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Diverse Genetic Regulon of the Virulence-Associated Transcriptional Regulator MucR in *Brucella abortus* 2308

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The Ros-type regulator MucR is one of the few transcriptional regulators that have been linked to virulence in *Brucella*. Here, we show that a *Brucella abortus* in-frame *mucR* deletion strain exhibits a pronounced growth defect during *in vitro* cultivation and, more importantly, that the *mucR* mutant is attenuated in cultured macrophages and in mice. The genetic basis for the attenuation of *Brucella mucR* mutants has not been defined previously, but in the present study the genes regulated by MucR in *B. abortus* have been elucidated using microarray analysis and real-time reverse transcription-PCR (RT-PCR). In *B. abortus* 2308, MucR regulates a wide variety of genes whose products may function in establishing and maintaining cell envelope integrity, polysaccharide biosynthesis, iron homeostasis, genome plasticity, and transcriptional regulation. Particularly notable among the MucR-regulated genes identified is *arsR6* (*nolR*), which encodes a transcriptional regulator previously linked to virulence in *Brucella melitensis* 16 M. Importantly, electrophoretic mobility shift assays (EMSAs) determined that a recombinant MucR protein binds directly to the promoter regions of several genes repressed by MucR (including *arsR6* [*nolR*]), and in *Brucella*, as in other alphaproteobacteria, MucR binds to its own promoter to repress expression of the gene that encodes it. Overall, these studies have uncovered the diverse genetic regulon of MucR in *Brucella*, and in doing so this work has begun to define the MucR-controlled genetic circuitry whose misregulation contributes to the virulence defect of *Brucella mucR* mutants.

Human infections by *Brucella* spp. represent the most common zoonosis worldwide (1). These Gram-negative bacteria naturally infect a variety of wild and domestic animals, and humans become infected through exposure to infected animals and animal products (2). The *Brucella* spp. are members of the α2 subclass of proteobacteria, which includes bacteria that are symbionts and pathogens of plants and mammalian pathogens (3). Very often, the bacteria in this group reside within or in close association with the cells of their host, and these interactions with the eukaryotic host cell are essential for the life of the bacteria. Due to the close phylogenetic relatedness of the α2-proteobacteria, these organisms use common genes and strategies for facilitating their interactions with their specific host (4), and the gene encoding the transcriptional regulator Ros/MucR is one of the genes conserved in the α2-proteobacteria that is important for host-bacterium interactions.

In *Agrobacterium tumefaciens*, *ros* (for rough outer surface) was identified as a gene whose inactivation results in small, nonmucoid colonies (compared to the normally larger, mucoid colonies of the wild-type strain) (5), and while virulence-associated genes (e.g., *virC* and *virD*) are disregulated in this mutant, a *ros* mutant maintains wild-type virulence (6, 7). In *Sinorhizobium meliloti*, mutation of the *mucR* gene leads to a slight growth defect compared to the parental strain, and overexpression of *mucR* in the parental strain results in a significant increase in colony mucoidy; however, the *S. meliloti mucR* mutant strain is not defective in nodule occupancy or its ability to fix nitrogen (8). Conversely, a *Rhizobium etli rosR* mutant exhibits altered colony morphology compared to the parental strain, and this mutant is defective in nodulation competitiveness and competitive growth in the rhizosphere (9, 10).

While early studies genetically linked *ros* and *mucR* mutations to growth defects and differences in colony mucoidy, the mechanism of action of the Ros/MucR proteins was not known at the time; however, it has since been determined that Ros/MucR proteins are transcriptional repressors that regulate numerous genes, including those involved in polysaccharide synthesis, motility, and quorum sensing (11, 12, 13, 14, 15, 16, 17, 18). Ros/MucR-type regulators are unusual in that they contain a Zn finger motif that is uncommon in prokaryotes, whereas transcriptional regulators with this type of motif are commonly found in eukaryotes (19). In fact, the origin of the Zn finger motif-containing proteins has been the source of some debate in recent years. Due to the close association of the alphaproteobacteria with eukaryotic host cells, it has been suggested that an ancestral alphaproteobacterium acquired a gene encoding the Zn finger protein from a eukaryotic host (19, 20); however, others have proposed that Zn finger proteins are of bacterial origin (21, 22). Regardless of their origin, the Zn finger motif-containing proteins, such as Ros and MucR, are essential for the biology of many members of the alphaproteobacteria.

A MucR ortholog was recently identified in the *Brucella* spp., and this regulator is essential for the virulence of *Brucella melitensis* 16 M (23). Additionally, a *B. melitensis mucR* mutant shows

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TABLE 1 Oligonucleotide primers used in this study

Designation	Sequence (5′→3′)[a]
Δ*mucR*-Up-For	GCGGATCCGATGAAATAGGGCTGTTCGC
Δ*mucR*-Up-Rev	GGTGCTTTCGTCGTTCGTT
Δ*mucR*-Down-For	TGATTCTTCAGCGAGTGAATCACG
Δ*mucR*-Down-Rev	GCCTGCAGCATTCCCAGCGATGTATGGG
mucR-comp-For	CTCAATTTTCTTGCGGTGC
mucR-comp-Rev	AGGCAGGACTGTCAGGAGAA
mucR-lacZ-For	GCAAGCTTTTCTATAAATCATATTTGTCTTGG
mucR-lacZ-Rev	GCGGATCCTTCCATAAGTTTTCCTTTT
rMucR-For	GCGGTCTCCGCGCGAAAATCTGGAAACGAACG ACGAAA
rMucR-Rev	GCGGTCTCCTATCAGGCGTCCTTCGGCTTGCGG
babR-RT-For	AAGAATTATGCGCATGACCT
babR-RT-Rev	GTTCCCACCCATCTGGAAAT
BAB1_0746-RT-For	AGGGAAGCAAAAGCGACATGT
BAB1_0746-RT-Rev	AAAGCTAGACTGGTGCCGGTACG
BAB1_0747-RT-For	CATGGAAACGTTTGGCAAATG
BAB1_0747-RT-Rev	CTGAAGTTCACTGCAGCTGTAATGG
BAB1_1035-RT-For	GTTCTTTGTCGGCTCCTTAACCTT
BAB1_1035-RT-Rev	TCCGAAATCAAGATGCTTGAGG
BAB1_1605-RT-For	TGGCAAAAGCTATTGATCTCAGCC
BAB1_1605-RT-Rev	GGCGTGGTGTGGGACTCATATA
BAB1_1893-RT-For	CATATGACCTGCCAGCCGC
BAB1_1893-RT-Rev	CCTGGTCGTCGCATCCTATG
BAB1_2012-RT-For	AACGGGAGAAACTTGTAAGGCAG
BAB1_2012-RT-Rev	GCAGCCTATGGTGTTTGTAGCG
BAB1_2041-RT-For	TCTTCTTCAGCCAGACATTCGAC
BAB1_2041-RT-Rev	TCAGCAGGCCGGATGTAAGCT
BAB2_0143-RT-For	GTTTTCTCGTTCCACTTCGCC
BAB2_0143-RT-Rev	CGGGTACAACCACGCTTGAA
BAB2_0807-RT-For	TGCTGGAGCTTGGTTTCAGG
BAB2_0807-RT-Rev	GGTTGTCTTCCCGCAATTCC
BAB2_1072-RT-For	GGTCGTGATCTTGTCCACCATAA
BAB2_1072-RT-Rev	GACGATCGATTTGATGCTACGG
BAB1_0746-EMSA-For	TTGAAATTGCCAACGAGCTTG
BAB1_0746-EMSA-Rev	GCCTGTTATTTCTTCATGGTCGC
BAB1_1035-EMSA-For	CGCCATAAAACGAACCTCA
BAB1_1035-EMSA-Rev	CGTCGGCAGAAGTAATTTT
BAB1_1605-EMSA-For	ATGATTTAGTAGAAAACGCAGA
BAB1_1605-EMSA-Rev	TGGCCAGATGTTGTGAAAG
BAB1_1893-EMSA-For	GCTGTCTTGTTCATTCTGTC
BAB1_1893-EMSA-Rev	CGGAATCATCTGTACCACC
BAB2_0840-EMSA-For	GCGGATCCGACAAAAATTGTTTAGAA
BAB2_0840-EMSA-Rev	TAAGATCACTCTTTCAAAGGCGGCT
mucR-EMSA-For	CTCAATTTTCTTGCGGTGCCCTG
mucR-EMSA-Rev	CGCAGCGGCTGACAATGGCAA

[a] Underlined sections indicate restriction endonuclease recognition sequences.

TABLE 2 Plasmids used in this study

Plasmid name	Description	Reference or source
pBBR1MCS-4	Broad-host-range cloning vector; Amp^r	27
pNPTS138	Cloning vector; contains *sacB* gene; Kan^r	26
pMR15	Broad-host-range vector containing a promoterless *lacZ* gene; Kan^r	29
pC^{3}029	In-frame deletion of *mucR* plus 1 kb of each flanking region in pNPTS138	This study
pC^{3}030	*mucR* locus including the entire promoter region in pBBR1MCS-4	This study
pC^{3}031	*mucR* promoter region cloned into pMR15	This study

promise as a potential candidate vaccine against *Brucella* infections (24). While it is clear that *mucR* is important for the pathogenesis of *Brucella*, the genes regulated by MucR in *Brucella* remain undefined. In the present study, an isogenic *mucR* deletion strain was derived from *Brucella abortus* 2308 in an attempt to define the *Brucella* MucR regulon, as well as to assess the phenotype of a *B. abortus mucR* mutant.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Brucella abortus* 2308 and derivative strains were routinely grown on Schaedler blood agar (SBA), which is Schaedler agar (Becton, Dickinson and Co., Franklin Lakes, NJ) containing 5% defibrinated bovine blood (Quad Five, Ryegate, MT), or in brucella broth (Becton, Dickinson and Co., Franklin Lakes, NJ). For cloning and recombinant protein production, *Escherichia coli* strains (DH5α and BL21) were grown routinely on tryptic soy agar or in Luria-Bertani broth. When appropriate, growth media were supplemented with ampicillin (100 μg/ml) or kanamycin (45 μl/ml).

Construction and genetic complementation of a *mucR* mutant. The *mucR* locus (*bab1_0594*) in *Brucella abortus* 2308 was mutated using a nonpolar, unmarked gene excision strategy described previously (25). An approximately 1-kb fragment representing the region upstream of the gene extending to the second codon of the coding region was amplified by PCR using primers Δ*mucR*-Up-For and Δ*mucR*-Up-Rev (Table 1), genomic DNA from *Brucella abortus* 2308 as a template, and *Pfx* polymerase (Invitrogen). Similarly, a fragment containing the last two codons of the coding region extending to approximately 1 kb downstream of *mucR* was amplified with primers Δ*mucR*-Down-For and Δ*mucR*-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 presence of ATP. Both of the DNA fragments were included in a single ligation mix with BamHI/PstI-digested pNPTS138 (Table 2) (26), which contains a kanamycin resistance marker and *sacB* gene for counterselection with sucrose. The resulting plasmid (pC^{3}029) (Table 2) was introduced into *B. abortus* 2308, and merodiploid transformants were obtained by selection on SBA plus kanamycin. A single kanamycin-resistant clone was grown for ~6 h in brucella broth and then plated onto SBA containing 10% sucrose. Genomic DNA from sucrose-resistant, kanamycin-sensitive colonies was isolated and screened by PCR for loss of the *mucR* gene, and an isogenic *mucR* mutant derived from *B. abortus* 2308 was named CC092. The *mucR* mutation in this strain was verified by DNA sequence analysis and Southern hybridization.

Genetic complementation of the *mucR* mutation in CC092 was achieved by expressing the wild-type *mucR* allele from its native promoter in pBBR1MCS-4 (Table 2) (27). The *mucR* gene, along with the native *mucR* promoter, was amplified by PCR using primers *mucR*-comp-For and *mucR*-comp-Rev (Table 1) and *Pfx* polymerase (Invitrogen). The resulting DNA fragment was treated with polynucleotide kinase and then ligated into SmaI-digested pBBR1MCS-4. This construct, pC^{3}030 (Table 2), was introduced into the *B. abortus mucR* mutant strain CC092 by electroporation.

All *Brucella* strains generated during this study were tested by the crystal violet exclusion assay in order to assess whether a given strain produced a smooth or rough form of lipopolysaccharide (LPS) (28). Briefly, *Brucella* strains were grown on tryptic soy agar for 72 to 96 h, and the plates were flooded with a dilute (1:1,000) solution of crystal violet for ~25 s. The parental strains *B. abortus* 2308 and *B. melitensis* 16 M were included as smooth LPS-producing controls, while *B. abortus* RB51 served as a rough LPS-producing control.

Construction of a transcriptional *mucR-lacZ* promoter fusion and β-galactosidase assays. The promoter region of the *B. abortus* 2308 *mucR* gene was fused to a *lacZ* reporter as a transcriptional fusion. Approximately 400 bp of the region upstream of *mucR* was amplified by PCR using primers *mucR-lacZ*-For and *mucR-lacZ*-Rev (Table 1) and *Brucella abortus* 2308 genomic DNA as a template. The amplified DNA fragment was sequentially digested with BamHI and HindIII and subsequently ligated into BamHI/HindIII-digested pMR15 (Table 2) (29), which contains a promoterless *lacZ* gene. The *lacZ* promoter fusion plasmid (pC^{3}031) (Table 2) was then electroporated into *Brucella abortus* 2308 and its derivative

strains. *Brucella abortus* strains harboring the *lacZ* fusion construct were grown in brucella broth, and β-galactosidase assays were performed as described previously (30).

Microarray analysis. Total RNA was isolated from *Brucella* cultures grown to late exponential phase as described previously (25), and contaminating genomic DNA was removed by treatment with RNase-free DNase I (Ambion). RNA was prepared from at least two biological replicates for each strain. Ten micrograms of each RNA sample was reverse transcribed, fragmented, and 3′ biotinylated as described previously (31). The labeled cDNA (1.5 μg) was hybridized to custom-made *Brucella abortus* GeneChips (PMD2308a520698F) by following the manufacturer's recommendations for antisense prokaryotic arrays (Affymetrix, Inc., Santa Clara, CA). Signal intensities were normalized to the median signal intensity value for each GeneChip, averaged, and analyzed with GeneSpring software, version 11.3. Transcripts exhibiting ≥2-fold changes in expression between the parental strain 2308 and the *mucR* mutant strain (CC092), as determined by Affymetrix algorithms for statistically significant differential expression (t test; $P \leq 0.05$), were reported. The microarrays used in this study were developed based on the *B. abortus* 2308 genome sequence (accession numbers AM040264 [chromosome 1] and AM040265 [chromosome 2]) and all *B. abortus* GenBank entries that were available at the time of design. In total, 3,019 predicted *Brucella* open reading frames and 1,892 intergenic regions greater than 50 bp in length are represented on PMD2308a520698F.

Real-time RT-PCR. Real-time reverse transcription-PCR (RT-PCR) was performed as described previously (25). Briefly, total *Brucella* RNA was isolated as above and treated with RNase-free DNase I (Ambion) to remove genomic DNA. cDNA was generated from the final RNA preparation using the SuperScript III cDNA synthesis system (Invitrogen, Carlsbad, CA) by following the manufacturer's protocol, and this cDNA was used for real-time PCR employing a 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 for 5 min at 95°C, followed by 40 cycles (denature for 15 s at 95°C, anneal for 15 s at 50°C, and extend for 15 s at 72°C) of amplification. 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 (32).

Purification of recombinant MucR. The *Strep*-tagII system (IBA, Göttingen, Germany) was used to produce recombinant *Brucella* MucR in *E. coli* strain BL21. The coding region of the *mucR* gene (*bab1_0594*) was amplified using the primers rMucR-For and rMucR-Rev (Table 1), *B. abortus* 2308 chromosomal DNA as a template, and *Taq* polymerase. The amplified DNA fragment was digested with BsaI and ligated into BsaI-digested pASK-IBA6, which encodes an amino-terminal *Strep*-tagII on the protein of interest. The resulting plasmid, prMucR, was transformed into *E. coli* strain BL21, and the strain harboring this plasmid was grown to an optical density at 600 nm (OD_{600}) of approximately 0.6 before recombinant gene expression was induced by addition of anhydrotetracycline (200 μg/ml, final concentration). Following 3 h of incubation at 37°C, the cells were collected by centrifugation (4,200 × g for 10 min at 4°C) and lysed by treatment with CelLytic B cell lysis reagent (Sigma, St. Louis, MO) in the presence of the protease inhibitor phenylmethanesulfonyl fluoride. The supernatant from the suspension of lysed cells was collected by centrifugation (14,000 × 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 and 300 mM NaCl), and recombinant protein was eluted with 2.5 mM desthiobiotin in buffer W. The degree of purity of recombinant MucR was high as judged by SDS-PAGE.

EMSAs. All recombinant MucR (rMucR) electrophoretic mobility shift assay (EMSA) experiments were carried out 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, 50 μg/ml bovine serum albumin, and 50 μg/ml salmon sperm DNA. DNA fragments corresponding to the promoter regions of various genes were amplified by PCR from *Brucella abortus* 2308 chromosomal DNA using specific primer sets (Table 1). The amplified DNA fragments were purified by agarose gel electrophoresis, and the fragments were end labeled with [γ-^{32}P]ATP (PerkinElmer, San Jose, CA) and polynucleotide kinase (Promega, Madison, WI). Increasing amounts of recombinant MucR were mixed with the radiolabeled DNA fragments in binding buffer, and the reaction mixtures were incubated at room temperature for 20 min. As controls, 25× molar concentrations of nonradiolabeled specific DNA (specific competitor) or nonradiolabeled nonspecific DNA (nonspecific competitor; coding region of *bab2_0350* [*ohrR*]) were added to some reaction mixtures. In other reaction mixtures, 0.5 mM chelator EDTA was included. The binding reaction mixtures were subjected to electrophoresis on 6% native polyacrylamide gels in 0.5× Tris-borate-EDTA (TBE) running buffer for approximately 1 h. Following electrophoresis, gels were dried onto 3-mm Whatman paper using a vacuum gel dryer system and visualized by autoradiography.

Analysis of *Brucella* LPS. *Brucella* strains were grown in brucella broth to stationary phase, and LPS was isolated by hot phenol extraction as described previously (33). Briefly, 50 ml of hot phenol (45%) was added to 50 ml of *Brucella* culture, and the mixture was incubated at ~65°C for 20 min. The organic phase was isolated and was incubated overnight with 3 volumes of saturated sodium acetate in methanol at −20°C. Following precipitation, the samples were subjected to centrifugation at 10,000 × g for 20 min, and the pellet was suspended in 50 mM sodium phosphate buffer containing 20 mM $MgCl_2$ and 5 mM EDTA. The samples were then treated with DNase and RNase (2 μg/ml of each) at 37°C for 2 h. Subsequently, proteinase K (20 μg/ml) was added, and the samples were incubated at ~65°C for 8 h. The LPS samples were then extracted with an equal volume of phenol, and the interface was removed. Both the organic and aqueous phases were incubated overnight with 3 volumes of saturated sodium acetate in methanol at −20°C. Following centrifugation at 10,000 × g for 20 min, the pellet was suspended in distilled water. Purified LPS was separated in 15% SDS-PAGE gels containing 3 M urea, and the gels were silver stained as described previously (34).

Western blot analyses of LPS were performed using monoclonal antibodies specific for the O chain (A78 12G12 F12) or the LPS core (A68 24G12 A8). Following separation by SDS-PAGE as described above, the LPS samples were transferred to a nitrocellulose membrane. 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 were incubated with the membranes at room temperature in 5% skim milk. Secondary antibodies (anti-mouse antibodies conjugated to horseradish peroxidase [HRP]) were used at a 1:20,000 dilution. All washing steps were performed with TBST. Development of the HRP signal was performed using WestPico (Pierce [Thermo Scientific], Rockford, IL).

Sensitivity of *B. abortus* strains to SDS and polymyxin B in a disk assay. *Brucella* strains were grown on SBA at 37°C under 5% CO_2 for 48 to 72 h, and the bacterial cells were harvested into phosphate-buffered saline (PBS) and suspended at a concentration of ~3.33 × 10^7 CFU/ml in brucella broth containing 0.5% agar (maintained at 55°C). Four milliliters of this suspension was overlaid onto brucella agar plates, and after solidification of the overlay, a sterile 7-mm Whatman disk was placed in the center of each plate. Seven microliters of either a 20% sodium dodecyl sulfate (SDS) or 10-mg/ml polymyxin B solution was applied to each filter disk, and the plates were incubated at 37°C with 5% CO_2 for 72 h. The zones of inhibition around each disk were then measured in millimeters.

Iron utilization assay. *Brucella* strains were grown in low-iron minimal medium (35) from a starting inoculum of 10^6 CFU/ml. Following incubation for 72 h at 37°C, bacterial suspensions were prepared in phosphate-buffered saline (pH 7.2) to a final concentration of 10^9 CFU/ml. Aliquots of 100 μl of the bacterial cell suspensions were mixed with 4 ml of

0.8% Noble agar, and the mixtures were overlaid onto tryptic soy agar (TSA) plates containing 300 μM ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid) (EDDHA). In addition, plates used for the ferrous iron utilization assay contained 2 mM sodium ascorbate in both the Noble agar overlay and the TSA plates to ensure that the iron was maintained in the ferrous form. Seven-millimeter sterile filter paper (Whatman no. 3) disks were placed on the plates, and 10 μl of a 50 mM solution of $FeCl_3$ or 50 mM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ was added to the filter disks. The plates were subsequently incubated for 72 h at 37°C with 5% CO_2, and following this incubation period, the diameter (in millimeters) of the zone of bacterial growth around each filter disk was measured and recorded.

Virulence studies. Experiments to test the intracellular survival and replication of *Brucella* strains in primary peritoneal murine macrophages were carried out as described previously (36). Briefly, resident peritoneal macrophages were isolated from mice and seeded in 96-well plates in Dulbecco's modified Eagle's medium with 5% fetal bovine serum, and the following day, the macrophages were infected with opsonized brucellae at a multiplicity of infection (MOI) of 50:1. After 2 h of infection, extracellular bacteria were killed by treatment with gentamicin (50 μg/ml). In some wells, the macrophages were then lysed with 0.1% deoxycholate in PBS, and serial dilutions were plated on SBA. In other wells, the macrophages were washed with PBS following gentamicin treatment, and fresh cell culture medium containing gentamicin (20 μg/ml) was added to the monolayer. After 24 and 48 h of infection with *Brucella* strains, the macrophages were lysed, and serial dilutions were plated on SBA. Triplicate wells were used for each *Brucella* strain tested.

The experimental methods for assessing the chronic infection of mice by *Brucella* strains were described previously (36). C57BL/6 mice (5 per *Brucella* strain) were infected intraperitoneally with ~5 × 10^4 CFU of each *Brucella* strain in sterile PBS. The mice were sacrificed at 1 and 4 weeks postinfection, and serial dilutions of spleen homogenates were plated on SBA.

Microarray data accession number. The GenBank accession number for the microarray data described in this report is GSE40532.

RESULTS

The *Brucella abortus mucR* mutant exhibits a significant growth defect during *in vitro* cultivation. The *mucR* gene of *Brucella abortus* 2308 is located on chromosome I and designated *bab1_0594* in the genome sequence, and *mucR* is flanked by the *ptrB* gene, encoding oligopeptidase B, and a *hyp* gene, encoding a small hypothetical protein (Fig. 1A). Importantly, this genetic organization is conserved among other *Brucella* spp., including *B. melitensis*, *B. suis*, and *B. ovis*. The most striking feature of the *B. abortus mucR* mutant observed during its phenotypic characterization was the slow growth of this mutant in brucella broth compared to that displayed by the parental 2308 strain (Fig. 1B) and the smaller-size colonies it produced when grown on SBA (Fig. 1C). The generation time for *B. abortus* 2308 grown in brucella broth was calculated to be 2.3 h, while the *mucR* mutant grown in the same medium exhibited a generation time of 8.0 h. Neither of the growth defects is observed in a derivative of the *B. abortus mucR* mutant carrying a copy of *mucR* on a pBBR1MCS-based plasmid (Fig. 1B and C). Despite the delayed growth exhibited by the *B. abortus mucR* mutant, this strain eventually attains the same cell density as the parental strain as it reaches stationary phase in brucella broth, and even with its growth defect, the *B. abortus mucR* mutant exhibits the same cellular morphology as parental strain 2308, as shown when these strains are examined by phase-contrast microscopy following growth on SBA (data not shown).

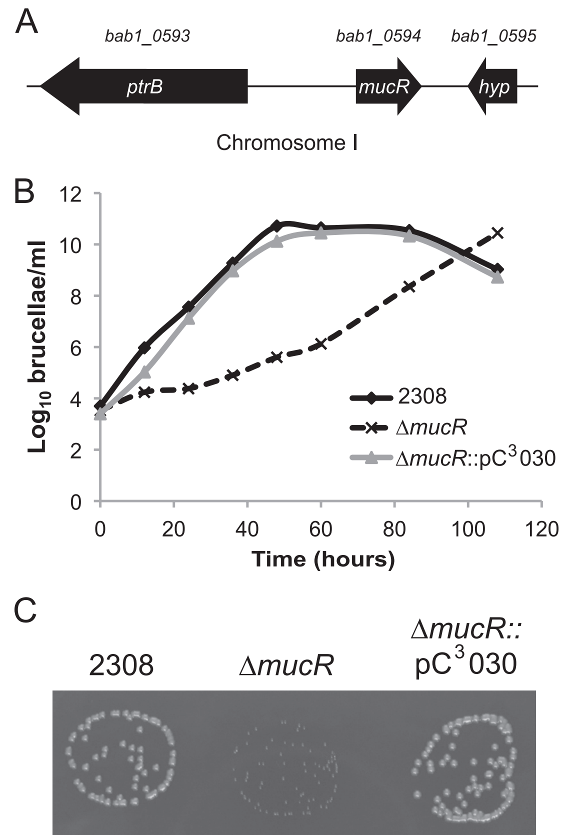

FIG 1 Deletion of *mucR* in *Brucella abortus* 2308 results in a growth deficiency. (A) Genetic organization of the *mucR* locus. The *mucR* gene is found on chromosome I and is designated *bab1_0594* in the *B. abortus* 2308 genome sequence. *mucR* is flanked by *ptrB* (*bab1_0593*), encoding oligopeptidase B, and by a small, hypothetical-protein-encoding gene. (B) Growth curves of *Brucella abortus* strains in a rich medium. *B. abortus* 2308, the isogenic *mucR* mutant (Δ*mucR*), and the complemented *mucR* mutant (Δ*mucR*::pC^{3}030) strains were grown in brucella broth, and the growth of each culture was monitored by plating serial dilutions on SBA to determine the number of CFU/ml. (C) Photograph of *Brucella abortus* colonies on blood agar after 60 h of growth.

Deletion of *mucR* leads to severe attenuation of *B. abortus* 2308 in mice. The *mucR* gene was previously reported to be important for the virulence of *Brucella melitensis* 16 M (23, 24); however, the role of *mucR* in *B. abortus* 2308 virulence has not been reported. Therefore, the *B. abortus mucR* mutant was tested for its ability to survive and replicate in cultured murine macrophages and for its capacity to produce chronic infections in C57BL/6 mice (Fig. 2). Compared to the parental strain, 2308, the *mucR* mutant strain was attenuated at both 24 and 48 h postinfection in macrophages. Complementation of *mucR* expression in the mutant strain restored its virulence in these cultured phagocytes to parental levels (Fig. 2A). Similarly, the *B. abortus mucR* mutant exhibited significantly reduced spleen colonization levels at 1 week and 4 weeks postinfection compared to 2308 when C57BL/6 mice were infected with these strains via the intraperitoneal route (Fig. 2B). These data are in line with the significant attenuation of a *B. melitensis mucR* mutant described previously (23) and suggest that the link between MucR and virulence is a conserved relationship in *Brucella* strains.

In *Brucella* strains, such as *B. melitensis* and *B. abortus*, that naturally produce the smooth form of lipopolysaccharide (S-LPS), the maintenance of an intact LPS O chain is critical for virulence (37). One of the prominent characteristics of the *B.*

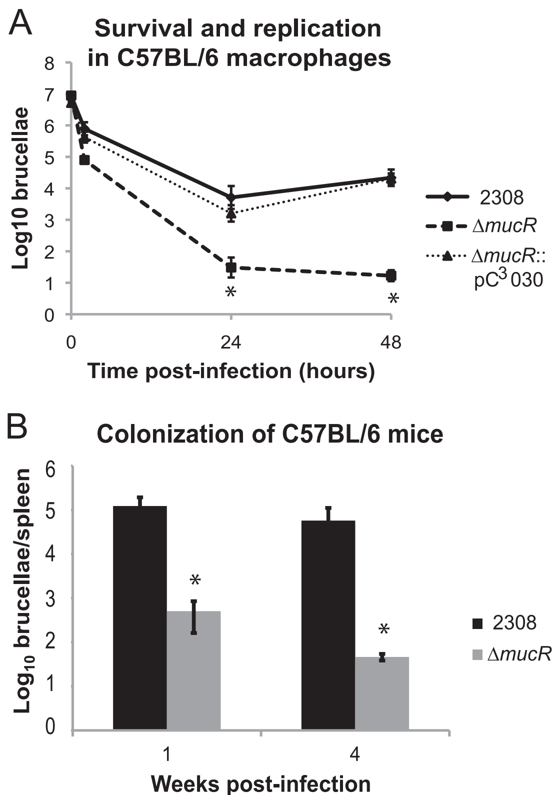

FIG 2 A *Brucella abortus mucR* mutant is significantly attenuated in the mouse model. (A) *Brucella abortus* 2308, the *mucR* isogenic mutant strain (Δ*mucR*), and the complemented *mucR* mutant strain (Δ*mucR*::pC^{3}030) were tested for survival and replication in primary murine macrophages. Macrophages were isolated from the peritoneal cavities of C57BL/6 mice, and the cells were infected with opsonized *Brucella* strains. Extracellular bacteria were killed by gentamicin treatment. At the indicated times, the macrophages were lysed, and serial dilutions were plated on blood agar to determine the number of viable intracellular brucellae. The asterisks indicate significant differences in survival and replication between parental strain 2308 and the *mucR* mutant strain (*t* test; $P < 0.05$). (B) Spleen colonization of mice experimentally infected with *Brucella* strains. C57BL/6 mice were infected intraperitoneally with approximately 5×10^4 CFU of *B. abortus* 2308 or the *mucR* mutant. At 1 and 4 weeks postinfection, the mice were sacrificed, and the serial dilutions of spleen homogenates were plated on blood agar to determine the number of brucellae colonizing the spleen. The asterisks indicate significant differences in spleen colonization between parental strain 2308 and the *mucR* mutant strain (*t* test; $P < 0.05$).

abortus mucR mutant that was observed during its initial phenotypic characterization was that the colonies produced by this mutant take up crystal violet, suggesting that it might have a rough (e.g., O-chain-deficient) LPS (28). Genetic complementation of the *B. abortus mucR* mutant, however, converted it to an S-LPS phenotype in the crystal violet assay, but as shown in Fig. 3A, the silver-stained SDS-PAGE gel profile of an LPS preparation from the *B. abortus mucR* mutant indicates that the O chain is intact. Additionally, immunoblot analysis with O-chain-specific antibodies confirmed that the *B. abortus mucR* mutant produces an intact O antigen (Fig. 3B). Conversely, immunoblot analysis with LPS core-specific antibodies revealed that the LPS core of the *mucR* mutant is altered compared to those of parental strain 2308 and the *mucR* complemented strain (Fig. 3C). It is currently unknown why the *B. abortus mucR* mutant displays a "false" rough LPS phenotype in the crystal violet assay, but the results of the SDS-PAGE and immunoblot analyses of the LPS from this strain suggest that complete loss of the LPS O chain is not a major contributor to the attenuation it exhibits in the mouse model. On the other hand, modification of the LPS core may contribute to the attenuation of the *mucR* mutant, but more work is needed to fully characterize the defect in the LPS core of the *B. abortus mucR* mutant strain.

The *B. abortus mucR* mutant exhibits a cell envelope defect. Due to the results obtained with crystal violet assays showing that the *B. abortus mucR* mutant aberrantly takes up crystal violet, we hypothesized that the *mucR* mutant has a generalized envelope defect that allows for the increased uptake of the dye. To test this hypothesis, we employed a disk diffusion assay to assess the sensitivity of the *B. abortus mucR* mutant to polymyxin B and sodium dodecyl sulfate (SDS) (Fig. 4A). All of the strains tested exhibited similar sensitivities to polymyxin B. However, compared to parental strain 2308, the *mucR* mutant showed a significantly increased sensitivity when exposed to SDS, and this defect was reversed when *mucR* was expressed from a plasmid in the *mucR* mutant strain. Altogether, these data indicate that MucR is important for the stability of the cell envelope in *B. abortus* 2308, but it remains unclear how deletion of *mucR* results in cell envelope abnormalities.

Definition of the MucR regulon in *B. abortus* 2308. To gain insight into the spectrum of *Brucella* genes regulated by MucR, as well as the genetic basis for the distinctive phenotypic properties exhibited by the *B. abortus mucR* mutant strain, microarray analysis was performed using RNA isolated from parental strain 2308 and the *mucR* mutant strain grown in brucella broth to early stationary phase. In all, 91 genes that exhibited altered expression (>2-fold difference) were identified in the *mucR* mutant (Table 3). The majority (76/91) of the altered genes were upregulated in the mutant strain, suggesting that MucR may serve predominately as a transcriptional repressor in *Brucella* strains, as it does in other alphaproteobacteria (16). The link between MucR and altered expression of a selected subset of the *Brucella* genes identified in the microarray analysis was independently confirmed by real-time PCR (Table 3).

The large majority of the genes whose expression levels were increased in the *mucR* mutant are predicted to encode hypothetical proteins. Other genes displaying increased expression in the *mucR* mutant included those encoding membrane proteins and transporters (*bab1_0115*, *bab1_0793*, and *bab2_0055*), putative polysaccharide biosynthesis and modification proteins (*bab1_0326*, *bab1_0560*, *bab1_1465*, and *bab2_0133* to *bab2_0135*), proteins potentially linked to genomic plasticity (i.e., transposases and phage remnants; *bab1_0554*, *bab1_0555*, *bab1_0746*, and *bab1_0747*), and transcriptional regulators (*babR* [*blxR*; *bab1_0190*], *arsR6* [*nolR*; *bab1_1605*], *bab2_0143*, *bab2_0806*, and *bab2_0807*). While the expression of the Crp family regulator encoded by *bab2_0807* was shown to be significantly disregulated in the *mucR* mutant strain by microarray analysis, an independent assessment of gene expression by real-time PCR determined that expression of *bab2_0807* is increased by only ~1.5-fold in the *mucR* mutant compared to parental strain 2308 (Table 3). It is noteworthy that the gene *bab1_0512*, encoding a cyclic di-GMP phosphodiesterase, was overexpressed by more than 4-fold in the *mucR* mutant strain. This gene in *B. melitensis* 16 M (*bmeI1448*) was recently named *bpdB*, and it was reported that deletion of

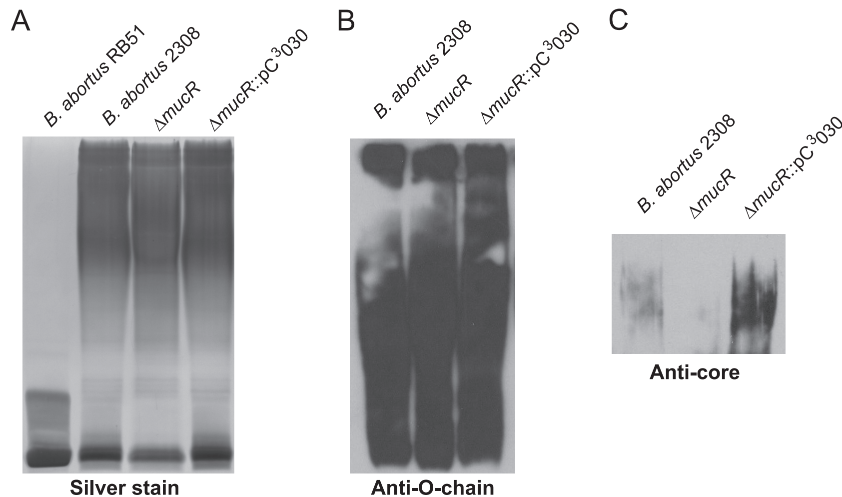

FIG 3 The lipopolysaccharide (LPS) core is modified in the *B. abortus mucR* mutant. *Brucella* strains were grown in brucella broth to stationary phase, and LPS was isolated by hot phenol extraction as described previously (33). Purified LPS from the specified *Brucella* strains was separated on an SDS-PAGE gel containing 3 M urea, and the LPS was then visualized by silver staining (A), Western blot analysis with an anti-O-chain monoclonal antibody (B), and Western blot analysis with an anticore monoclonal antibody (C).

bpdB results in attenuation of *B. melitensis* in mice (38). It is equally notable that the gene *bab1_0115*, encoding Omp25d, displays altered expression (~4-fold increase) in the *B. abortus mucR* mutant. The Omp25 family of proteins is important for the wild-type properties of the *Brucella* cell envelope (39), but to date, clear-cut associations between these proteins and virulence have been established only for *B. ovis*, which naturally lacks its LPS O chain (37).

The genes that were downregulated in the *mucR* mutant include genes putatively involved in polysaccharide biosynthesis and iron transport. The *Brucella abortus* genes *bab1_1973*, *bab1_1974*, and *bab1_1975* are orthologs of the *Sinorhizobium meliloti* genes *SMb20940*, *SMb20939*, and *SMb20938*, respectively, and in *S. meliloti*, these genes are involved in succinoglycan synthesis (40). While the function of the *bab1_1973-bab1_1975* operon in *B. abortus* 2308 is not currently known, it is interesting that MucR regulates the expression of these genes, because the MucR regulator in other alphaproteobacteria is known to control the production of succinoglycan (12, 41).

MucR indirectly regulates iron acquisition genes in *B. abortus* 2308. One set of four genes that displayed reduced expression in the *B. abortus mucR* mutant is designated *bab2_0837-0840* in the *B. abortus* 2308 genome sequence. Experimental evidence indicates that these genes encode a ferrous iron (Fe^{2+}) transporter (A. Elhassanny, unpublished data; 42), and when the *B. abortus mucR* mutant was examined for its ability to use $FeCl_3$ (a ferric iron source) and $(NH_4)_2Fe(SO_4)_2$ (a ferrous iron source) in an *in vitro* iron source utilization assay, this strain exhibited a reduced ability to use these iron sources compared to the parental 2308 strain or a complemented version of this mutant (Fig. 4B). A recombinant version of MucR did not bind to the promoter region of *bab2_0840*, which is the first gene of this four-gene operon, in an EMSA (Fig. 5A), indicating that the regulatory link between MucR and these iron transport genes is indirect.

MucR binds directly to the promoters of *bab1_0746*, *bab1_1035*, *bab1_1605*, and *bab1_1893*. *bab1_0746*, *bab1_1035*, and *bab1_1893* are among the most strongly MucR-regulated genes identified in *B. abortus* 2308 by the microarray analysis, and the regulatory link between MucR and wild-type expression of these genes has been independently verified by real-time RT-PCR analysis (Table 3). Although the *arsR6* (*nolR*; *bab1_1605*) gene appears to be less strongly regulated by MucR, this gene encodes an important virulence determinant in *B. melitensis* 16 M (BMEI0430) (43), and the regulatory relationship between MucR and wild-type *arsR6* (*nolR*) expression in *B. abortus* 2308 has been verified by real-time RT-PCR analysis, similar to its verification for *bab1_0746*, *bab1_1035*, and *bab1_1893* (Table 3). Recombinant MucR exhibited specific interactions with the promoter regions of all four of these genes in EMSAs (Fig. 5A), indicating that the regulatory links between MucR and these genes are direct.

MucR requires divalent cations for its binding to the *bab1_0746* and *nolR* promoters. Divalent cations are required for binding of the *S. meliloti* MucR to DNA. Bertram-Drogatz and colleagues reported that several different divalent cations, including Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}, could be incorporated in EMSAs to facilitate binding between *S. meliloti* MucR and promoter DNA, but exclusion of metal ions from the binding reactions abolished these interactions (12). Accordingly, the addition of the chelator EDTA results in inhibition of *S. meliloti* MucR binding to DNA (16). Zn^{2+} was included in the binding reactions shown in Fig. 4A, and no chelator, such as EDTA, was used in these experiments. Therefore, to determine if MucR from *Brucella abortus* requires divalent cations for binding to DNA, EMSAs were performed in the presence of EDTA (Fig. 5B). Binding of MucR to DNA fragments corresponding to the promoter regions of *bab1_0746* and *nolR* was abolished by the addition of 0.5 mM EDTA. From this, it is concluded that, similar to MucR-type regulators from other bacteria, the *Brucella* MucR also requires metal ions in order to bind to DNA and regulate gene expression.

MucR regulates the expression of *mucR* in *B. abortus* 2308. In other alphaproteobacteria, including *Sinorhizobium meliloti* and *Agrobacterium radiobacter*, MucR orthologs regulate the expression of the *mucR* genes, and this autoregulation is mediated by

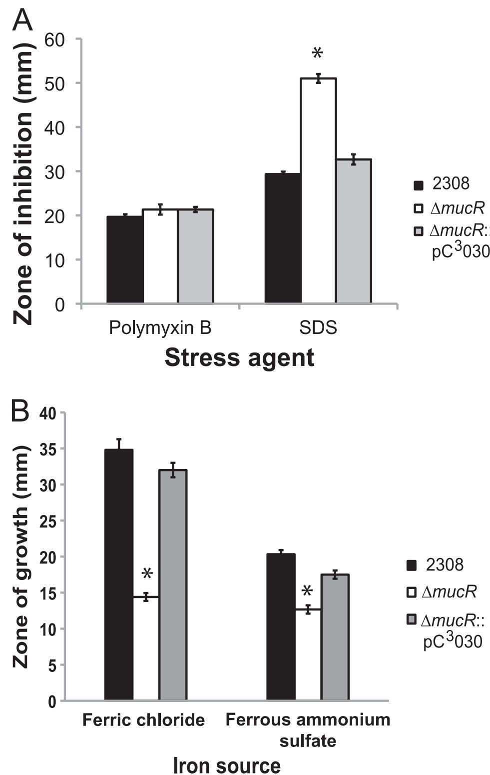

FIG 4 The *Brucella abortus mucR* mutant exhibits a cell envelope defect and an iron acquisition defect *in vitro*. (A) *Brucella* strains were tested in a disk diffusion assay for their comparative susceptibilities to polymyxin B and sodium dodecyl sulfate (SDS). The results are plotted as the average diameters (±standard deviations) of the zones of inhibition around disks containing the indicated stress agents, and the results are from single experiment that was repeated in triplicate. Asterisks denote statistically significant differences (*t* test; $P < 0.05$) between a mutant strains and parental strain 2308. (B) *Brucella abortus* 2308, the *mucR* mutant strain (Δ*mucR*), and the complemented *mucR* mutant strain (Δ*mucR*::pC[3]030) were tested for their ability to utilize ferric (Fe^{3+}) or ferrous (Fe^{2+}) iron in a disk diffusion assay. Iron sources (50 mM $FeCl_3$ or 50 mM ferrous ammonium sulfate) were applied to sterile Whatman paper disks on plates containing the chelator EDDHA, and following incubation at 37°C for 72 h, the diameter (in millimeters) of the zone of bacterial growth around each filter disk was measured. The data are represented as the average and standard deviation of the zones of growth recorded for each strain in triplicate, and asterisks denote statistically significant differences (*t* test; $P < 0.05$) between the *mucR* mutant strain and parental strain 2308.

MucR binding directly to the *mucR* promoter (12, 16, 44). Similarly, a plasmid-borne *mucR-lacZ* transcriptional fusion displayed elevated *mucR* expression the *B. abortus mucR* mutant compared to the parental 2308 strain or a derivative of this mutant carrying *mucR* on a plasmid (Fig. 6A), and recombinant MucR bound in a specific manner to the *B. abortus mucR* promoter region in an EMSA (Fig. 6B). These experimental findings indicate that, like its counterparts in the other alphaproteobacteria, expression of the *Brucella mucR* gene is regulated by an autoregulatory mechanism.

DISCUSSION

One of the striking phenotypic properties exhibited by the *B. abortus mucR* deletion strain is its pronounced growth defect during *in vitro* cultivation in either broth culture or on a solid medium (Fig. 1). The slow growth of the *B. abortus mucR* mutant is intriguing given that a *B. melitensis mucR* mutant does not have the same overt growth defect (data not shown) (23). Recent experiments do, however, indicate that a *B. melitensis* 16 M-derived *mucR* mutant has a more subtle growth defect than its *B. abortus* counterpart. Specifically, the *B. melitensis mucR* mutant enters stationary phase more rapidly and at a lower cell density than the 16 M parental strain (45). The genetic basis for the growth defect exhibited by the *B. abortus mucR* mutant is not readily apparent from the set of MucR-regulated genes identified in this study. The *bab2_1072* gene, which is >7-fold overexpressed in the *B. abortus mucR* mutant, is predicted to encode a PemK-like protein, and these types of toxins are involved in mRNA degradation and have been proposed to play a role in maintaining balanced growth in bacterial cell populations (46). Given this relationship between PemK family proteins and balanced cell growth, we hypothesized that overexpression of *pemK* in the *mucR* mutant could be responsible for the observed growth defect; however, deletion of *pemK* in the *mucR* mutant did not alleviate its slow-growth phenotype (data not shown). A more thorough comparative analysis of the phenotypic properties of the *B. abortus* and *B. melitensis mucR* mutants and the transcriptomes of these mutants and their parental strains will be needed to explain the basis for the differential effects that the *mucR* mutation has on the physiology of *B. abortus* 2308 and *B. melitensis* 16 M. Nevertheless, the experimental findings presented are consistent with the proposed role of MucR as a master transcriptional regulator of genes that perform multiple important metabolic and/or physiologic functions in *Brucella*.

Like the *B. melitensis mucR* mutant (23, 24), the *B. abortus mucR* mutant exhibits striking attenuation in the mouse model of chronic infection (Fig. 2). The growth defect of the *B. abortus mucR* mutant likely contributes to its attenuation in macrophages and mice, but there are several other genes whose altered expression in the *mucR* mutant may also contribute to the attenuation. The loss of either *arsR6* (*nolR*) (43) or *bpdB* (38), for example, results in the attenuation of *B. melitensis* 16 M in mice. Like MucR, ArsR6/NolR is a transcriptional regulator that is widely conserved in the alphaproteobacteria (47), and NolR appears to be required for the proper expression of the genes encoding the type IV secretion system in *B. melitensis* 16 M (43). BpdB is a cyclic di-GMP phosphodiesterase that works together with other enzymes of this type and cyclic di-GMP synthases to control the levels of this important secondary signaling molecule in *Brucella* strains (38), and the attenuation exhibited by *B. melitensis bpdA* and *bpdB* mutants in mice indicates that cyclic di-GMP-mediated signaling plays an important role in proper virulence gene expression. Consequently, further investigation of the role that MucR plays in regulating the expression of the *nolR* and *bpdB* genes should provide us with important insight into the basis for the attenuation exhibited by the *B. abortus mucR* mutant.

The observation that the *B. abortus mucR* mutant exhibits a defect in its capacity to use nonheme iron sources *in vitro* is consistent with the reduced expression of a set of genes (*bab2_0837-0840*) predicted to encode a ferrous iron transporter (42) in this mutant. The lack of direct binding of MucR to the promoter upstream of these genes indicates that this regulatory link is indirect. Direct regulation of the *bab2_0837-0840* operon may be carried out by one or more of the transcriptional regulators whose gene expression is repressed by MucR, such as BAB1_0190 (BabR/BlxR), BAB1_1605 (ArsR6/NolR), BAB2_0143, or BAB2_0806, or alternatively, the metabolic alterations in the *mucR* mutant may influence its iron homeostasis, leading to altered *bab2_0837-0840*

TABLE 3 Genes regulated by MucR in *Brucella abortus* 2308

Function and designation	Description	Fold change (Δ*mucR* strain vs 2308) in gene expression by: Microarray[a]	RT-PCR
Membrane proteins and transport systems			
BAB1_0115	Outer membrane protein Omp25d	3.97	
BAB1_0793	Membrane-bound proton-translocating pyrophosphatase	2.02	
BAB1_1893	Hyp[b] (DME[c] family transporter)	14.69	
BAB1_2138	Rare lipoprotein A	−2.07	
BAB2_0055	Amino acid transporter	3.27	
BAB2_0837	Hyp (polyferredoxin)	−2.34	
BAB2_0838	Iron permease FTR1	−3.58	
BAB2_0839	Hyp	−4.55	
BAB2_0840	Membrane antigen (iron transport)	−3.03	−7.10
Transcription and translation			
BAB1_0190	Transcriptional regulator, LuxR family protein BabR	21.06	73.82
BAB1_0460	Ribosomal protein P2	4.24	
BAB1_0594	Transcriptional regulatory protein MucR	−99.95	
BAB1_1605	Transcriptional regulator, LysR family protein ArsR6/NolR	2.10	2.36
BAB2_0143	AsnC family regulatory protein	2.02	2.62
BAB2_0806	LuxR family regulatory protein	2.69	
BAB2_0807	Crp family regulatory protein	2.04	1.53
Polysaccharide biosynthesis and modification			
BAB1_0326	Glycosyl transferase family protein	5.07	
BAB1_0560	Phosphoglucomutase/phosphomannomutase	2.34	
BAB1_1465	Glycoside hydrolase family protein	2.37	
BAB1_1973	ExsB protein	−3.75	−3.31
BAB1_1974	Putative 6-pyruvoyl tetrahydropterin synthase	−2.98	
BAB1_1975	Proline-rich extensin; radical SAM[d] family protein	−2.73	
BAB2_0133	Glycosyltransferase family protein	2.96	
BAB2_0134	Glu/Leu/Phe/Val dehydrogenase	2.99	
BAB2_0135	Dolichyl-phosphate-mannose-protein mannosyltransferase	2.57	
Genome plasticity			
BAB1_0554	IS5 family transposase OrfA	2.81	104.68
BAB1_0555	IS5 family transposase OrfB	3.68	
BAB1_0746	Hyp	91.29	1841.68
BAB1_0747	Integrase catalytic subunit	61.91	
Metabolism, signaling, and enzymatic processes			
BAB1_0459	Transglycosylase-associated protein	7.96	
BAB1_0512	EAL[e] domain-containing protein BpdB	4.30	
BAB1_0655	Antifreeze protein	2.08	
BAB1_0738	L-Lactate permease	−2.07	
BAB1_1035	Proline-rich extensin	12.81	23.55
BAB1_1099	Patatin	3.20	
BAB1_1206	7-Cyano-7-deazaguanine reductase	−2.38	
BAB1_1511	Nudix hydrolase	2.13	
BAB1_1535	C_5 cytosine-specific DNA methylase	4.11	
BAB1_2001	Aquaporin Z	3.28	
BAB1_2010	Glyceraldehyde-3-phosphate dehydrogenase	14.23	
BAB1_2041	ATP/GTP-binding domain-containing protein	21.01	120.32
BAB2_0196	Nickel-dependent hydrogenase b-type cytochrome subunit	2.82	
BAB2_0257	Beta-lactamase	12.72	
BAB2_0348	Protein kinase	−2.07	
BAB2_0607	Twin-arginine translocation pathway signal	6.94	
BAB2_0846	Frizzled protein	2.54	
BAB2_0865	Pyridoxal-dependent decarboxylase	2.52	
BAB2_0866	Glutamate decarboxylase alpha	2.21	
BAB2_1072	PemK family protein	7.23	
BAB2_1107	Aminoacyl-tRNA synthetase class I	9.88	7.21

(Continued on following page)

TABLE 3 (Continued)

Function and designation	Description	Fold change (Δ*mucR* strain vs 2308) in gene expression by: Microarray[a]	RT-PCR
Hypothetical			
BAB1_0013	Hyp	3.29	
BAB1_0043	Hyp	3.68	
BAB1_0069	Hyp	6.57	
BAB1_0070	Hyp	3.79	
BAB1_0087	Hyp	2.19	
BAB1_0189	Hyp	27.68	
BAB1_0198	Hyp	2.31	
BAB1_0265	Hyp	2.85	
BAB1_0271	Hyp	4.47	
BAB1_0324	Hyp	9.18	
BAB1_0745	Hyp	7.09	
BAB1_0750	Hyp	10.25	
BAB1_0751	Hyp	2.31	
BAB1_0893	Hyp	2.06	
BAB1_1125	Hyp	5.91	
BAB1_1352	Hyp	2.21	
BAB1_1398	Hyp	−2.65	
BAB1_1487	Hyp	2.62	
BAB1_1489	Hyp	2.22	
BAB1_1509	Hyp	3.47	
BAB1_1529	Hyp	19.56	
BAB1_1536	Hyp	2.48	
BAB1_1604	Hyp	4.88	
BAB1_1689	Hyp	2.34	
BAB1_1854	Hyp	2.73	11.41
BAB1_2000	Hyp	3.15	
BAB1_2002	Hyp	3.22	
BAB1_2011	Hyp	10.97	573.77
BAB1_2012	Hyp	74.13	
BAB1_2015	Hyp	−3.80	
BAB1_2021	Hyp	7.01	
BAB2_0092	Hyp	3.85	
BAB2_0132	Hyp	2.79	
BAB2_0258	Hyp	7.81	
BAB2_0450	Hyp	2.84	
BAB2_0613	Hyp	2.55	
BAB2_0861	Hyp	6.12	
BAB2_0867	Hyp	2.61	
BAB2_0873	Hyp	2.04	
BAB2_0887	Hyp	5.23	
BAB2_1151	Hyp	−2.56	

[a] Microarray analysis was performed using total cellular RNA from *Brucella* strains grown in brucella broth to late exponential phase, and values for genes whose expression was shown to be more than 2-fold altered in the *mucR* mutant compared to strain 2308 are shown.
[b] Hyp, hypothetical.
[c] DME, drug/metabolite exporter.
[d] SAM, *S*-adenosylmethionine.
[e] EAL, cyclic di-GMP phosphodiesterase.

expression mediated by the iron-responsive regulators Irr or RirA (48). Defining the role that MucR plays in regulating *bab2_0837-0840* expression in *B. abortus* 2308 will be an important aspect of gaining a better understanding of the basis for the attenuation of *Brucella mucR* mutants, because experimental evidence indicates that the ferrous iron transporter encoded by these genes plays a critical role in virulence in the mouse model (Elhassanny, unpublished).

ros and *mucR* mutants in other alphaproteobacteria exhibit altered mucoidy. For example, a naturally mucoid colony of *Agrobacterium tumefaciens* loses its mucoid appearance when *ros* is mutated (5), and a *mucR* mutation in *Sinorhizobium meliloti* results in a similar loss of colony mucoidy (8). Importantly, these mucoidy-related phenotypes have been linked to the disregulation of genes encoding proteins involved in exopolysaccharide synthesis (11, 12, 13, 14, 15). The *B. abortus mucR* mutant does not appear to be any more or less mucoid than parental strain 2308 based on macroscopic observations, but it is interesting that MucR controls the expression of genes (*bab1_1973* to *bab1_1975*) homologous to the *exsB*, *-C*, and *-D* genes of *S. meliloti*, which are

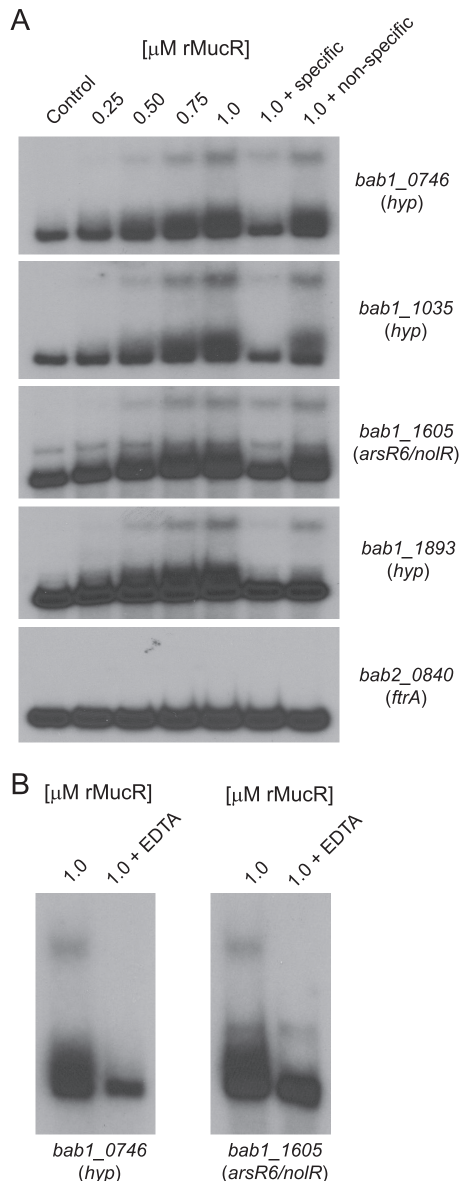

FIG 5 *Brucella abortus* MucR binds directly to the promoter regions of MucR-regulated genes. (A) Recombinant MucR (rMucR) protein was tested for binding to the promoter regions of MucR-regulated genes using an electrophoretic mobility shift assay (EMSA). Increasing concentrations of rMucR were incubated with radiolabeled DNA corresponding to the promoter regions of several different genes, and in some binding reactions, unlabeled specific and nonspecific competitor DNA fragments were included as controls. The binding reactions were resolved in 6% native polyacrylamide gels, and the reaction products were visualized by autoradiography. (B) Effect of EDTA on the binding of MucR to the promoter regions of MucR-regulated genes. EMSAs were performed as described for panel A, but here, 0.5 mM EDTA was included in some binding reactions. *hyp*, hypothetical.

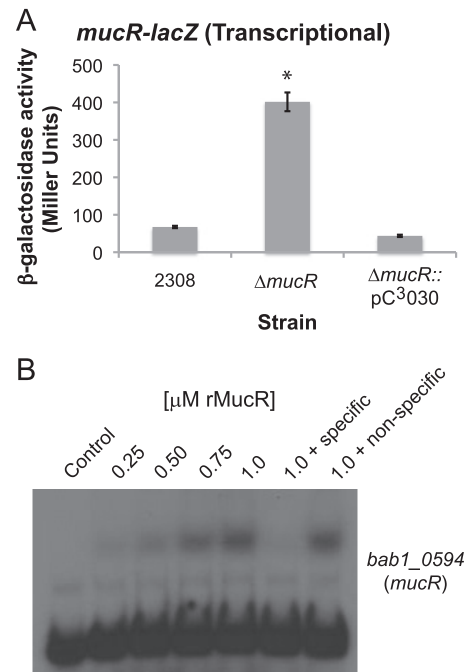

FIG 6 Autoregulation of *mucR* expression in *Brucella abortus* 2308. (A) β-Galactosidase activity produced by a *mucR-lacZ* transcriptional fusion. The activity of a *mucR-lacZ* transcriptional fusion was tested in *B. abortus* 2308, the *mucR* isogenic mutant strain (Δ*mucR*), and the *mucR* mutant complemented in *trans* (Δ*mucR*::pC^{3}030). β-Galactosidase activity is shown as average Miller units ± standard deviations, and the results shown are from a single experiment that was repeated in triplicate. The asterisk indicates a significant difference in β-galactosidase activity between the parental strain 2308 and the *mucR* mutant strain (*t* test; $P < 0.05$). (B) Recombinant MucR (rMucR) protein was tested for binding to the *mucR* promoter region using an EMSA. Similar to the EMSA experiments in Fig. 3A, increasing concentrations of rMucR were incubated with radiolabeled DNA corresponding to the *mucR* promoter region, and in some binding reactions, unlabeled specific or nonspecific competitor DNA fragments were included as controls. The binding reactions were resolved in 6% native polyacrylamide gels and visualized by autoradiography.

involved in succinoglycan production (40). Are these genes merely remnants from a common evolutionary ancestor, or do these genes play a yet-undefined role in exopolysaccharide synthesis in *Brucella*? Historically, exopolysaccharides have not been thought to play an important role in the biology of *Brucella*, but it has been reported that deletion of the LuxR-type regulator VjbR or overproduction of the acyl-homoserine lactone (AHL) acylase AiiD leads to production of a mannose-based exopolysaccharide in *B. melitensis* 16 M, which normally does not produce it (49, 50). Thus, *Brucella* strains apparently have the capacity to produce exopolysaccharides, but under what circumstances and for what reasons these polysaccharides are produced during the natural life of the brucellae remain unknown. Furthermore, if and how MucR is related to exopolysaccharide production in *Brucella* are currently not known.

Another facet of the present story that warrants further discussion is the possible relationship between MucR and proper regulation of the *Brucella* genes involved in lipopolysaccharide (LPS) biosynthesis. The *manB* gene (*bab1_0560*), for example, which encodes a phosphomannomutase known to be involved in the biosynthesis of the *Brucella* LPS O chain and linked to virulence (51), is repressed by MucR. Another MucR-regulated gene that potentially fits into this category is *bab2_0132*. This gene is predicted to encode a hypothetical protein with a conserved "GtrA" domain, which is notable because GtrA modifies the LPS O chain in *Shigella* strains (52). It is also interesting that two of the MucR-regulated genes in *B. abortus* 2308, *bab1_0554* and *bab1_0555*, which are predicted to encode transposases, are situated in the *wbk* locus, which is a large group of genes (>20 genes) whose products

function in the biosynthesis of LPS (53). Transposase-encoding genes residing in close proximity to *bab1_0554* and *bab1_0555* (e.g., *bab1_0556* and *bab1_0557*) have also been implicated in the spontaneous conversion of *Brucella* strains from the smooth to rough LPS phenotype (54). As mentioned previously in this report, although the LPS moieties of both the *B. abortus* and *B. melitensis mucR* mutants retain their O chains, experimental evidence has been obtained with both of these mutants suggesting that these LPS molecules are not entirely "wild type" in structure, as there is a defect in the core of the LPS produced by *Brucella mucR* mutants (Fig. 3C) (45). Thus, another important component of understanding the role of MucR in the virulence of *Brucella* strains will be to determine precisely what role this transcriptional regulator plays in the proper expression of their LPS biosynthesis and modification genes.

In addition to shedding light on the genes being regulated by MucR in *B. abortus* 2308, these and other recent studies have also begun to provide information regarding how *mucR* expression and MucR activity are controlled. The studies presented here, for instance, demonstrate that *mucR* expression in *B. abortus* 2308 is controlled by repressive autoregulation, and studies by Mirabella et al. suggest that *mucR* expression in *B. melitensis* 16 M may be induced by osmotic stress (45). The results presented in this report also support the contention that the activity of MucR-type transcriptional regulators is dependent upon the availability of divalent cations (22). Given the link between MucR and iron utilization in *Brucella*, it is possible that iron availability plays a role in the regulation of MucR activity. However, more work will be needed to define the specific environmental stimuli that control *mucR* expression and MucR activity in the *Brucella* spp.

In conclusion, the present study provides further evidence that MucR is a major regulator of virulence genes in *Brucella* spp., and furthermore, it has defined the genes that comprise the MucR regulon in *B. abortus* 2308. Altogether, this study paves the way for future work aimed at further characterizing the role of MucR in the biology and pathogenesis of *Brucella* spp.

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REFERENCES

1. **Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV.** 2006. The new global map of human brucellosis. Lancet Infect. Dis. **6**:91–99.
2. **Pappas G, Akritidis N, Bosilkovski M, Tsianos E.** 2005. Brucellosis. N. Engl. J. Med. **352**:2325–2336.
3. **Moreno E, Stackebrandt E, Dorsch M, Wolters J, Busch M, Mayer H.** 1990. *Brucella abortus* 16S rRNA and lipid A reveal a phylogenetic relationship with members of the alpha-2 subdivision of the class Proteobacteria. J. Bacteriol. **172**:3569–3576.
4. **Batut J, Andersson SG, O'Callaghan D.** 2004. The evolution of chronic infection strategies in the alpha-proteobacteria. Nat. Rev. Microbiol. **2**:933–945.
5. **Close TJ, Tait RC, Kado CI.** 1985. Regulation of Ti plasmid virulence genes by a chromosomal locus of *Agrobacterium tumefaciens*. J. Bacteriol. **164**:774–781.
6. **Close TJ, Rogowsky PM, Kado CI, Winans SC, Yanofsky MF, Nester EW.** 1987. Dual control of *Agrobacterium tumefaciens* Ti plasmid virulence genes. J. Bacteriol. **169**:5113–5118.
7. **Tait RC, Kado CI.** 1988. Regulation of the *virC* and *virD* promoters of pTiC58 by the *ros* chromosomal mutation of *Agrobacterium tumefaciens*. Mol. Microbiol. **2**:385–392.
8. **Zhan HJ, Levery SB, Lee CC, Leigh JA.** 1989. A second exopolysaccharide of *Rhizobium meliloti* strain SU47 that can function in root nodule invasion. Proc. Natl. Acad. Sci. U. S. A. **86**:3055–3059.
9. **Araujo RS, Robleto EA, Handelsman J.** 1994. A hydrophobic mutant of *Rhizobium etli* altered in nodulation competitiveness and growth in the rhizosphere. Appl. Environ. Microbiol. **60**:1430–1436.
10. **Bittinger MA, Milner JL, Saville BJ, Handelsman J.** 1997. *rosR*, a determinant of nodulation competitiveness in *Rhizobium etli*. Mol. Plant Microbe Interact. **10**:180–186.
11. **Becker A, Rüberg S, Küster H, Roxlau AA, Keller M, Ivashina T, Cheng HP, Walker GC, Pühler A.** 1997. The 32-kilobase *exp* gene cluster of *Rhizobium meliloti* directing the biosynthesis of galactoglucan: genetic organization and properties of the encoded gene products. J. Bacteriol. **179**: 1375–1384.
12. **Bertram-Drogatz PA, Quester I, Becker A, Pühler A.** 1998. The *Sinorhizobium meliloti* MucR protein, which is essential for the production of high-molecular-weight succinoglycan exopolysaccharide, binds to short DNA regions upstream of *exoH* and *exoY*. Mol. Gen. Genet. **257**:433–441.
13. **Rüberg S, Pühler A, Becker A.** 1999. Biosynthesis of the exopolysaccharide galactoglucan in *Sinorhizobium meliloti* is subject to a complex control by the phosphate-dependent regulator PhoB and the proteins ExpG and MucR. Microbiology **145**:603–611.
14. **Lloret J, Martín M, Oruezabal RI, Bonilla I, Rivilla R.** 2002. MucR and MucS activate *exp* genes transcription and galactoglucan production in *Sinorhizobium meliloti* EFB1. Mol. Plant Microbe Interact. **15**:54–59.
15. **Janczarek M, Skorupska A.** 2007. The *Rhizobium leguminosarum bv. trifolii* RosR: transcriptional regulator involved in exopolysaccharide production. Mol. Plant Microbe Interact. **20**:867–881.
16. **Bahlawane C, McIntosh M, Krol E, Becker A.** 2008. *Sinorhizobium meliloti* regulator MucR couples exopolysaccharide synthesis and motility. Mol. Plant Microbe Interact. **21**:1498–1509.
17. **McIntosh M, Krol E, Becker A.** 2008. Competitive and cooperative effects in quorum-sensing-regulated galactoglucan biosynthesis in *Sinorhizobium meliloti*. J. Bacteriol. **190**:5308–5317.
18. **Mueller K, González JE.** 2011. Complex regulation of symbiotic functions is coordinated by MucR and quorum sensing in *Sinorhizobium meliloti*. J. Bacteriol. **193**:485–496.
19. **Chou AY, Archdeacon J, Kado CI.** 1998. *Agrobacterium* transcriptional regulator Ros is a prokaryotic zinc finger protein that regulates the plant oncogene *ipt*. Proc. Natl. Acad. Sci. U. S. A. **95**:5293–5298.
20. **Bouhouche N, Syvanen M, Kado CI.** 2000. The origin of prokaryotic C2H2 zinc finger regulators. Trends Microbiol. **8**:77–81.
21. **Moreira D, Rodríguez-Valera F.** 2000. A mitochondrial origin for eukaryotic C2H2 zinc finger regulators? Trends Microbiol. **8**:448–450.
22. **Baglivo I, Russo L, Esposito S, Malgieri G, Renda M, Salluzzo A, Di Blasio B, Isernia C, Fattorusso R, Pedone PV.** 2009. The structural role of the zinc ion can be dispensable in prokaryotic zinc-finger domains. Proc. Natl. Acad. Sci. U. S. A. **106**:6933–6938.
23. **Wu Q, Pei J, Turse C, Ficht TA.** 2006. Mariner mutagenesis of *Brucella melitensis* reveals genes with previously uncharacterized roles in virulence and survival. BMC Microbiol. **6**:102. doi:10.1186/1471-2180-6-102.
24. **Arenas-Gamboa AM, Rice-Ficht AC, Kahl-McDonagh MM, Ficht TA.** 2011. Protective efficacy and safety of *Brucella melitensis* 16MΔ*mucR* against intraperitoneal and aerosol challenge in BALB/c mice. Infect. Immun. **79**:3653–3688.
25. **Caswell CC, Gaines JM, Roop RM, II.** 2012. The RNA chaperone Hfq independently coordinates expression of the VirB type IV secretion system and the LuxR-type regulator BabR in *Brucella abortus* 2308. J. Bacteriol. **194**:3–14.
26. **Spratt BG, Hedge PJ, te Heesen S, Edelman A, Broome-Smith JK.** 1986. Kanamycin-resistant vectors that are analogues of plasmids pUC8, pUC9, pEMBL8 and pEMBL9. Gene **41**:337–342.
27. **Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, II, Peterson KM.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166**:175–176.
28. **White PG, Wilson JB.** 1951. Differentiation of smooth and nonsmooth colonies of brucellae. J. Bacteriol. **61**:239–240.
29. **Bellaire BH, Elzer PH, Baldwin CL, Roop RM, II.** 2003. Production of the siderophore 2,3-dihydroxybenzoic acid is required for wild-type

growth of *Brucella abortus* in the presence of erythritol under low-iron conditions in vitro. Infect. Immun. **71**:2927–2932.

30. **Miller JH.** 1972. Experiments in molecular genetics, p 352–355. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
31. **Beenken KE, Dunman PM, McAleese F, Macapagal D, Murphy E, Projan SJ, Blevins JS, Smeltzer MS.** 2004. Global gene expression in *Staphylococcus aureus* biofilms. J. Bacteriol. **186**:4665–4684.
32. **Pfaffl MW.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29**:e45. doi:10.1093/nar/29.9.e45.
33. **Leong D, Diaz R, Milner K, Rudbach J, Wilson JB.** 1970. Some structural and biological properties of *Brucella* endotoxin. Infect. Immun. **1**:174–182.
34. **Tsai CM, Frasch CE.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
35. **López-Goñi I, Moriyón I, Neilands JB.** 1992. Identification of 2,3-dihydroxybenzoic acid as a *Brucella abortus* siderophore. Infect. Immun. **60**:4496–4503.
36. **Gee JM, Valderas MW, Kovach ME, Grippe VK, Robertson GT, Ng WL, Richardson JM, Winkler ME, Roop RM, II.** 2005. The *Brucella abortus* Cu,Zn superoxide dismutase is required for optimal resistance to oxidative killing by murine macrophages and wild-type virulence in experimentally infected mice. Infect. Immun. **73**:2873–2880.
37. **Vizcaíno N, Cloeckaert A.** 2012. Biology and genetics of the *Brucella* outer membrane, p 133–161. *In* López-Goñi I, O'Callaghan D (ed), *Brucella*: molecular microbiology and genomics. Caister Academic Press, Norfolk, United Kingdom.
38. **Petersen E, Chaudhuri P, Gourley C, Harms J, Splitter G.** 2011. *Brucella melitensis* cyclic di-GMP phosphodiesterase BpdA controls expression of flagellar genes. J. Bacteriol. **193**:5683–5691.
39. **Guzmán-Verri C, Manterola L, Sola-Landa A, Parra A, Cloeckaert A, Garin J, Gorvel JP, Moriyón I, Moreno E, López-Goñi I.** 2002. The two-component system BvrR/BvrS essential for *Brucella abortus* virulence regulates the expression of outer membrane proteins with counterparts in members of the Rhizobiaceae. Proc. Natl. Acad. Sci. U. S. A. **99**:12375–12380.
40. **Becker A, Küster H, Niehaus K, Pühler A.** 1995. Extension of the *Rhizobium meliloti* succinoglycan biosynthesis gene cluster: identification of the *exsA* gene encoding an ABC transporter protein, and the *exsB* gene which probably codes for a regulator of succinoglycan biosynthesis. Mol. Gen. Genet. **249**:487–497.
41. **Keller M, Roxlau A, Weng WM, Schmidt M, Quandt J, Niehaus K, Jording D, Arnold W, Pühler A.** 1995. Molecular analysis of the *Rhizobium meliloti mucR* gene regulating the biosynthesis of the exopolysaccharides succinoglycan and galactoglucan. Mol. Plant Microbe Interact. **8**:267–277.
42. **Roop RM, II, Anderson E, Ojeda J, Martinson D, Menscher E, Martin DW.** 2012. Metal acquisition by *Brucella* strains, p 179–199. *In* López-Goñi I, O'Callaghan D (ed), *Brucella*: molecular microbiology and genomics. Caister Academic Press, Norfolk, United Kingdom.
43. **Haine V, Sinon A, Van Steen F, Rousseau S, Dozot M, Lestrate P, Lambert C, Letesson JJ, De Bolle X.** 2005. Systematic targeted mutagenesis of *Brucella melitensis* 16M reveals a major role for GntR regulators in the control of virulence. Infect. Immun. **73**:5578–5586.
44. **Hussain H, Johnston AW.** 1997. Iron-dependent transcription of the regulatory gene *ros* of *Agrobacterium radiobacter*. Mol. Plant Microbe Interact. **10**:1087–1093.
45. **Mirabella A, Terwagne M, Zygmunt MS, Cloeckaert A, De Bolle X, Letesson JJ.** 2013. *Brucella melitensis* MucR, an orthologue of *Sinorhizobium meliloti* MucR, is involved in resistance to oxidative, detergent, and saline stresses and cell envelope modifications. J. Bacteriol. **195**:453–465.
46. **Condon C.** 2006. Shutdown decay of mRNA. Mol. Microbiol. **61**:573–583.
47. **Cren M, Kondorosi A, Kondorosi E.** 1995. NolR controls expression of the *Rhizobium meliloti* nodulation genes involved in the core Nod factor synthesis. Mol. Microbiol. **15**:733–747.
48. **Johnston AW, Todd JD, Curson AR, Lei S, Nikolaidou-Katsaridou N, Gelfand MS, Rodionov DA.** 2007. Living without Fur: the subtlety and complexity of iron-responsive gene regulation in the symbiotic bacterium *Rhizobium* and other alpha-proteobacteria. Biometals **20**:501–511.
49. **Uzureau S, Godefroid M, Deschamps C, Lemaire J, De Bolle X, Letesson JJ.** 2007. Mutations of the quorum sensing-dependent regulator VjbR lead to drastic surface modifications in *Brucella melitensis*. J. Bacteriol. **189**:6035–6047.
50. **Godefroid M, Svensson MV, Cambier P, Uzureau S, Mirabella A, De Bolle X, Van Cutsem P, Widmalm G, Letesson JJ.** 2010. *Brucella melitensis* 16M produces a mannan and other extracellular matrix components typical of a biofilm. FEMS Immunol. Med. Microbiol. **59**:364–377.
51. **Allen CA, Adams LG, Ficht TA.** 1998. Transposon-derived *Brucella abortus* rough mutants are attenuated and exhibit reduced intracellular survival. Infect. Immun. **66**:1008–1016.
52. **Guan S, Bastin DA, Verma NK.** 1999. Functional analysis of the O antigen glucosylation gene cluster of *Shigella flexneri* bacteriophage SfX. Microbiology **145**:1263–1273.
53. **Godfroid F, Cloeckaert A, Taminiau B, Danese I, Tibor A, de Bolle X, Mertens P, Letesson JJ.** 2000. Genetic organisation of the lipopolysaccharide O-antigen biosynthesis region of *Brucella melitensis* 16M (*wbk*). Res. Microbiol. **151**:655–668.
54. **Mancilla M, López-Goñi I, Moriyón I, Zárraga AM.** 2010. Genomic island 2 is an unstable genetic element contributing to *Brucella* lipopolysaccharide spontaneous smooth-to-rough dissociation. J. Bacteriol. **192**:6346–6351.