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Characterization of the Organic Hydroperoxide Resistance System of *Brucella abortus* 2308

Clayton C. Caswell, John E. Baumgartner, Daniel W. Martin, and R. Martin Roop II

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

The organic hydroperoxide resistance protein Ohr has been identified in numerous bacteria where it functions in the detoxification of organic hydroperoxides, and expression of *ohr* is often regulated by a MarR-type regulator called OhrR. The genes annotated as BAB2_0350 and BAB2_0351 in the *Brucella abortus* 2308 genome sequence are predicted to encode OhrR and Ohr orthologs, respectively. Using isogenic *ohr* and *ohrR* mutants and *lacZ* promoter fusions, it was determined that Ohr contributes to resistance to organic hydroperoxide, but not hydrogen peroxide, in *B. abortus* 2308 and that OhrR represses the transcription of both *ohr* and *ohrR* in this strain. Moreover, electrophoretic mobility shift assays and DNase I footprinting revealed that OhrR binds directly to a specific region in the intergenic region between *ohr* and *ohrR* that shares extensive nucleotide sequence similarity with so-called "OhrR boxes" described in other bacteria. While Ohr plays a prominent role in protecting *B. abortus* 2308 from organic hydroperoxide stress in *in vitro* assays, this protein is not required for the wild-type virulence of this strain in cultured murine macrophages or experimentally infected mice.

Brucella spp. are Gram-negative bacteria that belong to the α2 subclass of proteobacteria (30), and these bacteria infect a variety of wild and domestic animals, including cattle, sheep and goats, and swine, leading to abortions in pregnant females and sterility in males. Some *Brucella* strains also produce a zoonotic, debilitating febrile illness in humans (31). In areas of the world where brucellosis is endemic in food animals, including regions of Asia, Africa, and the Middle East, *Brucella* strains represent a major public health concern (1).

Within their mammalian hosts, the brucellae infect and reside within cells of the reticuloendothelial system, particularly macrophages (5). In these cells, the brucellae encounter a variety of harsh conditions, including nutrient deprivation, decreased pH, and exposure to reactive oxygen and nitrogen species (ROS and RNS, respectively) (33). ROS, most notably O_2^- and H_2O_2, are produced by macrophages as a defense mechanism against invading pathogens (12), and these molecules can cause DNA damage in the bacterium by participating in Fenton chemistry, leading to death of the bacterium (17, 18). ROS and RNS can also be harmful by causing the peroxidation of membrane lipids, leading to decreased membrane fluidity and subsequent dysfunction of membrane proteins (33).

Pathogenic bacteria, including *Brucella* strains, have evolved multiple strategies to resist the microbicidal activities of ROS and RNS produced by professional phagocytes in the host. Although the brucellae produce many enzymes capable of directly or indirectly detoxifying ROS and RNS (19, 35), only the periplasmic Cu,Zn superoxide dismutase SodC has been shown to play a role in protecting the intracellular brucellae from the oxidative killing pathways of host phagocytes (15).

The organic hydroperoxide resistance protein Ohr has been identified and characterized in several bacteria, including *Actinobacillus pleuropneumoniae* (37), *Agrobacterium tumefaciens* (6), *Bacillus subtilis* (14), *Mycoplasma genitalium* (36), *Pseudomonas aeruginosa* (2, 23), *Francisella tularensis* (24), and *Sinorhizobium meliloti* (11). These proteins belong to a family of peroxiredoxins that detoxify organic peroxides such as *tert*-butyl hydroperoxide or cumene hydroperoxide but are incapable of detoxifying H_2O_2 (7, 26). Organic peroxides are found widely in plants, where resistance to these compounds has been proposed to be an important determinant of the successful interactions between bacterial pathogens and symbionts and their plant hosts (20). In contrast, lipid peroxides formed by oxidative damage to host cell fatty acids have been proposed to be the only biologically relevant type of organic peroxides found in mammalian cells (16), and the importance of being resistant to organic peroxides for bacteria that are mammalian pathogens is unresolved (2, 8, 37).

Expression of bacterial *ohr* genes is typically controlled at the transcriptional level by the MarR-type regulator OhrR (9, 28). Under uninduced conditions, OhrR is in a reduced form that tightly binds to the *ohr* promoter, inhibiting *ohr* transcription (14). Upon exposure to organic peroxides, a conserved cysteine residue in OhrR becomes oxidized, and this transcriptional repressor loses its DNA binding activity, leading to elevated *ohr* transcription (10, 13). In many bacteria, OhrR also binds to the *ohrR* promoter region, leading to an autoregulatory circuit (2, 6, 14, 29).

The genes annotated as BAB2_0350 and BAB2_0351 in the *B. abortus* 2308 genome sequence are predicted to encode OhrR and Ohr orthologs, respectively. The studies described in this report were carried out to confirm the biological functions of these proteins and determine to what extent, if any, they contribute to the virulence of *B. abortus* 2308 in the mouse model of infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Brucella abortus* 2308 and derivative strains were routinely grown on Schaedler blood agar (SBA; BD,

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Address correspondence to R. Martin Roop II, roopr@ecu.edu.
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TABLE 1 Plasmids used in this study

Plasmid name	Description	Source or reference
pBBR1MCS-4	Broad-host-range cloning vector; Amp^r	21
pNPTS138	Cloning vector; contains *sacB* gene; Kan^r	38
pMR15	Broad-host-range vector containing a promoterless *lacZ* gene; Kan^r	3
pC^3015	In-frame deletion of *ohr* plus 1 kb of each flanking region in pNPTS138	This study
pC^3016	In-frame deletion of *ohrR* plus 1 kb of each flanking region in pNPTS138	This study
pC^3018	*ohr* locus including the entire promoter region in pBBR1MCS-4	This study
pC^3019	*ohrR* locus including the entire promoter region in pBBR1MCS-4	This study
pC^3020	*ohr* promoter region cloned into pMR15	This study
pC^3021	*ohrR* promoter region cloned into pMR15	This study

Franklin Lakes, NJ) containing 5% defibrinated bovine blood (Quad Five, Ryegate, MT) or in brucella broth (BD). For cloning and recombinant protein expression, *Escherichia coli* strains (DH5α and BL21) were grown on tryptic soy agar (BD) or in Luria-Bertani (LB) broth. When appropriate, growth media were supplemented with ampicillin (100 μg/ml) or kanamycin (45 μl/ml).

Construction and genetic complementation of *B. abortus ohr* and *ohrR* mutants. The *ohrR* and *ohr* genes (BAB2_0350 and BAB2_0351, respectively) in *Brucella abortus* 2308 were mutated using a nonpolar, unmarked gene excision strategy. An approximately 1-kb fragment of the upstream region of each gene to the second codon of the coding region was amplified by PCR using primers Δ*ohr*-Up-For and Δ*ohr*-Up-Rev (*ohr* mutant) or Δ*ohrR*-Up-For and Δ*ohrR*-Up-Rev (*ohrR* mutant) and genomic DNA from *Brucella abortus* 2308 as a template. Similarly, a fragment containing the last two codons of the coding region to approximately 1 kb downstream of each open reading frame (ORF) was amplified with primers Δ*ohr*-Down-For and Δ*ohr*-Down-Rev (*ohr* mutant) or Δ*ohrR*-Down-For and Δ*ohrR*-Down-Rev (*ohrR* mutant). The sequences of all oligonucleotide primers used in this study can be found in Table S1 in the supplemental material. 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 pNTPS138 (38), which contains an *nptI*-type kanamycin resistance gene and *sacB* for counterselection with sucrose. The resulting plasmids (pC^3015 [Δ*ohr*] and pC^3016 [Δ*ohrR*]) (Table 1) were introduced into *B. abortus* 2308, and merodiploid transformants were obtained by selection on SBA plus kanamycin. Kanamycin-resistant transformants were grown for 6 to 8 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 *ohr* or *ohrR* gene. The isogenic *ohr* mutant derived from *B. abortus* 2308 was named JB15, and the isogenic *ohrR* mutant derived from *B. abortus* 2308 was named JB16. The genotypes of the mutant strains were verified by DNA sequence analyses and confirmed by Southern hybridizations.

Plasmid pC^3015 was also introduced into the previously described *B. abortus ahpC* mutant KH40 (39) by electroporation, and selection for an *ahpC ohr* double mutant was performed as described above. Following confirmation of its genotype by DNA sequence analysis and Southern hybridization, the resulting *B. abortus ahpC ohr* double mutant was named CC104.

Genetic complementation of the *B. abortus ohr* and *ohrR* mutants was achieved by in *trans* complementation using pBBR1MCS-4 (21). The *ohr* gene, along with the native *ohr* promoter, was amplified by PCR using primers *ohr*-comp-For and *ohr*-comp-Rev (see Table S1 in the supplemental material). The resulting DNA fragment was digested with PstI, followed by treatment with polynucleotide kinase, and the digested/treated fragment was ligated into PstI/HincII-digested pBBR1MCS-4, yielding the plasmid pC^3017. The *ohrR* gene, along with the native *ohrR* promoter, was amplified by PCR using primers *ohrR*-comp-For and *ohrR*-comp-Rev (see Table S1), and this fragment was cloned into pBBR1MCS-4, producing pC^3018. These plasmids were introduced into the corresponding *B. abortus ohr* and *ohrR* mutants by electroporation.

Construction of *ohr-lacZ* and *ohrR-lacZ* transcriptional fusions and β-galactosidase assays. The promoter regions of *ohr* and *ohrR* were fused to a *lacZ* reporter as transcriptional fusions. For the *ohr* promoter fusion, approximately 200 bp of the *ohr* upstream region was amplified by PCR using primers *ohr-lacZ*-For and *ohr-lacZ*-Rev (see Table S1 in the supplemental material) and *Brucella abortus* 2308 genomic DNA as a template. For the *ohrR* promoter fusion, approximately 200 bp of the *ohrR* upstream region was amplified by PCR using primers *ohrR-lacZ*-For and *ohrR-lacZ*-Rev (see Table S1) and *Brucella abortus* 2308 genomic DNA as a template. The amplified DNA fragments were sequentially digested with BamHI and HindIII and subsequently ligated into BamHI/HindIII-digested pMR15 (3), which contains a promoterless *lacZ* gene. The resulting plasmids, pC^3020 and pC^3021, carrying the *ohr-lacZ* and *ohrR-lacZ* transcriptional fusions, respectively, were introduced into *B. abortus* 2308 and the isogenic *ohr* and *ohrR* mutants by electroporation. β-Galactosidase production by the *Brucella* strains carrying the *ohr-lacZ* and *ohrR-lacZ* fusions was determined using the methods described by Miller (25).

Real-time reverse transcriptase PCR (RT-PCR). *Brucella abortus* 2308 was grown in brucella broth to late exponential phase (~10^9 CFU/ml), and the cultures were treated with 5 mM H_2O_2, 5 mM cumene hydroperoxide, and 5 mM *tert*-buytl hydroperoxide or left untreated for 20 min with constant shaking at 37°C. Total RNA was isolated from the cultures, and genomic DNA was removed as described previously (4). cDNA was generated from the final RNA preparation using the SuperScript III cDNA synthesis system (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and this cDNA was used for real-time PCR employing a SYBR green PCR supermix (Roche, Mannheim, Germany). For these experiments, primers for 16S rRNA were used as a control, while gene-specific primers were used for evaluating relative levels of *ohr* and *ohrR* mRNA (see Table S1 in the supplemental material). 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).

Expression and purification of recombinant OhrR. The Strep-tag II system (IBA, Göttingen, Germany) was used to produce a recombinant version of the *Brucella* OhrR protein (rOhrR) in *E. coli* strain BL21. The coding region of the *ohrR* gene (BAB2_0350) was amplified using the primers rOhrR-For and rOhrR-Rev (see Table S1 in the supplemental material), *B. abortus* 2308 chromosomal DNA as a template, and *Taq* polymerase. The amplified DNA fragment was digested with BbsI and ligated into BsaI-digested pASK-IBA6, which is designed to produce an amino-terminal Strep-tag II "tagged" version of the protein of interest. The resulting plasmid, prOhrR, was transformed into *E. coli* strain BL21, and the strain harboring the recombinant protein expression plasmid was grown to an optical density at 600 nm (OD_{600}) of approximately 0.6 in LB broth before production of the recombinant protein was initiated by the addition of anhydrotetracycline (final concentration, 200 μg/ml) to the growth medium. Following further incubation for 2 h at 37°C, the bacterial cells were collected by centrifugation (4,200 × *g* for 10 min at 4°C) and lysed by treatment with CelLytic B (Sigma, St. Louis, MO) in the presence of the protease inhibitor phenylmethylsulfonyl fluoride. The supernatant from the suspension of lysed cells was cleared by centrifugation (14,000 × *g* for 10 min at 4°C), and the clarified supernatant was passed through an

affinity column packed with Strep-Tactin Sepharose. The column was washed extensively with buffer W (100 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA [pH 8.0]), and recombinant protein was eluted with 2.5 mM desthiobiotin in buffer W. The degree of purity of recombinant OhrR was high as judged by visualization of a single major band on SDS-PAGE.

Determination of the transcriptional start site for *ohr* by primer extension. Total RNA was extracted from *B. abortus* grown in brucella broth to late exponential phase as described previously (4). The oligonucleotide primer *ohr*-PE was end labeled with [γ-^{32}P]ATP (PerkinElmer, San Jose, CA) and polynucleotide kinase (Promega, Madison, WI), and the labeled primer was annealed to 50 μg of *Brucella* RNA. Primer extension analysis was carried out using the AMV RT primer extension system (Promega, Madison, WI) according to the manufacturer's instructions. DNA sequence analysis was performed using the same radiolabeled primer and the SequiTherm EXCEL II DNA sequencing kit (Epicentre, Madison, WI) by following the manufacturer's protocol. Primer extension products and DNA sequencing reaction products were separated on 6% denaturing polyacrylamide gels containing 7 M urea and visualized by autoradiography.

Determination of the transcriptional start site for *ohrR* by 5′ rapid amplification of cDNA ends (5′-RACE). 5′-RACE was carried out using the FirstChoice RLM-RACE kit (Ambion, Austin, TX) according to the manufacturer's instructions. *Brucella abortus* 2308 was grown in brucella broth to late exponential phase, and RNA was isolated and treated with DNase I as described previously (4). The *ohrR* gene-specific primer *ohrR*-RACE (see Table S1 in the supplemental material) was used in the PCR steps of the 5′-RACE protocol where *Taq* polymerase was also employed. The *ohrR* 5′-RACE product was gel purified and cloned into pGEM-T Easy. Plasmid DNA was purified from *E. coli* transformants, and DNA sequencing was performed to identify the *ohrR* transcriptional start site.

EMSAs. All rOhrR 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, 0.5 mM EDTA, 50 μg/ml bovine serum albumin, and 50 μg/ml salmon sperm DNA. A 205-bp DNA fragment corresponding to the *ohrR-ohr* intergenic region was amplified by PCR from *Brucella abortus* 2308 chromosomal DNA using primers Δ*ohrR*-Down-For and Δ*ohr*-Up-Rev (see Table S1 in the supplemental material) and chromosomal DNA from *B. abortus* 2308 as a template. The amplified DNA fragment was purified by agarose gel electrophoresis, and the fragment was end labeled with [γ-^{32}P]ATP (PerkinElmer, San Jose, CA) and polynucleotide kinase (Promega, Madison, WI). Increasing amounts of recombinant OhrR were mixed with radiolabeled *ohr-ohrR* intergenic region DNA in binding buffer, and the reaction mixtures were incubated at room temperature for 20 min. As controls, 50× molar concentrations of nonradiolabeled *ohr-ohrR* intergenic region DNA (specific competitor) or nonradiolabeled *ohrR* coding region DNA (nonspecific competitor) were added to some reaction mixtures. 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 at 4°C. Following electrophoresis, gels were dried onto Whatman 3MM filter paper using a vacuum gel dryer system and visualized by autoradiography.

DNase I footprint analysis. A DNA fragment corresponding to the *ohr-ohrR* intergenic region was used for DNase I footprint analysis. Oligonucleotide primers (Δ*ohrR*-Down-For and Δ*ohr*-Up-Rev) were individually end labeled with [γ-^{32}P]ATP (PerkinElmer, San Jose, CA) and polynucleotide kinase (Promega, Madison, WI), and the primers were used in individual PCRs with *Taq* polymerase to generate DNA fragments with a radiolabeled plus or minus strand. Following gel purification, approximately 10,000 cpm of each labeled DNA fragment was incubated separately in EMSA binding buffer (excluding EDTA) containing increasing concentrations of recombinant OhrR protein (total reaction volume, 20 μl). Following incubation at room temperature for 20 min, the binding reaction mixtures were treated with 0.25 U of RQ1 DNase I (Promega, Madison, WI) for 2 min at room temperature. The DNase I reactions were terminated by the addition of 0.5 M EDTA and incubation at 90°C for 5 min. The reaction mixtures were then cleaned by phenol-chloroform extraction, and following ethanol precipitation, the reaction mixture contents were suspended in formamide loading dye (STR 2× loading solution from Promega). DNA sequence analysis was performed using the same radiolabeled primers and the SequiTherm EXCEL II DNA sequencing kit (Epicentre, Madison, WI) by following the manufacturer's protocol. The digested DNA fragments and DNA sequencing reaction products were separated on 8% denaturing polyacrylamide gels containing 7 M urea and visualized by autoradiography.

Sensitivity of *B. abortus* strains to hydrogen peroxide and cumene hydroperoxide in a disk assay. *Brucella* strains were grown on Schaedler blood agar at 37°C under 5% CO_2 for 48 to 72 h, and the bacterial cells were harvested in 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). Three 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 a 30% H_2O_2 or 0.5 M cumene hydroperoxide solution was applied to each filter disk, and the plates were incubated at 37°C with 5% CO_2 for 72 h. The zone of inhibition around each disk was then measured in millimeters.

Virulence of *Brucella* strains in cultured murine macrophages and experimentally infected mice. Experiments to test the virulence of *Brucella* strains in primary, murine peritoneal macrophages were carried out as described previously (15). Briefly, resident peritoneal macrophages were isolated from mice and seeded into 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 of 50:1. After 2 h of infection, extracellular bacteria were killed by treatment with gentamicin (50 μg/ml). For the 2-hour time point, the macrophages were then lysed with 0.1% deoxycholate in PBS, and serial dilutions were plated on Schaedler blood agar (SBA). For the 24- and 48-hour time points, the cells were washed with PBS following gentamicin treatment, and fresh cell culture medium containing gentamicin (20 μg/ml) was added to the monolayer. At the indicated time point, the macrophages were lysed, and serial dilutions were plated on SBA. Triplicate wells were used for each *Brucella* strain tested.

The infection and colonization of mice by *Brucella* strains was as described previously (15, 34). 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.

RESULTS AND DISCUSSION

***B. abortus ohr* and *ohrR* mutants exhibit divergent sensitivities to cumene hydroperoxide.** To determine the roles of Ohr and OhrR in the response of *Brucella abortus* 2308 to different types of peroxide stress, isogenic *ohr* and *ohrR* mutants were constructed, and the sensitivities of these strains to hydrogen peroxide (H_2O_2) and cumene hydroperoxide (CuOOH), an organic hydroperoxide, were assessed. There was no significant difference in the zones of inhibition exhibited by these strains when they were exposed to H_2O_2 in a disk diffusion assay (Fig. 1). In contrast, the *B. abortus ohr* mutant JB15 exhibited a significantly larger zone of inhibition when exposed to CuOOH than the parental strain 2308, and this increased susceptibility to CuOOH was alleviated in a derivative of JB15 carrying the *ohr* gene on a pBBR1MCS-based plasmid. These data indicate that, similar to its orthologs in other bacteria, Ohr in *Brucella abortus* 2308 functions as a detoxifier of organic hydroperoxides but is not responsible for the detoxification of H_2O_2.

The sensitivity of the *B. abortus ohrR* mutant to organic peroxide was also assessed in the disk diffusion assay, and in contrast to the phenotype displayed by the isogenic *ohr* mutant, the *ohrR*

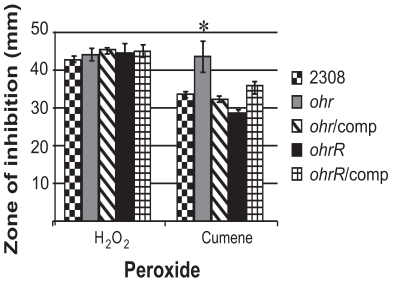
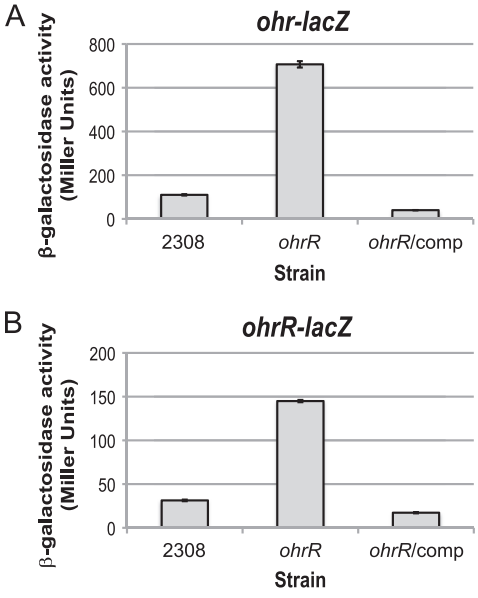


FIG 1 Ohr and OhrR are important determinants of resistance to organic hydroperoxide stress in *Brucella abortus* 2308. Isogenic *ohr* and *ohrR* mutants were constructed in *Brucella abortus* 2308, and each mutant strain was complemented with a plasmid-borne copy of the corresponding gene. These strains were tested in a disk diffusion assay for their comparative susceptibilities to hydrogen peroxide (H_2O_2) and the organic peroxide cumene hydroperoxide. The results are plotted as the average diameter (± standard deviation) of the zone of inhibition around a disk containing the indicated peroxide, and the results are from single experiment that was repeated in triplicate. An asterisk denotes a statistically significant difference ($P < 0.05$) between a given strain and the parental strain 2308.



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	Uninduced	Cumene	*tert*-butyl	H_2O_2
ohr	1.000	125.520	563.048	18.596
ohrR	1.000	7.137	31.628	1.913
16S rRNA	1.000	0.970	1.011	0.981

FIG 2 OhrR is a transcriptional repressor of *ohr* and *ohrR*, and *ohr* and *ohrR* expression is induced by organic hydroperoxide stress in *B. abortus* 2308. The promoter region of the *ohr* or *ohrR* gene was cloned as a transcriptional reporter fused to *lacZ*, and the activities of these fusions were tested in *B. abortus* 2308, the isogenic *ohrR* mutant JB16, and JB16 expressing *ohrR* from a plasmid. The *Brucella* strains harboring the promoter fusion plasmids were grown in brucella broth to late exponential phase. β-Galactosidase activity is shown as average Miller units ± the standard deviation, and the results shown are from a single experiment that was repeated in triplicate. (A) β-Galactosidase activity of the *ohr-lacZ* transcriptional fusion. (B) β-Galactosidase activity of the *ohrR-lacZ* transcriptional fusion. (C) Real-time RT-PCR analysis of *ohr* and *ohrR* expression in response to peroxide treatment. *Brucella abortus* 2308 was grown in liquid media, and cultures were treated with 5 mM H_2O_2 cumene hydroperoxide or *tert*-butyl hydroperoxide. An untreated culture (uninduced) was included as a control. Oligonucleotide primers specific for *ohr*, *ohrR*, or 16S rRNA were used to amplify the target genes by PCR, and quantification of the amplified DNA fragments was performed using SYBR green incorporation. The values represent the abundances of specific mRNAs relative to the level of mRNA in the uninduced culture (1.000).

mutant JB16 exhibited a decreased zone of inhibition around CuOOH in the disk assay compared to the parental 2308 strain (Fig. 1). Importantly, the decreased susceptibility of the *B. abortus ohrR* mutant to CuOOH was reversed when a plasmid-borne copy of *ohrR* was introduced into this strain. In other bacteria, OhrR has been shown to be a repressor of *ohr* transcription, and therefore, in the absence of OhrR (i.e., the *ohrR* mutant), levels of Ohr would be increased, even in a situation where no organic hydroperoxide stress is present. Consequently, the phenotype exhibited by the *B. abortus ohrR* mutant in this assay supports the proposition that OhrR represses *ohr* transcription in *B. abortus* 2308.

A *B. abortus ohrR* mutant exhibits elevated expression of both *ohr* and *ohrR*. Studies of the genetic loci encoding *ohr* and *ohrR* in other bacteria have shown that OhrR serves as a repressor for both *ohr* and its own gene, *ohrR* (6, 9, 14, 28). To test the hypothesis that OhrR controls the expression of these divergently transcribed genes in *B. abortus* 2308, the levels of β-galactosidase activities produced by derivatives of *B. abortus* 2308 and the isogenic *ohrR* mutant JB16 carrying *ohr-lacZ* and *ohrR-lacZ* transcriptional fusions were determined. As shown in Fig. 2, increased expression of both the *ohr-lacZ* and *ohrR-lacZ* fusions was observed in the *B. abortus ohrR* mutant compared to the parental 2308 strain, and in both cases, introduction of a plasmid-borne copy of *ohrR* into the *ohrR* mutant restored the activities of the *ohr-lacZ* and *ohrR-lacZ* transcriptional fusions in this strain to parental levels (Fig. 2). Additionally, it was found that the relative abundance of *ohr* transcripts is ~385-fold higher in the *B. abortus ohrR* mutant JB16 than it is in strain 2308 when total RNA from these strains was evaluated by real-time RT-PCR (data not shown), which provides an independent verification of the role of OhrR as a repressor of *ohr* expression in *Brucella*.

The expression of *ohr* and *ohrR* is induced by organic hydroperoxide stress in other bacteria, including, *Agrobacterium tumefaciens* (6), *Bacillus subtilis* (14), and *Pseudomonas aeruginosa* (2). The ability of peroxide treatment to induce the expression of *ohr* and *ohrR* in *Brucella abortus* 2308 was tested by treating cultures with 5 mM *tert*-butyl hydroperoxide, cumene hydroperoxide, or H_2O_2, and the levels of *ohr* and *ohrR* mRNA were assessed by real-time RT-PCR (Fig. 2C). Compared to untreated cultures, the expression of *ohr* was increased ~125-fold and ~563-fold by treatment with cumene hydroperoxide and *tert*-butyl hydroperoxide, respectively, and *ohrR* expression was elevated ~7-fold and ~31-fold under the same conditions. While exposure to H_2O_2 does not usually result in the induction of *ohr* and *ohrR* expression in other bacteria, there were slight elevations in *ohr* (~18-fold) and *ohrR* (~2-fold) mRNAs when *B. abortus* 2308 cultures were treated with H_2O_2. Additionally, elevation of *ohr* by H_2O_2 has also been observed by microarray analysis of H_2O_2-treated *B. abortus* 2308 cultures (data not shown). However, the increases in gene expression by organic hydroperoxides are substantially greater than the modest elevations observed during H_2O_2 stress, indicating that organic hydroperoxides are much more efficient at activating *ohr* and *ohrR* transcription than is H_2O_2. In all, these experiments show that, similar to the case with other bacteria, organic

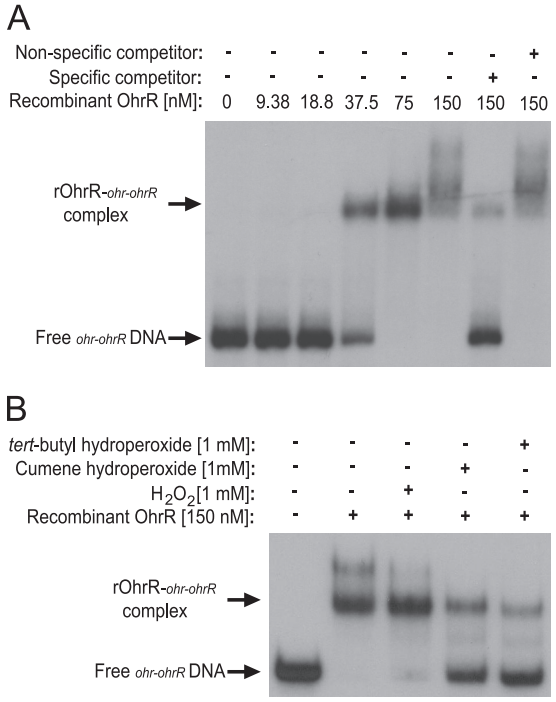


FIG 3 OhrR binds with high affinity to the *Brucella ohr-ohrR* intergenic region. (A) Recombinant OhrR (rOhrR) protein was tested for binding to the *ohr-ohrR* intergenic region using an electrophoretic mobility shift assay (EMSA). Increasing concentrations of rOhrR were incubated with radiolabeled *ohr-ohrR* intergenic region DNA, and in some binding reaction mixtures, unlabeled specific (*ohr-ohrR* intergenic region DNA) and nonspecific (*ohrR* coding sequence DNA) competitor DNA fragments were included as controls. The plus sign represents the inclusion of the specific component listed on the left side of the figure, and the minus sign indicates that the given component was not included in the binding reaction mixture. (B) EMSAs were performed with the *ohr-ohrR* intergenic region and 150 nM rOhrR protein in the presence and absence of different peroxides. A 1 mM final concentration of hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide, or cumene hydroperoxide was included in some of the binding reaction mixtures.

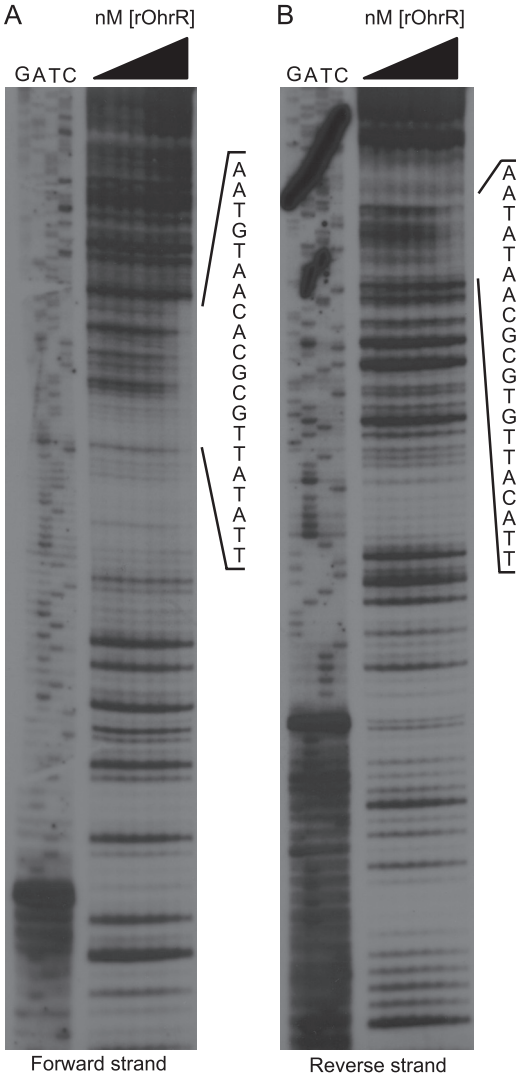


FIG 4 OhrR binds to an OhrR box in the *ohr-ohrR* intergenic region of *B. abortus* 2308. DNase I footprint analysis was employed to determine the exact nucleotides bound by OhrR in the *ohr-ohrR* intergenic region. The forward or reverse strands of *ohr-ohrR* intergenic region DNA were individually radiolabeled, and the labeled DNA fragments were incubated with increasing concentration of rOhrR protein. Following treatment with DNase I, the digested DNA fragments, along with the corresponding DNA sequencing reaction products (shown as G, A, T, or C along the top of the gel), were separated on 8% denaturing polyacrylamide gels and visualized by autoradiography. The region of the DNA fragment protected by rOhrR is listed vertically on the right side of each gel.

hydroperoxide stress induces the expression of *ohr* and *ohrR* in *B. abortus* 2308.

OhrR binds to an "OhrR box" in the *ohr-ohrR* intergenic region in *B. abortus* 2308. In order to determine if OhrR binds directly to the *ohr* and *ohrR* promoter regions to mediate transcriptional repression, electrophoretic mobility shift assays (EMSAs) were performed using purified OhrR and a DNA fragment corresponding to the *ohr-ohrR* intergenic region (Fig. 3). Recombinant OhrR protein bound to the *ohr-ohrR* DNA in a concentration-dependent manner, with significant binding observed at a 37.5 nM concentration of OhrR protein (Fig. 3A). The binding of OhrR to *ohr-ohrR* DNA was specific, because an excess of unlabeled *ohr-ohrR* DNA was able to compete for binding by OhrR; however, a nonspecific DNA fragment (the *ohrR* coding region) was not able to inhibit the binding between OhrR and *ohr-ohrR* intergenic region DNA. Notably, the addition of organic hydroperoxides (i.e., cumene and *tert*-butyl hydroperoxide), but not hydrogen peroxide (H_2O_2), to the EMSA reaction mixtures caused a significant inhibition of OhrR binding to the *ohr-ohrR* intergenic region (Fig. 3B). These experimental findings are consistent with the *Brucella* OhrR protein performing the same functions as its orthologs in other bacteria; that is, it serves as a transcriptional regulator that binds to the promoters of the *ohr* and *ohrR* genes and allows the maximum expression of these genes in response to exposure to organic peroxides but not hydrogen peroxide (2, 6, 14, 29).

DNase I footprinting was subsequently employed to identify the specific site within the *ohr-ohrR* intergenic region that is bound by OhrR (Fig. 4). These experiments revealed that OhrR binds to the 20-nucleotide sequence TTATATTGCGCACAATG TAA. This sequence is similar to OhrR boxes described in other bacteria (6, 9), where the consensus OhrR binding motif is made up of two AT-rich palindromic sequences of approximately 7 nucleotides separated by a variable-length spacer sequence consist-

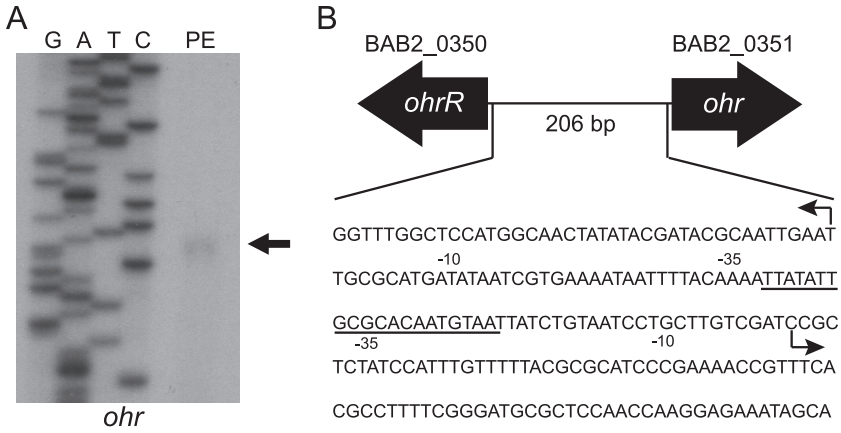


FIG 5 Organization of the *ohr-ohrR* locus in *Brucella abortus* 2308. (A) Primer extension analysis of the *ohr* mRNA was employed to determine the start site of transcription. The primer extension product, along with the corresponding DNA sequencing reaction products, was separated on 6% denaturing polyacrylamide gels and visualized by autoradiography. (B) Schematic representation of the *ohr-ohrR* locus. The transcriptional start sites of the *ohr* and *ohrR* mRNAs are denoted with an arrow, and the −10 and −35 elements of the promoters are shown. The region of the *ohr-ohrR* intergenic region that is bound by OhrR is underlined.

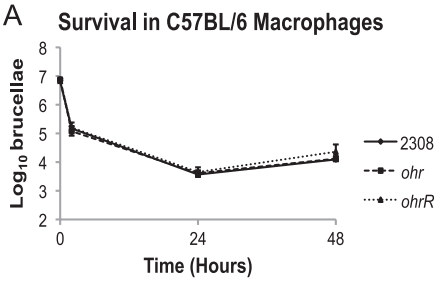
ing of 1 to 17 nucleotides, depending on the bacterial strain in question. The OhrR binding region in the *ohrR-ohr* intergenic region in *B. abortus* 2308 is similarly comprised of two 7-nucleotide palindromic sequences with a 6-nucleotide spacer region, and it is notable that the OhrR box is found only in the *ohr-ohrR* intergenic region and in no other place in the genome sequence of this strain. This suggests that *ohr* and *ohrR* may be the only *Brucella* genes regulated by OhrR.

Definition of the *ohr* and *ohrR* promoter elements and regulatory regions. In *B. abortus* 2308, *ohr* and *ohrR* are divergently oriented on chromosome 2 and separated by a 206-bp intergenic region (Fig. 5B), and a survey of the currently available *Brucella* genome sequences determined that this genetic organization of *ohr* and *ohrR* is a common feature among *Brucella* strains. In fact, *ohr* and *ohrR* are found intact (i.e., they do not appear to be pseudogenes) in all publicly available sequenced *Brucella* genomes, including *B. ovis* strains, which have undergone significant genome degradation compared to other *Brucella* strains (41). In order to gain additional insight into the regulation of *ohr* and *ohrR* expression by OhrR, it was important to define the promoters of *ohr* and *ohrR*. To do this, the transcriptional start site of the *ohr* mRNA was determined by primer extension (Fig. 5A). The *ohr* mRNA initiates 87 nucleotides upstream of the predicted *ohr* start codon, and importantly, the OhrR box overlaps the −35 element of the *ohr* promoter (Fig. 5B). Repeated attempts at defining the *ohrR* transcriptional start site by primer extension failed. Therefore, 5′-RACE was employed as an alternative approach, revealing that the *ohrR* transcript is initiated 41 nucleotides upstream of the putative *ohrR* coding region (Fig. 5B). The OhrR binding site also overlaps a portion of the −35 region of the *ohrR* promoter. Taken together with the data presented earlier in this report, this suggests that, by binding to a single site in the *ohr-ohrR* intergenic region, the *Brucella* OhrR protein can repress the expression of both of these genes until its DNA binding activity is alleviated by exposure to organic peroxides. This genetic organization of *ohr* and *ohrR* divergently transcribed and regulated by a single OhrR binding site has been observed in other bacteria, including the close phylogenetic relative of *Brucella*, *Agrobacterium tumefaciens* (6).

Neither the *Brucella ohr* mutant nor the *Brucella ohrR* mutant exhibits significant attenuation in the mouse model of infection. The experimental results reported so far demonstrate that Ohr plays an important role in protecting *B. abortus* 2308 from organic peroxides and that OhrR regulates the expression of the *ohr* gene in response to exposure to these compounds, but as noted in the introduction, it is unclear what role resistance to organic peroxides plays in the virulence of mammalian pathogens. To address this issue, the virulence properties of the *B. abortus ohr* and *ohrR* mutants were assessed in murine peritoneal macrophages and experimentally infected mice. The *B. abortus ohr* and *ohrR* mutants displayed the same intracellular survival and replication profiles in macrophages as that exhibited by the parental 2308 strain (Fig. 6A). Additionally, activation of the macrophages with gamma interferon did not result in differences in intracellular survival and replication of the *ohr* and *ohrR* mutant strains compared to 2308 (data not shown). Although the *B. abortus ohrR* mutant displayed considerable variability in its spleen colonization profiles in individual mice at 4 weeks postinfection compared to those exhibited by 2308 and the isogenic *ohr* mutant (Fig. 6B), statistical analysis failed to yield a significant difference between the groups of mice infected with *B. abortus* 2308, JB15, or JB16. Based on these findings, we conclude that neither Ohr nor OhrR plays an indispensable role in the virulence of the parent strain in mice. However, it is interesting to note that the levels of Ohr protein are significantly decreased during trafficking and residence of *B. abortus* 2308 in macrophages (22), suggesting that, while deletion of *ohr* does not result in overt attenuation, Ohr levels are responsive to intracellular signals encountered by the brucellae during the natural course of macrophage infection.

The data presented here show a clear role for Ohr in the biology of *Brucella abortus* 2308; however, several important questions remain unanswered. The most apparent question is where the brucellae would encounter organic hydroperoxides. Organic hydroperoxides are commonly produced by plants as a defense mechanism (27). Therefore, plant-associated bacteria, such as *Agrobacterium tumefaciens*, would benefit from a system to inactivate organic hydroperoxides. In mammalian pathogens, such as the brucellae, it is not clear why this type of system evolved, and

A



B

	Average Log_{10} CFU/spleen (+/-SD)	
Strain	Week 1	Week 4
2308	4.375 (0.31)	3.989 (0.56)
ohr	4.176 (0.34)	3.593 (0.48)
ohrR	3.651 (0.86)	3.952 (0.91)

FIG 6 Virulence of *B. abortus* 2308 and the isogenic *ohr* and *ohrR* mutants in murine peritoneal macrophages and mice. (A) Macrophage survival and replication experiments. Cultured resident peritoneal macrophages from C57BL/6 mice were infected with *B. abortus* 2308, JB15 (2308 *ohr*), and JB16 (2308 *ohrR*). At the indicated times postinfection, the macrophages were lysed, and the number of intracellular brucellae present in these phagocytes was determined by serial dilution, plating, and bacteriologic culture. (B) Mouse infection experiments. C57BL/6 mice (5 per strain) were infected intraperitoneally with *B. abortus* 2308, JB15 (2308 *ohr*), and JB16 (2308 *ohrR*). Mice were sacrificed at weeks 1 and 4 postinfection, and the number of brucellae colonizing the spleens was determined. The data are represented as the average number of CFU ± the standard deviation (SD) from the 5 mice colonized with a specific *Brucella* strain at a specific time point.

the results of these studies do not support a role for Ohr in virulence in a mouse model of infection. It is possible that the *ohr-ohrR* locus is an evolutionary remnant retained from a plant-associated alphaproteobacterial ancestor, but it is also conceivable that Ohr contributes to a phase of the life-style of the brucellae that is not simulated in the mouse model, such as an acute infection of a pregnant host animal (e.g., cattle, goats, or swine). Moreover, as discussed earlier, the *ohr ohrR* system is found intact in all *Brucella* strains whose genome sequences are publicly available, and from an evolutionary standpoint, this system should be retained only if it is beneficial for the bacterium. Therefore, it stands to reason that the genomic preservation of *ohr* and *ohrR* is advantageous to the life of the brucellae, but future work examining the role of Ohr and OhrR during the infection of natural host animals is needed in order to fully understand how these gene products benefit *Brucella*.

In regard to the conservation of the *ohr ohrR* system, these genes are also present in many other closely related members of the *Alphaproteobacteria*. In fact, the *ohr ohrR* systems of the alphaproteobacteria *Agrobacterium tumefaciens* (6) and *Sinorhizobium meliloti* (11) have been characterized and reported in the literature. In addition to these published examples, many other alphaproteobacteria possess the *ohr* and *ohrR* genes as determined from searches of publicly available genome sequence data. Using the online software available at http://www.microbesonline.org/, alignments of the *ohr* and *ohrR* loci were generated from *Brucella* strains and several other phylogenetically related alphaproteobacteria, including *Ochrobactrum* spp., *Bradyrhizobium* spp., *Sinorhizobium* spp., *Rhizobium* spp., and *Agrobacterium* spp. Among these alphaproteobacteria, all encode Ohr and OhrR, and the genetic organization of the *ohr* and *ohrR* genes is conserved and similar to that of *Brucella* strains; that is, *ohr* and *ohrR* are divergently oriented at a single genetic locus. However, the genomic context of the genes surrounding *ohr* and *ohrR* is strikingly different among the alphaproteobacteria, but the evolutionary implications of this variation remain unknown.

AhpC does not play a role in the detoxification of organic hydroperoxides in *B. abortus* 2308. The peroxiredoxin AhpC was originally identified based on its role in protecting *Salmonella enterica* serovar Typhimurium and *Escherichia coli* from organic peroxides (40). Subsequent studies with other bacterial AhpC orthologs have shown that, while some of these proteins detoxify both organic peroxides and hydrogen peroxide, others have specificity for one or the other of these compounds (9). It was noted in a previous study that a *B. abortus ahpC* mutant did not exhibit increased sensitivity to cumene hydroperoxide or *tert*-butyl hydroperoxide compared to the parental strain (39), and the *B. abortus ahpC ohr* double mutant constructed for the study described here showed the same level of susceptibility to cumene hydroperoxide in disk assays as the *ohr* mutant JB15 from which it was derived (data not shown). These experimental findings indicate that, like its counterpart in *Agrobacterium tumefaciens* (6), *Brucella* AhpC has specific activity for H_2O_2 and does not contribute to resistance to organic peroxides. Thus, there is a theme emerging that Ohr plays the role of the primary detoxifier of organic hydroperoxides in the alphaproteobacteria; however, further studies are needed to determine if Ohr is in fact the sole detoxifier of organic hydroperoxides in *Brucella* strains or if other gene products also contribute to organic hydroperoxide defense in these bacteria.

ACKNOWLEDGMENT

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