responsible for secreting protein-DNA complexes during plasmid conjugation and oncogenic transfer DNA (T-DNA) translocation, respectively (48, 54). Instead, other T4SSs, such as the *Helicobacter pylori* Cag system, the *Legionella pneumophila* Dot/Icm system, or the *Bordetella pertussis* Ptl system, have evolved to deliver protein substrates toward the eukaryotic host, where they subvert cellular processes and favor the development of the bacterial infection (8, 44). As in all these pathogenic bacteria, virulence of *Brucella* is also fully dependent on the integrity of a T4SS, since deletion of one or more T4SS-encoding *virB* genes completely abrogates the ability of this genus of facultative intracellular bacteria to colonize its eukaryotic host (30, 47).

*Brucella* comprises many species, which are the causative agents of a worldwide-distributed zoonotic disease known as brucellosis. The members of *Brucella* differ in their host preference, affecting mammals that range from rodents to cetaceans (52). *Brucella abortus*, the causal agent of bovine brucellosis, is one of the species that can be transmitted from infected animals to humans through inhalation, by contact with skin wounds or mucosa, or by consumption of contaminated dairy products. In the natural host, brucellosis produces sterility in males and abortion in pregnant females, whereas in humans it causes debilitating symptoms and complications that can lead to death (16).

The ability of *Brucella* to infect and persist within the mammalian host relies on different mechanisms that allow this bacterium to overcome the innate and adaptive immune host responses. Among all these strategies, bacterial survival and intracellular replication into the host professional phagocytes are key processes underlying brucellosis. After entering the host macrophages, the bacterium is located in the lumen of the so-called *Brucella*-containing vacuole (BCV) (32). During the first stages of the intracellular infection, this compartment undergoes sustained interactions with endoplasmic reticulum (ER) membranes. This process, which depends essentially on the activity of the VirB system, is necessary for the biogenesis of an ER-derived bacterial replication compartment, since abrogation of the early BCV-ER interactions prevents *Brucella* from multiplying intracellularly and leads to bacterial degradation in lysosomes (7). Recently, screening procedures based on different methods led to the identification of proteins that are translocated into the host cell cytoplasm in a VirB-dependent manner (10, 22). Until now, the function of these substrates in the establishment of the host cell infection has not

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This work is dedicated to the memory of Rodolfo A. Ugalde.

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been described except for RicA, a protein that interacts with and recruits the small GTPase Rab2 into BCVs during the intracellular trafficking of *Brucella* (9).

The first reported evidence of intracellular regulation of the VirB T4SS showed that expression of the *virB* genes is induced after internalization of the bacterium in macrophage cell lines (4). Such induction is dependent on the acidification of the BCV and reaches the maximal level of expression at 5 h postinfection. Subsequently, expression of the *virB* operon is rapidly turned off, which suggests that the system is controlled by regulatory mechanisms that respond to specific signals sensed by the bacterium within the host cell (46).

The main regulatory protein controlling expression of the *Brucella virB* genes is VjbR, a quorum sensing (QS)-related regulator that binds the *virB* promoter ( $P_{virB}$ ) at position -94 relative to the transcription start site (2, 12). In addition, many other regulators were shown to directly or indirectly influence *virB* expression, including the stringent response mediator Rsh and transcription factors belonging to different families (13, 18, 37). In addition, although their binding sites in the *virB* promoter have not yet been reported, it was recently shown that both the two-component regulator BvrR and the second QS-related transcription factor BabR/BlxR participate in regulation of *virB* expression by directly interacting with  $P_{virB}$ , as demonstrated by pulldown experiments and electrophoresis mobility shift assays (EMSAs), respectively (6, 25).

In previous works from our group, we identified two transcriptional regulators that directly contribute to induction of *virB* expression: (i) IHF, a nucleoid-associated protein that binds to  $P_{virB}$  at position -162.5 and induces a DNA bend that is necessary for the promoter activation (46); and (ii) HutC, a GntR family regulator that binds at position -188 and links *virB* expression to histidine catabolism (45). Here, we report the identification of MdrA, a third regulatory element that was isolated using the same screening procedure. Our experiments showed that this transcription factor binds at two different sites of  $P_{virB}$  and regulates *virB* expression in a growth phase-dependent manner. Analysis of promoter activities under different mutant backgrounds revealed that MdrA exerts its regulatory role in redundancy with the function of HutC. Besides, consistently with both the architecture and localization of the MdrA-binding sites within the promoter region, we also observed that this transcription factor competes with IHF and HutC for the binding to  $P_{virB}$ . As is usually the case with MarR family members, our experiments showed that the presence of a specific ligand dissociates MdrA from a target DNA binding site. Such ligand-induced disruption of promoter binding indicates that this process may allow MdrA to receive and respond to environmental signals.

## MATERIALS AND METHODS

**Media and culture conditions.** *Brucella* strains were grown in tryptic soy broth (TSB) or in Gerhardt-Wilson minimal medium (15) or MM1 (45) at pH 4.5, 5.5, or 7.0. Bacteria were cultured at 37°C in a rotary shaker (250 rpm). Media were supplemented with kanamycin (50  $\mu\text{g ml}^{-1}$ ) and/or nalidixic acid (5  $\mu\text{g ml}^{-1}$ ) as needed. Sodium deoxycholate (DOC) (Sigma) was eventually added to the media at a final concentration of 100, 250, 500, 750, or 1,000  $\mu\text{M}$ . *Escherichia coli* strains were cultured in Luria-Bertani (LB) broth at 37°C in a rotary shaker at 250 rpm. Media were supplemented with ampicillin (100  $\mu\text{g ml}^{-1}$ ) or kanamycin (25  $\mu\text{g ml}^{-1}$ ) as needed.

**Isolation and identification of MdrA.** The protein detected by EMSA was isolated from exponential-phase cultures (optical density at 600 nm [ $\text{OD}_{600}$ ],  $\sim 0.5$ ) of the avirulent *Brucella abortus*  $\Delta\text{pgm}$  strain (51). Bacteria were harvested and disrupted as described previously (46). After ultracentrifugation and filtration, the total protein extract was fractionated by ammonium sulfate precipitation. The different fractions were analyzed by EMSA using probe vir-up or the control probe B10. The positive fraction (0 to 45% ammonium sulfate saturation) was suspended in 35 mM phosphate buffer (pH 6.8) containing 3 mM  $\beta$ -mercaptoethanol and dialyzed against the same buffer overnight at 4°C. The solution was loaded onto a Mono-S column and eluted with a linear gradient of 35 mM phosphate buffer (pH 6.8), 3 mM  $\beta$ -mercaptoethanol, and 1 M NaCl. The DNA-binding activity of the fractions was again analyzed by EMSA using the probes described above. Positive fractions were subjected to affinity chromatography: the biotinylated probe biot-vir-up, which corresponds to the -430 to -202 region of  $P_{virB}$ , was constructed by PCR using *Taq*, the 5'-biotinylated primer pvd228, the primer pirup, and genomic DNA of *B. abortus* 2308 as the template. The biotinylated control probe was constructed as described previously (45). The biotinylated probes were bound to streptavidin paramagnetic spheres (Promega), and a binding reaction was performed using binding buffer and the positive fractions of the Mono-S column. After two washes with binding buffer with 0.2 M NaCl, the DNA-bound proteins were eluted with 0.85 M NaCl and analyzed by 12.5% SDS-PAGE. The gel was silver stained with a mass spectrometry-compatible method for visualizing the protein bands. A band that was observed with the probe biot-vir-up but was absent in samples from the control probe was excised from the gel and analyzed by mass spectrometry by Vital Probes Inc. (Mayfield, PA).

**Construction of plasmids.** For construction of pK18mob-*sacB*- $\Delta\text{mdrA}$ , two PCRs were carried out using *Pfx* (Invitrogen), genomic DNA of *B. abortus* 2308 as the template, and primers SpeMarA (5'-GGACTAGTTGACGATA TGTTCGCCGCAT-3') and COMMarA (5'-CTGCAAGAAGGAGAGTA TCGAATGCAAGCGCTCGACAAGC-3') or SpeMarB (5'-GGACTAGT AACCGCTCTTGCGGCAATC-3') and COMMarB (5'-TCGATACT CTCCTTCTGCAGTTTATGGCGATGAACAAGTCG-3'). Both PCR products, corresponding to two 400-bp flanking regions of *mdrA*, were annealed and used as the templates for a PCR performed with primers SpeMarA and SpeMarB. The product was digested with SpeI and cloned into the kanamycin-resistant ( $\text{Kan}^r$ ) plasmid pK18mob-*sacB* (42).

For construction of the complementation plasmid pK18mob-*sacB*-Knock-in-*mdrA*, a PCR was carried out using *Pfx*, genomic DNA of *B. abortus* 2308 as the template, and primers SpeMarA (5'-GGACTAGTTG ACGATATGTTTCGCCGCAT-3') and SpeMarB (5'-GGACTAGTAACC GCTCTTGCGGCAATC-3'). The PCR product, which contained the wild-type sequence of the *mdrA* gene and two 400-bp flanking regions, was digested with SpeI and cloned into the kanamycin-resistant plasmid pK18mob-*sacB*.

For construction of the expression vector pQE-31-*mdrA*, a PCR was performed using *Pfx*, genomic DNA of *B. abortus* 2308 as the template, and primers BamMarII (5'-CGGGATCCGACCAATACCCAGCGCAAG AT-3') and HindMar (5'-GGTACCTCAGCCGCGAGATGGCGT-3'). The PCR products were digested with BamHI and HindIII and cloned into plasmid pQE-31 (Qiagen).

**Construction of *B. abortus*  $\Delta\text{mdrA}$  strain.** Plasmid pK18mob-*sacB*- $\Delta\text{mdrA}$  was transferred to *B. abortus* 2308 by biparental conjugation.  $\text{Kan}^r$  colonies were selected as single-homologous recombinants. Selection with sucrose, excision of plasmids, and generation of deletion mutants were performed as described previously (46). PCR analyses of kanamycin-susceptible ( $\text{Kan}^s$ ) colonies were carried out with primers SpeMarA and SpeMarB to identify clones that contained the deletion of *mdrA*.

**Construction of the double mutant strain *B. abortus*  $\Delta\text{hutC}$   $\Delta\text{mdrA}$ .** Plasmid pK18mob-*sacB*- $\Delta\text{mdrA}$  was transferred to *B. abortus*  $\Delta\text{hutC}$  (45) by biparental conjugation.  $\text{Kan}^r$  colonies were selected as single-homologous recombinants. After selection with sucrose and excision of the plas-

mid, a PCR analysis of Kan<sup>r</sup> colonies was carried out with primers SpeMarA and SpeMarB to identify clones that contained the deletion of *mdrA* in the  $\Delta$ *hutC* mutant background.

**Construction of the knock-in complemented strain *B. abortus*  $\Delta$ *hutC*  $\Delta$ *mdrA*-KI.** Plasmid pK18mob-*sacB*-Knock-in-*mdrA* was transferred to the *B. abortus*  $\Delta$ *hutC*  $\Delta$ *mdrA* strain by biparental conjugation. Kan<sup>r</sup> colonies were selected as single-homologous recombinants. After selection with sucrose and excision of the plasmid, a PCR analysis of Kan<sup>r</sup> colonies was carried out with primers SpeMarA and SpeMarB to identify clones that incorporated the wild-type *mdrA* gene.

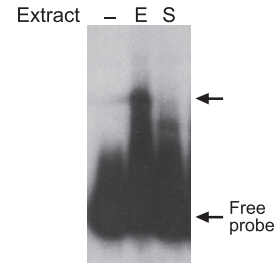
**Construction of strains containing a single P<sub>*virB*</sub>-*lacZ* chromosomal transcriptional fusion.** Plasmid pK18mob-P<sub>*virB*</sub>-*lacZ* was transferred by biparental conjugation into the wild-type strain *B. abortus* 2308 or into *B. abortus*  $\Delta$ *mdrA*, *B. abortus*  $\Delta$ *hutC*, *B. abortus*  $\Delta$ *hutC*  $\Delta$ *mdrA*, or *B. abortus*  $\Delta$ *hutC*  $\Delta$ *mdrA*-KI. Kan<sup>r</sup> colonies were selected as single-homologous recombinants.

**Electrophoresis mobility shift assays.** <sup>32</sup>P labeling of probes, incubations, and running conditions were as described previously (46). The radiolabeled probe *vir-up* was constructed using *Taq* (Invitrogen), primers *pvirup* (5'-ATGACAGGCATATTTCAAC-3') and *pvd228* (5'-GTGATTTCATATTTTTGCGG-3'), and genomic DNA of *B. abortus* 2308 as the template. The radiolabeled probes *vir-down* and *vir-down-ihf*<sup>-</sup> were constructed by PCR using primers *pvd229* and *pvirdownI*, and genomic DNA of *B. abortus* 2308 or plasmid pBluescript-P<sub>*virB*</sub>-*IHF*-*sacB*/R as the template, respectively, as described previously (46). The radiolabeled control probe B10 was constructed with primers B10Qu and B10d2, as described previously (45). The radiolabeled probe 286/59 was constructed using primers *pvu144* (5'-CGGGATCCGATTCTTGGTCGGGTTAC-3') and *pvir-downIII* (5'-TGTTTAAGCCGATATATGGAC-3') and genomic DNA of *B. abortus* 2308 as the template.

The unlabeled competitors were constructed by PCR using *Taq*, genomic DNA of *B. abortus* 2308 as the template, and primers *pvirup* and *pvd228* for competitor up, primers *pvu77* (5'-CGGGATCCGATGCCGCCTAATGGAGC-3') and *pvd228* for competitor 77, primers *pvu144* and *pvd228* for competitor 144, and primers *pvu229* and *pvir-downI* for competitor down. The unlabeled competitors A1 and B1 were constructed as described previously (46). Binding reactions were performed in binding buffer (15 mM Tris-HCl [pH 8.0], 0.5 mM EDTA, 10  $\mu$ g ml<sup>-1</sup> bovine serum albumin [BSA], 1 mM dithiothreitol [DTT], 30 mM KCl, 6% glycerol, 50  $\mu$ g ml<sup>-1</sup> salmon sperm DNA).

**DNAse I footprinting.** Labeling reactions, purification of probes, binding reactions, DNase I digestion, purification of digested fragments, and electrophoresis conditions were performed as described previously (46). Probe *vir-upII* was constructed using *Taq*, primer *pvd228*, the <sup>32</sup>P-labeled primer *pvirup*, and genomic DNA of *B. abortus* 2308 as the template. Probe *vir-downII* was constructed using primer *pvu144*, the <sup>32</sup>P-labeled primer *pvir-downI*, and genomic DNA of *B. abortus* 2308 as the template. Probe *vir-upIII* was constructed using primer *pvirup*, the <sup>32</sup>P-labeled primer *pvir-downIII*, and genomic DNA of *B. abortus* 2308 as the template.

**Expression and purification of recombinant proteins.** Recombinant IHF and HutC were prepared as described previously (45, 46). Recombinant MdrA was prepared as follows. Plasmid pQE-31-*mdrA* was transferred into *E. coli* M15 (pREP4) (Qiagen). Transformed bacteria were cultured in LB until the OD<sub>600</sub> reached 0.6 and were induced with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). After 4 h, bacteria were harvested, suspended in lysis buffer (20 mM Tris-HCl [pH 7.6], 1 mM phenylmethylsulfonyl fluoride [PMSF]), and disrupted by sonication. After centrifugation, NaCl was added to a final concentration of 0.5 M, and the sample was loaded into a Hi-Trap nickel-chelating column (Amersham Biosciences). After washing with buffer B (20 mM Tris-HCl [pH 7.6], 0.5 M NaCl, 50 mM imidazole), the column was equilibrated with buffer A (20 mM Tris-HCl [pH 7.6], 0.5 M NaCl) and eluted with a linear gradient of buffer C (20 mM Tris-HCl [pH 7.6], 0.5 M NaCl, 1 M imidazole). Eluates were analyzed by 12.5% SDS-PAGE, and the fractions con-



**FIG 1** Identification of MdrA. EMSA performed with probe *vir-up* and total crude protein extracts from exponential (E) or stationary-phase (S) cultures of *B. abortus*. Arrows indicate the positions of the free probe and of a protein-DNA complex.

taining the recombinant protein MdrA (purity, ~95%) were pooled and dialyzed against buffer D (20 mM Tris-HCl [pH 7.6], 0.5 M NaCl, 3 mM  $\beta$ -mercaptoethanol).

**$\beta$ -Gal activity determinations.**  $\beta$ -Galactosidase ( $\beta$ -Gal) activity was determined in whole bacterial cells as described previously (46), and it was expressed in Miller units by the following formula: [ $A_{420}$ /(volume  $\times$  OD<sub>600</sub>)]  $\times$  100.

## RESULTS

**Isolation and identification of MdrA.** In previous studies, two transcription factors implicated in the control of expression of the *virB* operon were isolated by affinity chromatography from *B. abortus* total protein extracts (45, 46). Such analyses were performed by using DNA probes that contained sequences corresponding to positions -201 to +24 relative to the transcription start site of the *virB* promoter (P<sub>*virB*</sub>). In order to identify possible additional regulatory proteins interacting with further upstream regions, we performed EMSAs using crude protein extracts and a radioactively labeled probe corresponding to positions -430 to -202 of P<sub>*virB*</sub>. As shown in Fig. 1, a protein-DNA complex was detected using total protein extracts of *B. abortus* grown in rich medium (TSB) until exponential phase, whereas no signal was observed with stationary-phase extracts. To isolate the factor responsible for the observed complex, proteins were fractionated by ammonium sulfate precipitation and ion-exchange chromatography, followed by EMSA of each fraction during the purification process (see Materials and Methods). Subsequently, the partially purified protein fractions were subjected to affinity chromatography using a biotin-labeled probe, *vir-up*, or a nonrelated control probe bound to streptavidin-coated paramagnetic spheres, and the corresponding eluates were analyzed by SDS-PAGE. Using this procedure, we observed a specific band, which was excised from the gel and identified by mass spectrometry as YP\_414216, a protein belonging to the MarR family of transcriptional regulators (14). Because of its biochemical properties and its role on regulation of *virB* expression (see below), we called this protein MdrA (for MarR-like sodium deoxycholate-responsive activator). As revealed by BLAST analyses, MdrA shares 29% identity and 44% similarity with the *Escherichia coli* prototypical member MarR (24), whereas it displayed slightly higher homology with other representative MarR members such as PecS, SlyA, and RovA (identity and similarity, 33% and 54%, 30% and 49%, and 31% and 50%, respectively) (21, 27, 34). As shown in Fig. 2, a multiple sequence alignment indicated that MdrA displays a high degree of amino acid conservation at positions corresponding to the winged helix-turn-helix DNA-binding domain ( $\beta$ 1- $\alpha$ 3- $\alpha$ 4[recognition



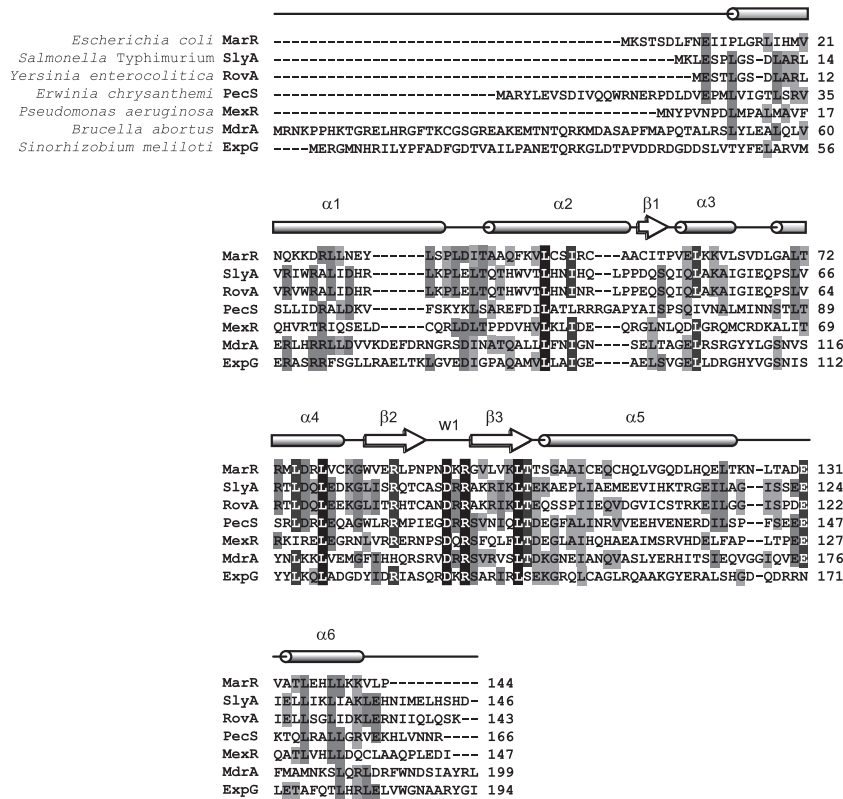


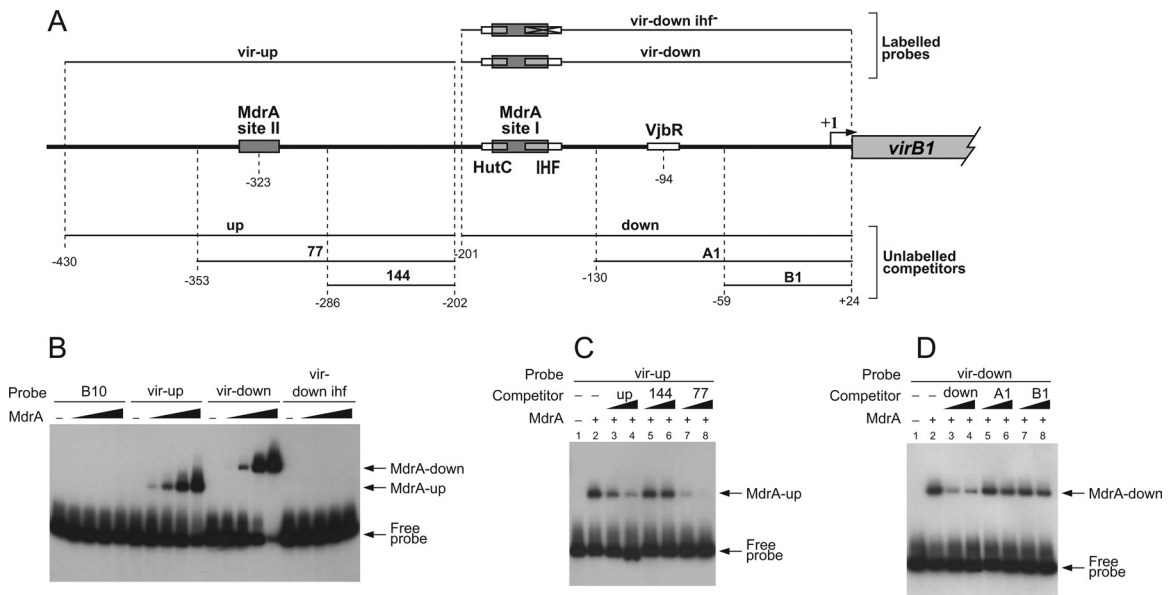
FIG 2 Sequence alignment of MdrA and different representative members of the MarR family of transcriptional regulators. Relative positions of secondary-structure elements are shown as a schematic representation of  $\alpha$ -helices,  $\beta$ -sheets, and the wing region (W1), according to the *Escherichia coli* MarR crystal structure described by Alekshun et al. (1). White letters identify residues identical in all (highlighted in black) or in six (highlighted in dark gray) of the seven MarR homologs considered. Black letters identify residues identical in five or four (highlighted in gray) or in three (highlighted in light gray) of the seven MarR homologs. The sequence alignment was performed using ClustalW (49).

helix]- $\beta$ 2-W1[wing]- $\beta$ 3), which is characteristic of this family of transcriptional regulators (1).

**MdrA interacts with  $P_{virB}$  at two different binding sites.** To study the MdrA- $P_{virB}$  interaction, a His-tagged recombinant protein was expressed and assayed by EMSA using different radioactively labeled probes (Fig. 3A). As shown in Fig. 3B, recombinant MdrA interacted with probe vir-up, showing an electrophoretic mobility similar to that of the native nucleoprotein complex, whereas no signal was observed with the control probe B10. Incubation of MdrA with probe vir-down also resulted in the formation of a protein-DNA complex, which indicated that this regulator specifically recognizes two separated regions of the promoter (Fig. 3B). On the other hand, incubation of MdrA with a probe that lacks the IHF-binding site produced no signal by EMSA (Fig. 3B), suggesting that the MdrA-binding site overlaps that of IHF. Competition experiments performed in the presence of an excess of different unlabeled fragments indicated that the sequences recognized by MdrA in  $P_{virB}$  are located at two regions residing between positions -353 and -286 and between -201 and -130 (Fig. 3C and D). Here, we will refer to the downstream- and upstream-located elements recognized by this protein in  $P_{virB}$  as the MdrA-binding sites I and II, respectively.

**Analysis of the interaction of MdrA, IHF, and HutC with the downstream region of  $P_{virB}$ .** The above-described experiments showed that MdrA specifically binds to a 71-bp region of the promoter that also contains the binding sites for IHF and HutC. In

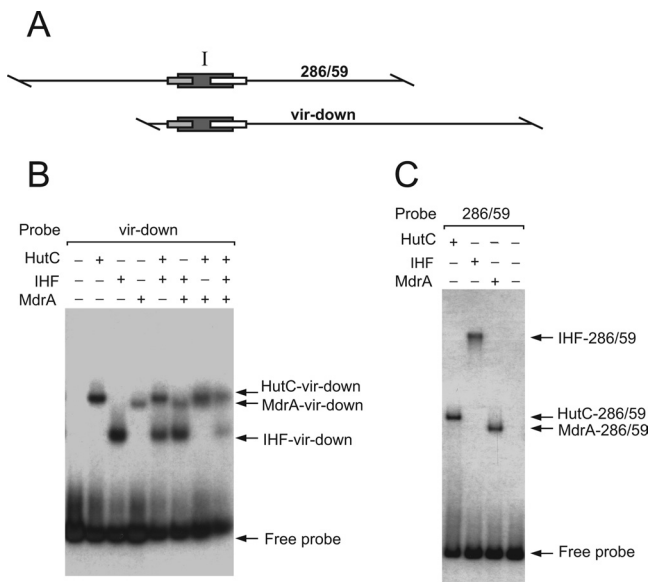
previous works, we found that IHF and HutC compete with one another during binding to  $P_{virB}$  because their binding sites overlap (45, 46). Accordingly, we next asked if binding of MdrA to  $P_{virB}$  affects the interaction of IHF and HutC with the promoter. To address this question, we incubated probe vir-down with each of the three pairs of proteins or with all three proteins together. We then carried out EMSAs on the different protein combinations. Figure 4B shows that, separately, each of the regulators generated a signal corresponding to a binary protein-DNA complex, whereas no ternary complexes were observed with any of the combinations of two or more proteins. These results indicated that MdrA competes with IHF and HutC for the binding to the probe vir-down, suggesting that the MdrA-binding site I overlaps those of the two other regulators. The same results were obtained when EMSAs were carried out with probe 289/59, whose length is the same as that of probe vir-down but in which the location of the binding sites for MdrA, HutC, and IHF are close to the center rather than near one end (data not shown). However, the electrophoretic mobility of the complex between IHF and probe 289/52 was substantially lower than that of the complex between IHF and probe vir-down (Fig. 4B and C). The reduced mobility of the IHF-289/52 complex is due to the IHF-induced bend of DNA, and the effect of bending on migration is more pronounced when the binding site is located near the center of the molecule (38). On the other hand, unlike IHF, the relative mobility of the MdrA- and HutC-nucleoprotein complexes remained constant regardless



**FIG 3** Analysis of the interaction between MdrA and the *virB* promoter. (A) Schematic representation of genomic sequences corresponding to the regulatory region of the *virB* operon, radiolabeled probes used for EMSA, or unlabeled competitors. Positions relative to the transcription start site are indicated. MdrA-, HutC-, IHF-, and VjbR-binding sites are indicated as boxes. The diagonally crossed box indicates the nonrelated sequences that replace the IHF-binding site in probe *vir-down-ihf*. (B) EMSA performed with the indicated probes and 0, 10, 20, 40, or 60 nM MdrA. (C) EMSA performed with probe *vir-up*, MdrA, and the indicated unlabeled DNA fragments as competitors. Protein concentrations were as follows: line 1, no protein; lines 2 to 8, 30 nM. Concentrations of unlabeled DNA competitors were as follows: lanes 1 and 2, no competitor DNA; lanes 3, 5, and 7, 150 ng; lanes 4, 6, and 8, 360 ng. (D) EMSA performed with probe *vir-down*, MdrA, and the indicated unlabeled DNA fragments as competitors. The concentrations of protein and unlabeled DNA competitors were the same as for panel C.

of the probe used, indicating that the last two transcriptional regulators do not induce bending of DNA upon binding (Fig. 4B and C).

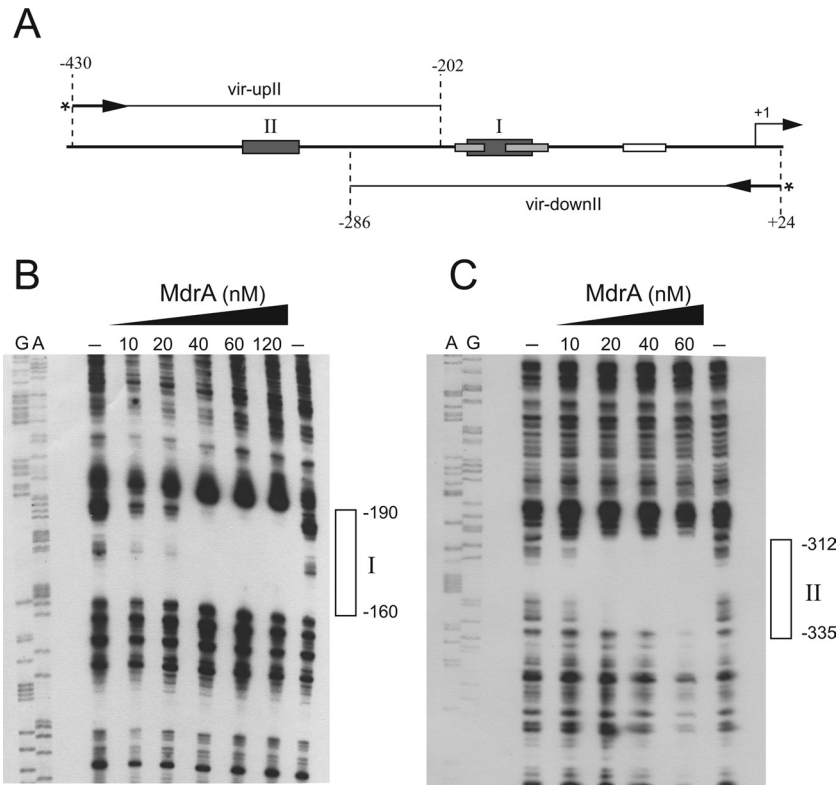
**Determination of the MdrA-binding sites in  $P_{virB}$ .** To identify



**FIG 4** Analysis of the interaction between MdrA, HutC, and/or IHF with the downstream region of the *virB* promoter. (A) Schematic representation of probes *vir-down* and 286/59. Rectangles indicate the positions of the binding sites of HutC, IHF, and MdrA. (B) Coincubation experiment performed by EMSA with probe *vir-down* and different combinations of 20 nM MdrA, 20 nM HutC, or 40 nM IHF. (C) Coincubation experiment performed by EMSA with probe 286/59 and different combinations of MdrA, HutC, or IHF. Protein concentrations were as described for panel B.

both MdrA-binding sites I and II, we performed DNase I footprinting experiments using probes corresponding to different regions of  $P_{virB}$ : (i) a 228-bp fragment containing the upstream region of the promoter that was labeled at the 5' end of the top strand and (ii) a 312-bp fragment containing the downstream region that was labeled at the 5' end of the bottom strand (Fig. 5A). As shown in Fig. 5B, it was observed that MdrA bound to a 30-bp region extending from position -190 to -160, which corresponds to the MdrA-binding site I (Fig. 5B). DNase I footprinting experiments performed with the probe that contains sequences of the upstream region of  $P_{virB}$  showed that MdrA protected a 24-bp region that extends from position -335 to -312, which corresponds to the MdrA-binding site II (Fig. 5C). When a 370-bp fragment was end labeled at the top strand and used as a probe (Fig. 6A), it was observed that MdrA protected two regions corresponding to the MdrA-binding sites I and II (Fig. 6B and C), which indicated that this transcription factor is able to bind simultaneously to both operator sequences.

The DNase I footprinting experiments showed that the MdrA-binding site I overlaps those of both HutC and IHF (Fig. 5A), consistent with the observation that MdrA competed with these two transcriptional regulators for the binding to probe *vir-down* (Fig. 4B). The analysis of the protected regions revealed the presence of two partially conserved 11-bp motifs arranged as inverted repeats in the MdrA-binding site II (Fig. 7B). In contrast, the analysis of sequences corresponding to the MdrA-binding site I showed no obvious dyad symmetry. However, alignment of the 11-bp motifs together with the downstream protected region revealed the presence of a similar sequence located at the center of the MdrA-binding site I (Fig. 7A), suggesting that MdrA specifically recognizes this sequence. In agreement with this hypothesis, MdrA was unable to bind a probe lacking a sequence that is within

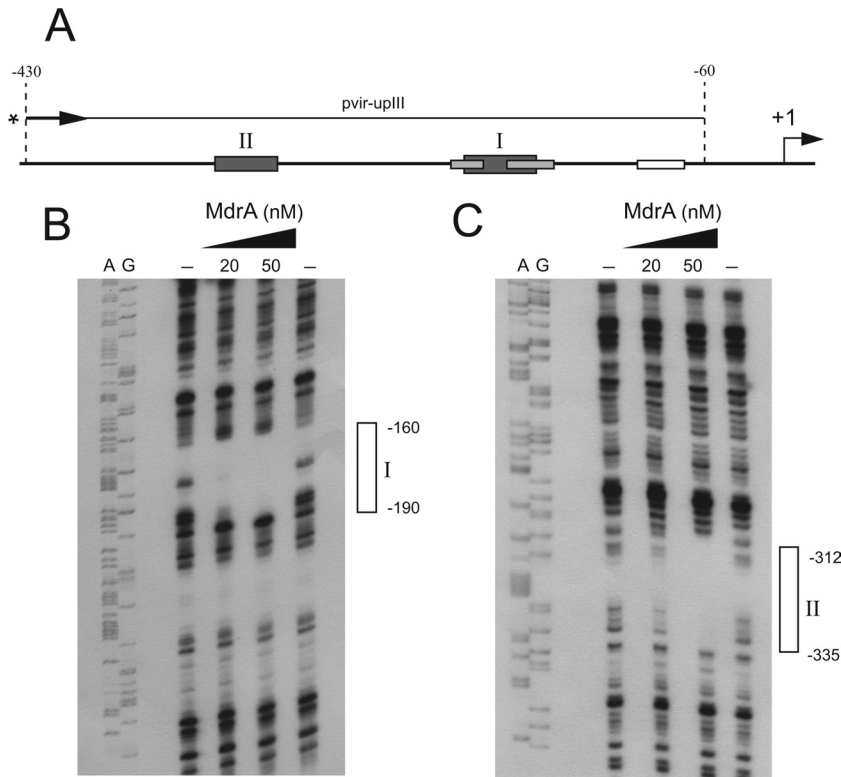


**FIG 5** Determination of the MdrA-binding sites in the *virB* promoter. (A) Schematic representation of probes *vir-upII* and *vir-downII* used for DNase I footprinting experiments. Positions relative to the transcription start site of  $P_{virB}$  are indicated. MdrA-, HutC-, IHF-, and VjbR-binding sites are indicated as boxes. An arrow and an asterisk indicate the primer used for construction of each 5' radiolabeled probe. (B) DNase I footprinting experiment performed with probe *vir-downII* and MdrA at the indicated concentrations. Lanes A and G show sequence reactions performed by the Sanger method with primer *vir-downI*. The protected region of the MdrA-binding site I is indicated with an open rectangle. (C) DNase I footprinting experiment performed with probe *vir-upII* and MdrA at the indicated concentrations. Lanes A and G show sequence reactions performed by the Sanger method with primer *vir-up*. The protected region of the MdrA-binding site II is indicated as described for panel B.

the 11-bp motifs found in both MdrA-binding sites (Fig. 7C), which supports the notion that the sequence TAAA is part of the motif recognized by MdrA in the promoter.

**MdrA dissociates from DNA in the presence of sodium deoxycholate.** Several members of the MarR family are capable of directly binding specific ligands, which dissociate the regulator from DNA with a consequent modulation of gene expression (17). In *Salmonella enterica* serovar Typhimurium, it was recently found that sodium deoxycholate (DOC), a component of bile, interacts with MarR and interferes with its ability to bind the promoter of the *marRAB* operon (35). Accordingly, we investigated whether the DNA-binding activity of MdrA is susceptible to being affected by total bile salts or bile components. We observed by EMSA that formation of the MdrA-*vir-down* complex was impaired in the presence of total bile salts (data not shown). When individual components of bile were tested, we observed that binding of MdrA to  $P_{virB}$  was impaired by 250  $\mu$ M DOC, whereas the binding of IHF or HutC was not affected by this bile salt at any of the assayed concentrations (Fig. 8). Therefore, these results demonstrated that, as is observed with other members of the MarR-family of transcriptional regulators, the interaction between MdrA and its operator sequences can be modulated by specific compounds, and such an ability may be involved in transduction of environmental stimuli to regulate MdrA-dependent gene expression.

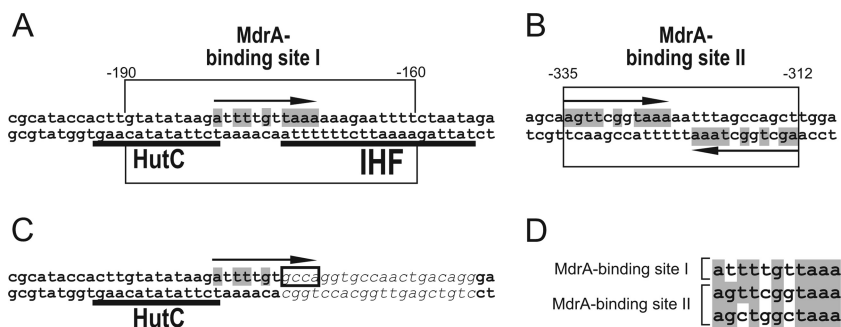
**MdrA and HutC exert redundant roles on regulation of *virB* expression.** In order to determine the role of MdrA on transcriptional regulation of the *virB* genes, we introduced transcriptional fusions between  $P_{virB}$  and the *lacZ* reporter gene into *B. abortus* wild-type strain 2308 or into a  $\Delta mdrA$  deletion mutant. Determination of  $\beta$ -galactosidase activities showed no differences between these strains after incubation of bacteria in rich medium (TSB) or in minimal media at different pH values in the presence of different carbon sources (data not shown). Based on these results, we hypothesized that the regulatory function of MdrA on  $P_{virB}$  is probably redundant with that of other transcription factors. Given that both MdrA and HutC bound to overlapping binding sites and that they appear to have similar structural roles, since neither of them induces DNA bending, we aimed to determine whether these two proteins exert redundant roles on *virB* expression. To this end,  $\beta$ -galactosidase activities of the  $P_{virB}$ -*lacZ* fusions were also assayed in both *B. abortus*  $\Delta hutC$  and the *B. abortus*  $\Delta hutC \Delta mdrA$  double deletion mutant strain. Using all these genetic backgrounds, we observed no  $\beta$ -galactosidase activity differences between strains either when bacteria were grown in TSB until stationary phase ( $OD_{600} \geq 3.5$ ) or when they were grown in the above-mentioned minimal culture media (data not shown). However, in bacteria grown in TSB until exponential phase ( $OD_{600} \sim 0.5$  to 1.0), the  $\Delta hutC \Delta mdrA$  double deletion mutant showed a decrease of about 45% in promoter activity, whereas both the



**FIG 6** MdrA binds simultaneously to binding sites I and II. (A) Schematic representation of probe *vir-upIII* used for DNase I footprinting experiments shown in panels B and C. Positions relative to the transcription start site of  $P_{virB}$  are indicated. Rectangles indicate MdrA-, HutC-, IHF-, and Vjbr-binding sites. The arrow and asterisk indicate the primer used for construction of the 5' radiolabeled probe *vir-upIII*. (B and C) Different runs of a DNase I footprinting experiment performed with probe *vir-upIII* and MdrA at the indicated concentrations. Lanes A and G show sequence reactions performed by the Sanger method with primer *virup*. The protected regions of the MdrA-binding sites I and II are indicated with open rectangles.

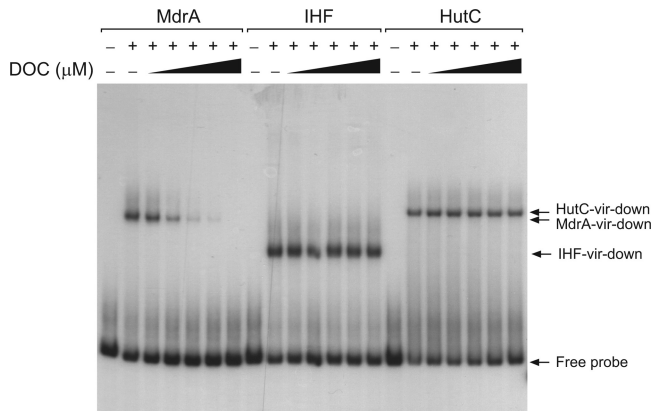
$\Delta hutC$  and the  $\Delta mdrA$  single mutants showed the same levels of  $\beta$ -galactosidase activity as the wild-type strain (Fig. 9). Complementation of *mdrA* by a knock-in strategy in the double mutant restored the wild-type  $\beta$ -galactosidase activity levels (Fig. 9), thus

confirming that MdrA exerts a growth-phase-dependent positive regulatory role on *virB* expression, functionally redundant with HutC. The analyses performed in the presence of DOC displayed the same pattern of  $\beta$ -galactosidase activity as that observed with-



**FIG 7** Sequence analysis of the MdrA-protected regions in the *virB* promoter. (A) Schematic representation of the MdrA-protected sequence in the binding site I. The rectangle indicates regions protected from DNase I cleavage in experiments performed with probe *vir-upIII* and *vir-downII*. Positions relative to the transcription start site are indicated. Sequences corresponding to the HutC- and IHF-binding sites are underlined. The arrow indicates the position of the 11-bp MdrA-binding consensus motif. Nucleotides that match the 11-bp MdrA-binding consensus motif are highlighted in gray. (B) Schematic representation of the MdrA-protected sequence in the binding site II. The open rectangle indicates the region protected from DNase I cleavage. Positions relative to the transcription start site are indicated. Arrows indicate the positions of the 11-bp MdrA-binding consensus motifs. Nucleotides that match the 11-bp MdrA-binding consensus motifs are highlighted in gray. Arrows indicate dyad symmetry. (C) Schematic representation of sequences corresponding to probe *vir-down-ihf*<sup>-</sup>. The HutC-binding site is indicated as described for panel A. Nucleotides that match the 11-bp MdrA-binding consensus motif are highlighted in gray. Nucleotides that replaced the IHF-binding site by a nonrelated sequence are indicated in italics. The position of nucleotides of the 11-bp MdrA-binding consensus motif that are not present in probe *vir-down-ihf*<sup>-</sup> is indicated by an open rectangle. (D) Alignment of sequences corresponding to the 11-bp MdrA-binding consensus motif found at the protected regions of the MdrA-binding sites I and II.





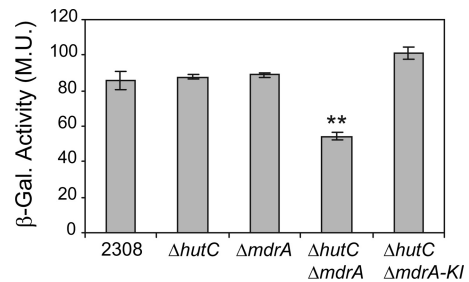
**FIG 8** Effect of DOC on the binding activity of MdrA. EMSA performed with probe vir-down and 30 nM MdrA, 50 nM IHF, or 30 nM HutC in the presence of 100, 250, 500, 750, or 1,000  $\mu$ M DOC.

out this bile salt (data not shown), even at concentrations 3-fold higher than that which dissociated MdrA from the promoter. Thus, the sole presence of this bile salt was not a sufficient stimulus for modulating *virB* expression under standard culture conditions, which suggests that the capability of the MdrA-mediated regulation to respond to environmental signals may be restricted to specific environmental conditions.

Taken together, these results revealed that MdrA controls expression of the *virB* genes in a growth-phase-dependent manner and exerts a regulatory role functionally redundant to that of a nonrelated factor.

## DISCUSSION

Expression of the *virB* operon is under the control of transcriptional regulatory mechanisms that involve perception of environmental conditions related to the intracellular lifestyle of *Brucella* (4). Over the last years, several studies revealed that activity of the *virB* promoter is modulated by the direct action of many transcriptional regulators belonging to different families, including a nucleoid-associated protein (IHF), QS-related transcription factors, and a regulator of histidine catabolism (6, 12, 25, 45, 46). Here, using an approach that previously allowed us to isolate IHF and HutC, we identified MdrA, a protein belonging to the MarR family of transcription factors involved in regulation of virulence genes, aromatic catabolic pathways, or bacterial responses to environmental stress (53). The first evidence of interaction between MdrA and  $P_{virB}$  indicated that this regulator binds to the promoter at sequences located far upstream of the transcription start site (positions  $-430$  to  $-202$ ). Further EMSA analyses showed that MdrA was also able to interact with the downstream region, indicating that this regulator recognizes two distinct binding sites at  $P_{virB}$  (Fig. 3). These observations were confirmed by DNase I footprinting analyses that showed that MdrA is able to bind simultaneously to both regions, which are centered at positions  $-175$  and  $-323.5$  (MarR-binding sites I and II, respectively). It is remarkable that all positive regulators characterized so far in the MarR family act by means of displacing repressors (19, 40) or through prototypical activation mechanisms by interacting with RNA polymerase (RNAP) (28, 50). The latter mode of regulation may not account for the activity of MdrA on the *virB* promoter, since it activates transcription from regions located far upstream of posi-



**FIG 9** Role of MdrA in regulation of the *virB* promoter activity. *B. abortus*  $P_{virB}$ -lacZ (wild type) (strain 2308), *B. abortus*  $\Delta hutC$   $P_{virB}$ -lacZ, *B. abortus*  $\Delta mdrA$   $P_{virB}$ -lacZ, *B. abortus*  $\Delta hutC \Delta mdrA$   $P_{virB}$ -lacZ, and *B. abortus*  $\Delta hutC \Delta mdrA-KI$   $P_{virB}$ -lacZ were grown in rich medium (TSB) until exponential phase ( $OD_{600}$ , 0.5 to 1). Subsequently, bacteria were harvested, and  $\beta$ -galactosidase activities were determined. Values are means  $\pm$  standard deviations in Miller units (M.U.) of duplicate wells from a representative of three experiments. \*\*,  $P < 0.01$ .

tion +1. We hypothesize that MdrA could interfere with the activity of an unknown repressor; a possible candidate may be BabR, which is a negative regulator that affects *virB* expression by 20% and whose binding site in the promoter has not yet been identified (6). Alternatively, contact between MdrA and RNAP may be mediated by additional elements that could introduce conformational changes in chromatin structure. Similar mechanisms were previously suggested for other positive regulators that bind far upstream from the transcription start site but whose mechanisms of activation have not yet been elucidated (e.g., EspR, RutR, TodT, and AlgR) (20, 26, 29, 39). It is worth mentioning that the possible involvement of elements that introduce modifications of the nucleoprotein structure of local sequences was also hypothesized for the LuxR-type regulator VjbR, since it binds at position  $-94$  and consequently could not activate transcription of the *virB* promoter in a prototypical manner (2).

Our experiments revealed that the MdrA-binding site I overlaps those previously identified for IHF and HutC, which was consistent with the fact that these three transcription factors compete for binding to the promoter (Fig. 4). Similar structures have been reported for other promoters regulated by members of the MarR family (i.e., CbaR, MarR, and RovA), wherein the regulators bind to two or more operator sites (19, 24, 36). Furthermore, competition with nucleoid-associated proteins (i.e., H-NS and IHF) or with other global regulators (cyclic AMP receptor protein [CRP]) due to overlapping binding sites has also been reported to be part of the regulatory mechanisms involving MarR-related regulators (19, 40, 43). An additional feature shared by MdrA and most of the MarR homologs is the ligand-mediated modulation of its DNA-binding activity. However, it is important to highlight that MdrA is the first ligand-responsive positive regulator characterized in this family, since no ligand has yet been found for the other MarR-type transcriptional activators described so far (14). Curiously, MdrA is the second positive regulator of the *virB* promoter that dissociates from DNA in response to specific signals, since VjbR was previously shown to respond to acyl-homoserine lactones (2, 12). Our results indicated that either bile or micromolar concentrations of DOC specifically dissociated MdrA from DNA without affecting either IHF or HutC (Fig. 8). These observations have raised the hypothesis that after ligand recognition, MdrA could act as a signal transducer that modulates expression



of the *virB* genes in response to exposure of *Brucella* to DOC within the host environment. Previous studies have described mechanisms in *Brucella* that confer resistance to DOC (11, 23, 33). Moreover, it was recently shown that *Brucella* is able to infect dendritic cells from murine Peyer's patches and to negatively modulate its activation through the action of the protein Btp1 (41). Although these lines of evidence support the notion that *Brucella* is able to overcome the action of bile salts and to invade antigen-presenting cells in the host's gut, we failed to observe any effect of DOC on *virB* expression in exponential-phase cultures under standard growth conditions. However, it cannot be excluded that, under some type of stress experienced by the bacterium within the host, this bile salt may permeate the bacterial membrane more easily and act as an environmental signal to induce an MdrA-mediated response. It is worth mentioning that it cannot be deduced that there exist any additional potential ligands for MdrA from the few MarR homologs reported so far in *Rhizobiales*, since their known ligands do not share structural similarity with DOC (5, 31). Therefore, further studies will be needed to determine whether this bile salt, or a structurally similar compound, can interact with MdrA *in vivo* and modulate *virB* expression.

The results presented here showed that MdrA and HutC, two independently isolated proteins belonging to different families of transcription factors, bind to P<sub>virB</sub> at overlapping target DNA sequences and positively modulate expression of the *virB* genes to similar extents. Moreover, both regulators have been shown to play redundant roles since the regulatory activity of MdrA could not be determined unless a  $\Delta mdrA \Delta hutC$  double deletion mutant background was used to measure *virB* promoter activity (Fig. 9). In addition, our EMSAs indicated that, unlike IHF, neither MdrA nor HutC induced DNA bending upon binding to P<sub>virB</sub> (Fig. 4), suggesting that these two transcriptional regulators exert similar structural roles in agreement with their redundant activity on modulation of *virB* expression.

The data obtained from the present work showed that the function of MdrA leads to a positive regulatory effect on *virB* expression, which does not take place in an all-or-nothing manner. Deletion of *mdrA* affected *virB* expression by 45%, which was a magnitude of difference detectable by quantitative measurements of  $\beta$ -Gal activity (Fig. 9) but not by Western blot experiments performed with an anti-VirB7 antibody (data not shown). Our results indicated that, similarly to what was previously observed with HutC, MdrA also acts as an activator that enhances *virB* promoter activity under defined conditions and appears to play an accessory regulatory role, probably acting to synchronize maximal *virB* expression with certain metabolic and/or environmental signals. It was previously observed that HutC activates intracellular expression of the *virB* genes within J774 macrophages (45). In contrast, MdrA does not appear to play any role in this experimental model, since deletion of *mdrA* did not produce any detectable intracellular effect and the  $\Delta mdrA \Delta hutC$  double mutant affected intracellular *virB* expression to the same extent as in the single  $\Delta hutC$  deletion background (see Fig. S1 in the supplemental material). On the other hand, MdrA was necessary for *virB* expression in cultured bacteria at the exponential phase of the growth in rich medium, whereas in stationary phase we did not observe any MdrA-dependent effect (Fig. 9). In addition, it is worth noting that the growth phase wherein MdrA exerts its regulatory activity is coincident with the only condition under which it could be

isolated (Fig. 1), indicating that this regulator is probably under the control of mechanisms that modulate its expression and/or its DNA-binding activity. Taken together, our observations suggest that the MdrA-mediated regulation of the *virB* genes may be a result of an adaptation to activate *virB* expression at stages of the infection process during which HutC is not acting. However, although functionality of MdrA was demonstrated, it still remains to be determined which are the conditions wherein MdrA-dependent expression is achieved *in vivo*. Besides, it will also be important to assess whether this MarR-related transcriptional regulator is able to perceive environmental signals in specific tissues/organs of the mammalian host during the course of the disease.

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