Comparative Study of the Roles of AhpC and KatE as Respiratory Antioxidants in *Brucella abortus* 2308\(^\text{v}\)

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*Brucella* strains are exposed to potentially toxic levels of H\(_2\)O\(_2\) both as a consequence of their aerobic metabolism and through the respiratory burst of host phagocytes. To evaluate the relative contributions of the sole catalase KatE and the peroxiredoxin AhpC produced by these strains in defense against H\(_2\)O\(_2\)-mediated toxicity, isogenic katE, ahpC, and *katE ahpC* mutants were constructed and the phenotypic properties of these mutants compared with those of the virulent parental strain *B. abortus* 2308. The results of these studies indicate that AhpC is the primary detoxifier of endogenous H\(_2\)O\(_2\) generated by aerobic metabolism. KatE, on the other hand, plays a major role in scavenging exogenous and supraphysiologic levels of H\(_2\)O\(_2\), although this enzyme can play a supporting role in the detoxification of H\(_2\)O\(_2\) of endogenous origin if AhpC is absent. *B. abortus ahpC* and *katE* mutants exhibit wild-type virulence in C57BL/6 and BALB/c mice, but the *B. abortus ahpC katE* double mutant is extremely attenuated, and this attenuation is not relieved in derivatives of C57BL/6 mice that lack NADPH oxidase (cybb) or inducible nitric oxide synthase (Nos2) activity. These experimental findings indicate that the generation of endogenous H\(_2\)O\(_2\) represents a relevant environmental stress that *B. abortus* 2308 must deal with during its residence in the host and that AhpC and KatE perform compensatory roles in detoxifying this metabolic H\(_2\)O\(_2\).
In this report, we present evidence that AhpC is the primary antioxidant used by *B. abortus* 2308 to detoxify endogenous \( \text{H}_2\text{O}_2 \) generated by respiratory metabolism during routine aerobic cultivation. KatE, on the other hand, plays a major role in scavenging exogenous and supraphysiologic levels of \( \text{H}_2\text{O}_2 \); although this enzyme can play a supporting role in the detoxification of \( \text{H}_2\text{O}_2 \) of endogenous origin if AhpC is absent. Interestingly, AhpC and KatE appear to play complementary roles in protecting *B. abortus* 2308 from \( \text{H}_2\text{O}_2 \) of metabolic origin during residence in mice, and the presence of either *Helicobacter pylori* (45), *Mycobacterium bovis* (72), and *Staphylococcus aureus* (15) but does not appear to be required for the virulence of *Salmonella enterica* serovar Typhimurium (68), *Mycobacterium tuberculosis* (64), *Legionella pneumophila* (51), or *Porphyromonas gingivalis* (32) in experimental models.

In this report, we present evidence that AhpC is the primary antioxidant used by *B. abortus* 2308 to detoxify endogenous \( \text{H}_2\text{O}_2 \) generated by respiratory metabolism during routine aerobic cultivation. KatE, on the other hand, plays a major role in scavenging exogenous and supraphysiologic levels of \( \text{H}_2\text{O}_2 \); although this enzyme can play a supporting role in the detoxification of \( \text{H}_2\text{O}_2 \) of endogenous origin if AhpC is absent. Interestingly, AhpC and KatE appear to play complementary roles in protecting *B. abortus* 2308 from \( \text{H}_2\text{O}_2 \) of metabolic origin during residence in mice, and the presence of either AhpC or KatE alone is sufficient to allow this strain to maintain a chronic infection.

### Materials and Methods

**Bacterial strains and growth conditions.** *Brucella abortus* 2308 and derivatives of this strain (Table 1) were cultivated on Schaedler agar (SA; Becton, Dickinson and Company), supplemented with 5% defibrinated bovine blood (SBA) at 37°C with 5% CO\(_2\) or in brucella broth (Becton, Dickinson and Company) at 37°C with shaking at 165 rpm. *Escherichia coli* strains were grown in Luria Bertani (LB) broth (SBA) at 37°C with 5% CO\(_2\) or in brucella broth (Becton, Dickinson and Company) at 37°C with shaking at 165 rpm. *Salmonella enterica* serovar Typhimurium S19 containing the katE gene from *B. abortus* S19 (18) was used to introduce defined mutations into the genome of *B. abortus* 2308 by the gene replacement strategy (19). The strategy (19) was used to introduce defined mutations into the genome of *B. abortus* 2308. ColEl1-based plasmids containing cat-disrupted versions of the *ahpCD* (pKHS3) and *katE* (pMEK7-9c) loci and a bla-disrupted *ahpCD* locus (pKHS5) (Table 1) were independently introduced into *B. abortus* 2308 by

### Table 1. Bacterial strains used in this study

| Strain or plasmid | Genotype or description | Reference or source |
|-------------------|-------------------------|---------------------|
| *Escherichia coli* DH5α | F\(^{-}\) d80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(k\(^+\) m\(^{6+}\)) phoA supE44 thi-1 gyrA96 relA1 | Invitrogen |
| *Brucella abortus* 2308 | Virulent challenge strain | Laboratory stock |
| KH16 | 2308 *ahpCD*:cat; Cm\(^{r}\) | This study |
| KH40 | 2308 *ahpCD*:bla; Ap\(^{r}\) | This study |
| KH2 | 2308 katE*:cat; Cm\(^{r}\) | This study |
| MEK6 | 2308 *katE*:ahph3A; Km\(^{r}\) | This study |
| KK9 | 2308 *ahpCD*:bla katE*:ahph3A; Ap\(^{r}\) Km\(^{r}\) | This study |
| KK21 | 2308 *ahpCD*:cat katE*:ahph3A; Cm\(^{r}\) Km\(^{r}\) | This study |
| **Plasmids** | | |
| pGEM-T Easy | ColE1-based cloning vector; Ap\(^{r}\) | Promega |
| pBC KS+ | ColE1-based cloning vector; Cm\(^{r}\) | Stratagene |
| pBRR1MCS-4 | pBBR-based broad-host-range cloning vector; moderate copy no. (10-14 copies per cell); Ap\(^{r}\) | 35 |
| pMR10-Ap | RK2-based broad-host-range cloning vector; low copy no. (2-4 copies per cell); Ap\(^{r}\) | 69 |
| pMR10 | RK2-based broad-host-range cloning vector; low copy no. (2-4 copies per cell); Kan\(^{r}\) | 22 |
| pBlue-CM2 | 656-bp cat gene from pBC cloned into the EcoRV site of pBluescript KS+ | 54 |
| pKS+ Kan | 794-bp *ahpA* gene from TnphoA cloned into Sall-HindIII-digested pBluescript II KS+ | 35 |
| pMWV19 | 2,072-bp genomic DNA fragment from *B. abortus* 2308 containing *ahpCD* (PCR primers: *ahpCD*-3F/*ahpCD*-3R) cloned into pGEM-T Easy | This study |
| pMEK21 | Derivative of pBRR1MCS-4 carrying the *katE* gene from *B. abortus* S19 | 21 |
| pMWV77 | 2,094-bp NotI fragment from pMWV19 containing *ahpCD* cloned into pMR10-Ap | This study |
| pKHS6 | 1,382-bp genomic DNA fragment from *B. abortus* 2308 containing *ahpCD* (PCR primers: *ahpCD*-2F/*ahpCD*-2R) cloned into pBRR1MCS-4 | This study |
| pKHS2 | 2,499-bp genomic DNA fragment from *B. abortus* 2308 containing *ahpCD* (PCR primers: *ahpCD*-1F/*ahpCD*-1R) cloned into pGEM-T Easy | This study |
| pKHS3 | Derivative of pKHS2 in which a 673-bp BsmBI/HindIII fragment internal to the *ahpC* and *ahpD* coding regions was replaced with the *cat* gene from pBluCM2 | This study |
| pKHS4 | 2,534-bp fragment from pKHS2 containing *ahpCD* cloned into pBC KS+ | This study |
| pKHS5 | 2,950-bp HindIII/HincII fragment internal to the *ahpC* and *ahpD* coding regions was replaced with the *bla* gene from pGEM-T Easy | This study |
| pKHS4 | 2,534-bp fragment from pKHS2 containing *ahpCD* cloned into pBC KS+ | This study |
| pMEK7-9 | 1,917-bp genomic DNA fragment from *B. abortus* S19 containing *katE* cloned into the PvuII site of pUC18 | This study |
| pMEK7-9c | Derivative of pMEK7-9 in which a 1-kb PstI/EcoRI fragment internal to the *katE* coding region was replaced with the *cat* gene from pBlue-CM2 | This study |
| pMEK7-9k | Derivative of pMEK7-9 in which a 1-kb PstI/EcoRI fragment internal to the *katE* coding region was replaced with the *aph3A* gene from pKS+ Kan | This study |
electroporation, and transformants were selected on SBA containing chloramphenicol or ampicillin. Putative aphCD-cat (designated KH16), aphCD-bla (designated KH40), and katE-cat (designated KH2) mutants were selected for further evaluation based on their failure to grow on SBA supplemented with ampicillin (KH16 and KH2) or chloramphenicol (KH40). The genotypes of KH16, KH40, and KH2 were confirmed by PCR analysis of genomic DNA from these strains by use of aphCD, katE, cat, and bla-specific primer sets as appropriate and Southern blot analysis with probes for aphCD, cat, and bla.

Plasmid pMEK7-9k, which contains an aphC-x-DraI disrupted version of katE (21), was introduced into the B. abortus aphCD mutant KH16 by electroporation, and transformants were selected on SBA supplemented with kanamycin. A putative B. abortus aphCD katE double mutant (designated KK21) was selected for further evaluation based on its resistance to kanamycin and chloramphenicol and its sensitivity to ampicillin. A two-step process was also used to construct a second B. abortus aphCD katE double mutant, designed to meet the regulatory requirement that Brucella strains engineered to possess resistance to chloramphenicol not be introduced into experimentally infected animals. First, pMEK7-9k was used in a gene replacement strategy as described above to construct a katE mutant (MEK6) from B. abortus 2308. Plasmid pKH5 was then used to introduce a bla disrupted version of the aphCD locus into MEK6, resulting in the construction of the B. abortus aphCD katE double mutant KK9.

The genotypes of B. abortus strains KK21 and KK9 were confirmed by PCR analysis of genomic DNA from these strains by use of aphCD, katE, cat, bla, and aph34-specific primer sets as appropriate and Southern blot analysis with probes for aphCD, cat, bla, and aph34.

Crystal violet exclusion was used to verify that the B. abortus aphCD and katE mutants and aphCD katE double mutants retain their smooth lipopolysaccharide phenotypes (1). A solution of 3% H2O2 was also placed on bacterial colonies grown on Schaedler agar to verify the presence or absence of visible catalase activity in the B. abortus strains used in this study.

Quantification of peroxide levels in Brucella abortus cell suspensions. B. abortus strains grown on SBA supplemented with the appropriate antibiotics for 48 h were inoculated into 3 ml brucella broth in 17- by 100-mm tubes and incubated at 37°C with shaking at 165 rpm. Following overnight incubation, the bacterial cells were harvested by centrifugation and resuspended in phosphate-buffered saline (PBS) to an optical density at 600 nm (OD600) of 1.0. A commercial peroxidase method for measuring hydrogen peroxide, and 10 μl of a fresh 0.5% solution of paraquat (PO4; Acrós Organics) was added to each tube. Plates were incubated for 3 days, and the zones of inhibition surrounding each disk were measured in millimeters.

This same assay was also used to measure the sensitivity of B. abortus strains to H2O2 with the exceptions that 10 μl of a 30% solution of H2O2 was added to the filter disks instead of PO4, and SA plates supplemented with bovine catalase were not used.

Growth characteristics of the Brucella abortus strains in rich and nutrient-limited media. B. abortus strains were grown overnight in 3 ml brucella broth in 17- by 100-mm culture tubes incubated at 37°C with shaking at 165 rpm. The resulting cultures were inoculated into either 500-ml flasks containing 100 ml of brucella broth at a cell density of approximately 109 CFU per ml or 500-ml flasks containing 100 ml Gerhardt’s minimal medium (GMM) (23) at a cell density of 109 CFU/ml and the flask incubated at 37°C with shaking at 165 rpm. The number of viable brucellae in these cultures was determined at selected time points by inoculation by serial dilution and plating on SBA or SBA containing the appropriate antibiotic.

Peroxynitrite resistance assay. B. abortus strains were grown on SBA at 37°C with 5% CO2 for 48 h. Bacterial cells were harvested and resuspended to a cell density of 109 in 1 ml PBS in 17- by 100-mm culture tubes. The peroxynitrite generator SIN-1 (3-morpholinosydnonimine HCl; Sigma Aldrich) at a final concentration of 15 mM and 1,000 U/ml of bovine catalase were added to the cell suspensions and the mixtures incubated for 1 h at 165 rpm at 37°C. The murine model of viable brucellae in these cultures and in parallel cultures that were not exposed to SIN-1 were then determined by serial dilution and plating on SBA.

Experimental infection of cultured murine macrophages. A modification of the methods described by Gee et al. (22) was used to evaluate the capacity of the B. abortus strains to survive and replicate in cultured murine resident peritoneal macrophages. Briefly, macrophages obtained from 6- to 8-week-old female BALB/c mice were seeded at a density of 1.5 × 106 cells per well in sterile 96-well plates in Dulbecco's modified Eagle's medium (ATCC) with fetal calf serum. After allowing phagocytosis to occur for 2 h, extracellular bacteria were removed by incubating the cell monolayers with 100 U/ml gamma interferon (IFN-γ; PeproTech) was added to cultured macrophages for 30 min before macrophages were washed and coincubated with the Brucella strains (multiplicity of infection [MOI], 50:1) that were previously opsonized with a subagglutinating concentration (1:1,000) of hyperimmune mouse serum. After allowing phagocytosis to occur for 2 h, extracellular bacteria were removed by incubating the cell monolayers with 100 U/ml gamma interferon (IFN-γ; PeproTech) was added to cultured macrophages for 30 min before macrophages were washed and coincubated with the Brucella strains (multiplicity of infection [MOI], 50:1) that were previously opsonized with a subagglutinating concentration (1:1,000) of hyperimmune mouse serum. After allowing phagocytosis to occur for 2 h, extracellular bacteria were removed by incubating the cell monolayers with 100 U/ml gamma interferon (IFN-γ; PeproTech) was added to cultured macrophages for 30 min before macrophages were washed and coincubated with the Brucella strains (multiplicity of infection [MOI], 50:1) that were previously opsonized with a subagglutinating concentration (1:1,000) of hyperimmune mouse serum. After allowing phagocytosis to occur for 2 h, extracellular bacteria were removed by incubating the cell monolayers with 100 U/ml gamma interferon (IFN-γ; PeproTech) was added to cultured macrophages for 30 min before macrophages were washed and coincubated with the Brucella strains (multiplicity of infection [MOI], 50:1) that were previously opsonized with a subagglutinating concentration (1:1,000) of hyperimmune mouse serum. After allowing phagocytosis to occur for 2 h, extracellular bacteria were removed by incubating the cell monolayers with 100 U/ml gamma interferon (IFN-γ; PeproTech) was added to cultured macrophages for 30 min before macrophages were washed and coincubated with the Brucella strains (multiplicity of infection [MOI], 50:1) that were previously opsonized with a subagglutinating concentration (1:1,000) of hyperimmune mouse serum. After allowing phagocytosis to occur for 2 h, extracellular bacteria were removed by incubating the cell monolayers with 100 U/ml gamma interferon (IFN-γ; PeproTech) was added to cultured macrophages for 30 min before macrophages were washed and coincubated with the Brucella strains (multiplicity of infection [MOI], 50:1) that were previously opsonized with a subagglutinating concentration (1:1,000) of hyperimmune mouse serum. After allowing phagocytosis to occur for 2 h, extracellular bacteria were removed by inc

| Designation | Sequence |
|-------------|----------|
| aphCD-1F    | 5′-GCCGAGAACCGGGAGACCAGGA-3′ |
| aphCD-1R    | 5′-TGGGGCAGTGCCGCTGATCC-3′ |
| aphCD-2F    | 5′-CCAGTGGCCAGAAAAATGTAAGGC-3′ |
| aphCD-2R    | 5′-GATCAAACCGAGCTGGCATTAGC-3′ |
| aphCD-3F    | 5′-GCCGAAGACCTTGCCGACAGA-3′ |
| aphCD-3R    | 5′-CATCGTACCGCTGGATCTG-3′ |
RESULTS

Identification of an alkyl hydroperoxide reductase complex (AhpCD) in B. abortus 2308. The genes designated BAB2_0531 and BAB2_0532 in the B. abortus 2308 genome sequence are annotated as ahpC and ahpD, respectively. The products of these two genes are predicted to be components of the alkyl hydroperoxide reductase complex AhpCD. In many bacteria, the peroxiredoxin AhpC serves as an important antioxidant that detoxifies hydrogen peroxide, organic peroxides, and/or peroxyxinitrite (9, 26, 61). AhpD is a peroxiredoxin reductase that uses reducing equivalents generated by cellular metabolism to recycle the enzymatic activity of AhpC (10, 26). The Brucella AhpC shares 47% amino acid identity with the Mycobacterium tuberculosis AhpC, and the Cys-61, Cys-174, and Cys-176 residues that have been shown to be important for activity in the latter protein (12, 26) are conserved as Cys-57, Cys-171, and Cys-173 in the Brucella AhpC ortholog. Likewise, Brucella AhpD displays 44% amino acid identity with its M. tuberculosis counterpart, and amino acid sequence alignment indicates that the Cys-131 and Cys-134 residues in this protein are equivalent to the Cys-130 and Cys-133 residues that are required for the peroxiredoxin reductase activity of mycobacterial AhpD (10, 26). Reverse transcriptase PCR analysis indicates that the ahpC and ahpD genes in B. abortus 2308 are cotranscribed as an operon (data not shown), which is consistent with the predicted function of their products in an enzymatic complex and the genetic organization of the ahpCD operons in other bacteria (10, 26).

A B. abortus ahpCD mutant exhibits higher levels of endogenous cellular peroxides than the parental strain. Studies performed with E. coli indicate that AhpC plays a major role in removing the H2O2 that is generated in the cytoplasm of this bacterium as a by-product of aerobic metabolism (61). Phenotypic analysis of the B. abortus mutant ahpCD KH16 suggests that Brucella the AhpC performs a similar function. Significantly higher levels of endogenous peroxides are detected in KH16 than in the parental 2308 strain following aerobic growth (Fig. 1), and the levels of these ROS are significantly diminished in a derivative of the ahpCD mutant carrying a plasmid-borne copy of the ahpCD locus. Endogenous peroxide levels also return to approximately wild-type levels in a derivative of KH16 carrying a plasmid that overexpresses katE (Fig. 1). Because monofunctional catalases such as the Brucella KatE detoxify H2O2 but not organic peroxides (13, 38), these findings indicate that the elevated levels of endogenous peroxides detected in the ahpC mutant are predominantly made up of H2O2. It is also important to note that overexpression of katE or the addition of exogenous catalase reduces the levels of endogenous peroxides detected in B. abortus 2308 cell suspensions below the baseline levels shown in Fig. 1 (data not shown), indicating that this assay provides a reliable indication of the levels of endogenous H2O2 generated by the Brucella strains examined in this study.

The biochemical properties of catalases allow these enzymes to degrade H2O2 across a broad range of concentrations (67). Accordingly, catalases often provide bacteria with a second line of defense against the buildup of endogenous H2O2 of metabolic origin when primary detoxifiers such as AhpC are absent (15, 61). The levels of peroxides detected in the B. abortus katE mutant KH2 are substantially lower than those detected in the isogenic ahpCD mutant and not significantly different from those detected in the parental 2308 strain. Moreover, although the levels of endogenous peroxides detected in the B. abortus ahpCD katE double mutant KK21 are consistently higher than those detected in the isogenic ahpC mutant KH16, these differences are not statistically significant. These experimental findings suggest that KatE plays a limited role in protecting B. abortus 2308 from the buildup of endogenous H2O2 during routine aerobic cultivation.

AhpCD is required for the wild-type resistance of B. abortus 2308 to endogenous H2O2 generated by the redox cycling agent paraquat. Paraquat (PQ) reacts with components of the respiratory chain in bacterial cells, leading to the univalent reduction of O2 and the generation of O2− in these cells (24). This O2− then serves as a substrate for cytoplasmic superoxide dismutases such as SodA, which can convert this ROS to H2O2 and O2 (20). Spontaneous nonenzymatic dismutation of O2− to H2O2 and O2 also occurs under physiologic conditions (27). Thus, one of the consequences of treating respiring bacterial cells with PQ is the generation of increased intracellular levels of H2O2. The B. abortus ahpCD mutant KH16 and the ahpCD katE mutant KK21 consistently and reproducibly exhibit larger zones of inhibition around disks containing PQ in a disk sensitivity assay than does the parental 2308 strain or the isogenic katE mutant (Fig. 2A). Introduction of a plasmid-borne copy of the ahpCD locus reduces the sensitivity of KH16 and KK21 to PQ to approximately the same levels displayed by B. abortus 2308.

H2O2 is an uncharged ROS and can readily cross cellular membranes by diffusion. This allows extracellular catalase to serve as an efficient detoxifier of intracellular H2O2 (62). Consequently, since the addition of paraquat results in the generation of both superoxide and hydrogen peroxide, an important
control in these assays is the addition of exogenous catalase to the test medium to relieve hydrogen peroxide toxicity. This allows for determination of whether or not the increased susceptibility of the *B. abortus* *ahpCD* and *ahpCD katE* mutants to PO is due to the increased intracellular accumulation of H$_2$O$_2$. As shown in Fig. 2B, the addition of exogenous catalase to the test medium reduces the zone of inhibition around disks containing PO exhibited by *B. abortus* KH16 (*ahpCD*) and KK21 (*ahpCD katE*) to the same size as those exhibited by the parental 2308 strain. This indicates that the increased sensitivity of the *B. abortus* *ahpCD* and *ahpCD katE* mutants to PO is due to the increased intracellular accumulation of H$_2$O$_2$ and not a differential sensitivity of these mutants to O$_2$. More importantly, these experimental findings further support the contention that AhpC serves as a primary detoxifier of endogenous H$_2$O$_2$ produced by respiratory metabolism in *B. abortus* 2308, while KatE plays a limited and secondary role in this regard.

The *ahpCD* locus is required for maintenance of stationary-phase viability of *B. abortus* 2308 during aerobic growth in a defined minimal medium. *B. abortus* 2308, KH16 (*ahpCD*), KH2 (*katE*), and KK21 (*ahpCD katE*) exhibit equivalent growth kinetics and viability during exponential growth and stationary phase when these strains are cultivated aerobically in brucella broth (Fig. 3A). When these strains are grown in Gerhardt’s minimal medium (GMM), however, the *B. abortus* *ahpCD* mutant KH16 and the isogenic *ahpCD katE* double mutant KK21 both exhibit a significant loss of stationary-phase viability compared to their respective parental strains 2308 and KH2. Similarly increased levels of endogenous peroxides are present in the *B. abortus* *ahpCD* mutant KH16 and the *ahpCD katE* double mutant KK21 compared to those present in 2308 and the katE mutant KH2 during growth in GMM (data not shown). The loss of stationary-phase viability in GMM exhibited by the *B. abortus* *ahpCD* mutant KH16 can also be rescued to a significant degree by the introduction of a plasmid containing either *ahpCD* or *katE* into this strain (Fig. 3C), and this phenotype in the *B. abortus* *ahpC katE* double mutant KK21 can be rescued by a plasmid carrying *katE*. These data suggest that AhpC plays a particularly important role in detoxifying endogenous H$_2$O$_2$ generated during stationary phase in *B. abortus* 2308 during *in vitro* cultivation under nutrient-limiting conditions.

**KatE is the major detoxifier of exogenous hydrogen peroxide in *B. abortus* 2308.** While AhpC appears to be the major detoxifier of endogenous H$_2$O$_2$ in *B. abortus* 2308, the results presented in Fig. 4 indicate that KatE is the major detoxifier of exogenous H$_2$O$_2$ in this strain. Even at levels of exogenous H$_2$O$_2$ as low as 5 μM, the *B. abortus* *katE* and *ahpCD katE* mutants exhibit a marked defect in their capacity to degrade exogenous H$_2$O$_2$ compared to their parental strains (Fig. 4), and these defects are much more dramatic when these strains are exposed to 50 and 100 μM H$_2$O$_2$ (Fig. 5). The role of KatE in the degradation of exogenous H$_2$O$_2$ is further reflected in the differences in the sensitivities to H$_2$O$_2$ exhibited by the *B. abortus* *ahpC katE* and *ahpCD katE* double mutant in a disk sensitivity assay (Table 3), where the strains lacking KatE display a much more pronounced phenotype than the *ahpCD* mutant.

The *B. abortus* *ahpCD* mutant KH16 displays increased sensitivity to peroxynitrite. Biochemical studies have shown that in addition to H$_2$O$_2$, AhpC can also detoxify organic peroxides such as tert-butyl hydroperoxide (t-BOOH) (26), cumene hydroperoxide (CHP) (28, 50), and peroxynitrite (ONOO$^-$) *in vitro* (9), and genetic studies have shown that this peroxiredoxin provides bacterial cells with an important defense against environmental exposure to these compounds (14, 39, 66). The results shown in Fig. 6 suggest that AhpC plays an important role in protecting *B. abortus* 2308 from exposure to ONOO$^-$. Compared to the parental strain, the *ahpCD* mutant KH16 displays an increased sensitivity to the ONOO$^-$ generator SIN-1 in an *in vitro* assay, and genetic complementation of KH16 with a plasmid-borne wild-type version of the *ahpCD* locus restores the resistance of the mutant to ONOO$^-$ to the same levels as those exhibited by the parent strain. In contrast, the extent to which AhpC contributes to the detoxification of organic peroxides in *B. abortus* 2308 is unclear. The *B. abortus* *ahpCD* mutant KH16 exhibits variable and inconsistent sensitivity to t-BOOH and CHP in *in vitro* assays, and the levels of lipid hydroperoxides present in *B. abortus* 2308 and
KH16 cells following aerobic growth are equivalent (data not shown).

The presence of either AhpC or KatE alone allows B. abortus strains to retain their virulence in the mouse model.

During residence in their mammalian hosts, Brucella strains are exposed to both exogenous ROS produced by the oxidative burst of host phagocytes and endogenous ROS arising as by-products of their own aerobic metabolism (57). To determine to what extent AhpC and KatE protect B. abortus 2308 from H₂O₂ of exogenous and endogenous origin in the host, the virulence properties of B. abortus 2308 and isogenic ahpCD, katE, and ahpCD katE mutants in cultured murine macrophages and experimentally infected mice were evaluated. Only the B. abortus ahpCD katE mutant KK9 exhibited significant and stable attenuation compared to the parental 2308 strain in cultured murine macrophages (Fig. 7), and this attenuation was consistently observed only when these phagocytes were stimulated with IFN-γ/H9253. Notably, the addition of apocynin (a NADPH oxidase inhibitor), L-NMMA (an iNOS inhibitor), or both of these inhibitors in combination to the phagocyte cultures failed to alleviate the attenuation exhibited by the B. abortus ahpCD katE mutant in the IFN-γ-treated macrophages (Fig. 7). These experimental findings suggest that neither AhpC nor KatE is playing a role in protecting B. abortus 2308 from exogenous ONOO⁻/H₂O₂ generated by the NADPH oxidase and iNOS activity of host macrophages. The B. abortus ahpCD katE double mutant KK9 was also the only mutant to display significant attenuation compared to the parental 2308 strain in C57BL/6 (Fig. 8A) or BALB/c (Fig. 8B) mice, and the severe attenuation exhibited by B. abortus 2308 from exogenous H₂O₂ produced as a result of the oxidative burst of these phagocytes. They also suggest that AhpC does not play a prominent role in protecting B. abortus 2308 from exogenous ONOO⁻ generated by the NADPH oxidase and iNOS activity of host macrophages. Instead, they support the contention that the buildup of endogenous H₂O₂ is a biologically relevant environmental stress encountered by B. abortus 2308 during its residence in the murine host (57). Moreover, they demonstrate that the presence of either AhpC or KatE alone is sufficient to alleviate this stress.
DISCUSSION

The experimental findings presented here show that AhpC plays a major role in scavenging H$_2$O$_2$ that is generated as a by-product of respiratory metabolism in *B. abortus* 2308. This function is similar to that reported for the *E. coli* AhpC and consistent with the reported biochemical properties of this class of peroxiredoxins in general, which work most efficiently on low levels of H$_2$O$_2$ (61). The capacity of AhpC to scavenge metabolic H$_2$O$_2$ appears to be especially important to *B. abortus* 2308 for the maintenance of stationary-phase viability when this strain is cultured under nutrient-limited conditions. This function is consistent with the observation that maximum expression of an *ahpC-lacZ* fusion is observed during stationary phase in *B. abortus* 2308 (K. Steele, unpublished observations), and AhpC has been proposed to be an important stationary-phase antioxidant (56, 60, 71). The basis for the H$_2$O$_2$-dependent loss of viability of the *B. abortus ahpC* mutant during stationary phase is not known. But the fact that the *ahpC* mutant does not exhibit this phenotype during growth in a nutritionally replete medium suggests that certain key biosyn-

![FIG. 5. KatE is the predominant detoxifier of 50 and 100 μM exogenous H$_2$O$_2$ in *B. abortus* 2308. Shown are the levels of peroxides present in *B. abortus* 2308 (A), KH16 (B), KH2 (C), and KK21 (D) cell suspensions at selected times following the addition of 50 μM (solid lines) and 100 μM (dashed lines) H$_2$O$_2$. Panel E shows levels of H$_2$O$_2$ detected in cell-free test medium at selected time points following the addition of 50 μM (solid lines) and 100 μM (dashed lines) H$_2$O$_2$. The data presented here are representative of multiple (≥3) experiments performed from which equivalent results were obtained. "EP" denotes the levels of endogenous peroxides detected prior to the addition of the H$_2$O$_2$; "↑" denotes the levels of intracellular peroxides detected immediately after the addition of the H$_2$O$_2$. Note that the time points after addition of the H$_2$O$_2$ differ for some of the panels in this figure.]

| TABLE 3. Sensitivity of *B. abortus* 2308, KH16 (2308 *ahpCD*), KH2 (2308 *katE*), and KK21 (2308 *ahpCD katE*) to H$_2$O$_2$ |
|-----------------------------------------------|
| Strain | Zone of inhibition (mm) |
|-------|-------------------------|
| 2308.. | 24 ± 0.58               |
| KH16.. | 27 ± 0.58*              |
| KH2... | 41 ± 1.5**              |
| KK21... | 41 ± 0.0**              |

* Zone of inhibition around disks containing 10 μl of a 30% solution of H$_2$O$_2$.
* P ≤ 0.05; ** P ≤ 0.005 for comparisons of 2308 versus KH16, KH2, or KK21.
thetic enzymes in *B. abortus* 2308 may be particularly susceptible to H$_2$O$_2$-mediated damage in the absence of AhpC. This phenotype could be masked if the products of the corresponding biosynthetic pathways can be readily obtained from the growth medium. H$_2$O$_2$-mediated damage of the 4Fe-4S clusters in isopropylmalate isomerase, a key enzyme in the leucine biosynthetic pathway, for instance, leads to growth arrest in *E. coli* katG katE ahpC mutants (29). In contrast to what was found for the ahpC mutant, the phenotypes exhibited by the *B. abortus* katE mutant and ahpCD katE mutant suggest that the sole catalase produced by this bacterium plays a minimal role in detoxifying endogenous H$_2$O$_2$ of metabolic origin during routine aerobic cultivation *in vitro*. These results are intriguing considering the compensa-
tory roles that AhpC and catalases have been reported to perform in scavenging cytoplasmic H$_2$O$_2$ of metabolic origin in ahpC mutants in other bacteria (15, 61). In many cases, the loss of either ahpC or a catalase gene alone does not produce an aerobic growth defect in bacteria, but the loss of both AhpC and a catalase does. The lack of an observable aerobic growth defect in the B. abortus katE mutant in vitro is also notable because KatG appears to be the major scavenger of endogenous H$_2$O$_2$ in Bradyrhizobium japonicum (46), a close phylogenetic relative of the brucellae. In fact, the observation that the B. abortus ahpCD katE double mutant does not exhibit a detectable defect in growth during routine aerobic cultivation in a rich medium or growth on agar plates suggests that this bacterium produces other antioxidants that are capable of compensating for the loss of AhpC’s capacity to detoxify H$_2$O$_2$ of metabolic origin. This proposition is further supported by the observation that introduction of the katE mutation into the B. abortus ahpC mutant did not increase this mutant’s loss of stationary-phase viability during aerobic culture in a minimal medium. Furthermore, the B. abortus ahpCD katE mutant can still degrade 50 μM H$_2$O$_2$, suggesting that other antioxidants are present to remove the H$_2$O$_2$. The products of the genes designated BAB1_0941 and BAB1_0504 in the B. abortus 2308 genome sequence would appear to be good candidates for this function. BAB1_0941 is predicted to encode a homolog of the bacterioferritin comigratory protein (Bcp) (30), and BAB1_0504 is predicted to encode an AhpC/TSA (thiol-specific antioxidant)-type peroxiredoxin that has sequence similarity to the PrxV-type peroxiredoxins that protect mammalian mitochondria from H$_2$O$_2$ damage (5). Whether or not the putative peroxiredoxins encoded by BAB1_0941 and/or BAB1_0504 can compensate for loss of AhpC activity in B. abortus 2308 remains to be determined experimentally.

Despite the fact that KatE appears to play a minimal role in protecting B. abortus 2308 from endogenous H$_2$O$_2$ during routine aerobic cultivation in vitro, the studies performed with experimentally infected mice suggest that this enzyme plays a pivotal backup role in protecting this bacterium from the metabolic H$_2$O$_2$ it generates during replication in the host. This proposition is based on two observations. First, although our in vitro studies suggest that the Brucella AhpC has the capacity to degrade H$_2$O$_2$, ONOO$^-$, and possibly organic peroxides (see below), the only described function for monofunctional catalases such as KatE that the authors are aware of is the degradation of H$_2$O$_2$. This strongly suggests that H$_2$O$_2$ toxicity plays a key role in the attenuation exhibited by the B. abortus ahpCD katE mutant. Second, this mutant displays the same level of attenuation in NADPH oxidase-deficient mice that it does in wild-type mice, indicating that AhpC and KatE do not provide protection from exogenous H$_2$O$_2$ produced as a by-product of the oxidative burst of host phagocytes. The fact that the presence of either AhpC or KatE alone allows B. abortus 2308 to maintain persistent infection in mice suggests that brucellae possess functionally redundant systems to protect themselves from the metabolic H$_2$O$_2$ they generate endogenously during replication in the host. This is perhaps to be expected of a bacterium that must deal with exposure to ROS of both endogenous origin as well as those generated by the NADPH oxidase and iNOS activity of host phagocytes (57) during residence in this environment.

The fact that neither AhpC nor KatE appears to play a role in protecting the brucellae from the oxidative burst of macrophages is intriguing, especially considering the role that ROS and IFN-γ have been proposed to play in the brucellacidal activity of these phagocytes (31). Moreover, the temporal nature of the attenuation exhibited by the B. abortus ahpC katE double mutant KK9 in cultured macrophages (e.g., 24 h p.i.) and the observation that attenuation of this mutant was observed only when these phagocytes were stimulated by IFN-γ are what would be predicted for a Brucella mutant with an increased sensitivity to the oxidative burst (31). The apparent lack of correlation between macrophage NADPH oxidase activity and the attenuation exhibited by the B. abortus ahpC katE double mutant is also perplexing given the documented role that Brucella SodC plays in detoxifying O$_2^-$ produced by host macrophages (22), a process that generates H$_2$O$_2$. One possibility is that the exogenous H$_2$O$_2$ produced as a by-product of the oxidative burst of host macrophages is less of a threat to the brucellae than the primary product of this reaction (e.g., O$_2^-$). This would be analogous to the situation observed for Salmonella strains, where mutants lacking SODs that detoxify exogenous O$_2^-$ (e.g., sodC mutants) are more attenuated in experimental hosts than the corresponding catalase- or AhpC-deficient mutants (17, 25, 68). Clearly, a more comprehensive evaluation of the gene products that protect the brucellae from the respiratory burst of host macrophages is warranted. It will also be important to define the Brucella cellular components that are prone to damage by exogenous and endogenous ROS during replication in host macrophages.

The increased sensitivity of the B. abortus ahpC mutant to the ONOO$^-$ generator SIN-1 in vitro assays suggests that the Brucella AhpC, like its counterparts in Salmonella enterica serovar Typhimurium, Mycobacterium tuberculosis, and Helicobacter pylori, has peroxynitrite reductase activity (9). This enzymatic activity has been proposed to be important as a bacterial defense against ONOO$^-$ production by host macrophages (39), but the results obtained in this study with the B. abortus ahpCD mutant KH40 and the ahpCD katE double mutant KK9 in iNOS-deficient mice suggest that AhpC does not play a prominent role in protecting the parental 2308 strain from ONOO$^-$ produced by host phagocytes.

In addition to their ability to detoxify H$_2$O$_2$ and ONOO$^-$, bacterial AhpC proteins have also been shown to be able to degrade organic peroxides. Indeed, the name alkyld hydroperoxide reductase reflects the fact that degradation of organic peroxides was the first property identified for many members of this class of bacterial enzymes (28). Thus, it is notable that no conclusive evidence was obtained from the studies described in this report supporting a role for AhpC in the detoxification of organic peroxides in B. abortus 2308. One possible explanation for these findings is that this bacterial strain also possesses the organic peroxide scavenger Ohr (40). Phenotypic analysis of an ohr mutant indicates a role for Ohr in the detoxification of the organic peroxides tert-butyl hydroperoxide and cumene hydroperoxide in B. abortus 2308 (J. Baumgartner, unpublished). Consequently, further phenotypic analysis of ahpC and ohr mutants and ahpC ohr double mutants will be needed to determine whether or not AhpC can detoxify organic peroxides in B. abortus 2308.

In summary, the results presented in this report indicate that
AfHC and KatE play distinct but complementary roles in protecting B. abortus 2308 from exposure to H₂O₂. In order to better understand the contributions of these antioxidant to the physiology and intracellular lifestyle of this bacterium, it will be important in future studies to examine the coordinate regulation of afhCD and katE during different stages of growth and in response to oxidative stress. It will also be important to determine how Brucella KatE is exported to the periplasm.

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