SodA is a major metabolic antioxidant in *Brucella abortus* 2308 that plays a significant, but limited, role in the virulence of this strain in the mouse model

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The gene designated BAB1_0591 in the *Brucella abortus* 2308 genome sequence encodes the manganese-cofactored superoxide dismutase SodA. An isogenic *sodA* mutant derived from *B. abortus* 2308, designated JB12, displays a small colony phenotype, increased sensitivity *in vitro* to endogenous superoxide generators, hydrogen peroxide and exposure to acidic pH, and a lag in growth when cultured in rich and minimal media that can be rescued by the addition of all 20 amino acids to the growth medium. *B. abortus* JB12 exhibits significant attenuation in both cultured murine macrophages and experimentally infected mice, but this attenuation is limited to the early stages of infection. Addition of the NADPH oxidase inhibitor apocynin to infected macrophages does not alleviate the attenuation exhibited by JB12, suggesting that the basis for the attenuation of the *B. abortus sodA* mutant is not an increased sensitivity to exogenous superoxide generated through the oxidative burst of host phagocytes. It is possible, however, that the increased sensitivity of the *B. abortus sodA* mutant to acid makes it less resistant than the parental strain to killing by the low pH encountered during the early stages of the development of the brucella-containing vacuoles in macrophages. These experimental findings support the proposed role for SodA as a major cytoplasmic antioxidant in brucella. Although this enzyme provides a clear benefit to *B. abortus* 2308 during the early stages of infection in macrophages and mice, SodA appears to be dispensable once the brucellae have established an infection.

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INTRODUCTION

Brucella abortus is a Gram-negative bacterium of the alphaproteobacteria family that produces disease in both cattle and humans (Roop *et al.*, 2009). Within their mammalian hosts, the brucellae invade macrophages and reside within specialized phagosomal compartments known as the replicative brucella-containing vacuoles (BCVs) (Celli *et al.*, 2003). Here, they encounter many environmental stresses, including exposure to reactive oxygen species (ROS) from the oxidative burst of macrophages, as well as exposure to ROS generated from their own aerobic metabolism (Roop *et al.*, 2009).

One of these ROS is superoxide ($O_2^{\cdot -}$) and brucella strains produce two superoxide dismutases (SODs), SodA (Sriranganathan *et al.*, 1991) and SodC (Beck *et al.*, 1990; Bricker *et al.*, 1990), that can directly detoxify this compound. SodA is a manganese-cofactored SOD that

resides in the cytoplasmic compartment of brucella strains (Stabel *et al.*, 1994). In contrast, the brucella SodC is a copper/zinc-cofactored SOD that resides in the periplasm (Stabel *et al.*, 1994). Because $O_2^{\cdot -}$ is a charged species, it does not easily cross cellular membranes, and consequently SODs almost exclusively detoxify $O_2^{\cdot -}$ in the intracellular bacterial compartments within which they reside (Sadosky *et al.*, 1994; St John & Steinman, 1996). Specifically, SodA usually detoxifies endogenously generated $O_2^{\cdot -}$ arising as a by-product of respiratory metabolism (Imlay, 2008), while SodC typically detoxifies $O_2^{\cdot -}$ of exogenous origin such as that generated by the respiratory burst of host phagocytes (De Groote *et al.*, 1997; Piddington *et al.*, 2001; Dunn *et al.*, 2003). Experimental evidence indicates that SodC plays this latter role in *B. abortus* 2308 (Gee *et al.*, 2005), but the biological function of SodA in this strain has not been examined. Consequently, the purpose of this study was to define the role of SodA in protecting *B. abortus* 2308 from the oxidative stresses encountered during *in vitro* growth as well as during its residence in a mammalian host.

Abbreviations: BCV, brucella-containing vacuoles; ROS, reactive oxygen species; SOD, superoxide dismutase.

METHODS

Bacterial strains, media and growth conditions. Routine cultivation of *Escherichia coli* strains was carried out in Luria–Bertani (LB) broth or on LB agar plates containing either 100 µg ampicillin ml⁻¹ (Sigma) or 45 µg kanamycin ml⁻¹ (Sigma), when appropriate. The *E. coli* strain DH5α was used as a host for procedures involving recombinant DNA. *B. abortus* strains were routinely grown in brucella broth or low iron minimal medium supplemented with 50 µM FeCl₃ (López-Goñi *et al.*, 1992) at 37 °C with aeration, or upon Schaedler agar (SA) supplemented with 5 % bovine blood (SBA) or Schaedler agar supplemented with 1000 U bovine liver catalase ml⁻¹ (Sigma) at 37 °C under 5 % CO₂. Frozen stocks of bacteria were maintained at -80 °C in a mixture of either 25 %, v/v, glycerol/75 % brucella broth (brucella strains) or 25 %, v/v, glycerol/75 % LB broth (*E. coli* strains).

Construction and genetic complementation of a *B. abortus* *sodA* mutant. The methods described by Elzer *et al.* (1994) were used to construct an isogenic *sodA* mutant from *B. abortus* 2308. PCR employing *Taq* polymerase (Invitrogen) with the oligonucleotide primers *sodA*UPfwd (5'-GCTCTAGAGGTCTTATTACGCCTAAAT-3') and *sodA*DOWNrev (5'-GCTCTAGATATGGATGCTGGACATGCAG-3') was used to amplify a 2355 bp fragment containing *sodA* and the downstream gene (BAB1_0592) from *B. abortus* 2308 genomic DNA. This fragment was then cloned into pGEM-T Easy (Promega), producing the plasmid designated pGEMsodA. Inverse PCR employing Herculase polymerase (Stratagene) with pGEMsodA as a template and the primers 5'-CGGGATCCCGGGATTACGTGCTGGAAATGTA-3' and 5'-GGAATTCCTCTTTTCTTTAA-GCTTGC-3' was used to generate a linearized derivative of pGEMsodA (designated pGEMΔsodA) from which all but 16 nt of the *sodA*-coding region were removed and replaced with the flanking *EcoRI* and *BamHI* sites. The resulting plasmid was recircularized by treatment with T4 DNA ligase. pGEMΔsodA was subsequently digested with *EcoRI* and *BamHI*, and ligated with the non-polar *aph3a*-based mutagenesis cassette from pUC18K (Ménard *et al.*, 1993). The resulting plasmid (designated pGEMΔsodAnpKn) was introduced into *B. abortus* 2308 by electroporation and transformants were selected on SBA supplemented with 45 µg kanamycin ml⁻¹. Putative *B. abortus* *sodA* deletion mutants were identified based on their resistance to kanamycin and sensitivity to ampicillin, and their genotypes were confirmed by Southern hybridization. Chromosomal DNA preparations from putative deletion mutants and strain 2308 were digested with *EcoRI* and *HindIII* and subjected to agarose gel electrophoresis. The DNA was transferred to nitrocellulose and the blots were hybridized with a *sodA*-specific probe that was generated using the *EcoRI* fragment bearing *sodA* from pJG39 (see below). The probe was labelled using the DIG-High Prime DNA Labelling and Detection Kit II (Roche) according to the manufacturer's instructions. One confirmed *B. abortus* *sodA* mutant, designated JB12, was selected for further phenotypic evaluation.

To facilitate complementation of the *B. abortus* *sodA* mutant, a 1105 bp fragment encompassing the *sodA* gene was amplified from *B. abortus* 2308 genomic DNA using the primers *sodA*ENGRSfwd (5'-CAGAAATCGCTATAAGCACCC-3') and *sodA*ENGNRSRev (5'-GCGTGCAAGACCCTGATA-3') and cloned into pGEM-T Easy, producing pJG39. A 1123 bp *EcoRI* fragment containing the *sodA* gene was then excised from pJG39 and cloned into the *EcoRI* site of pMR10-Ap (Valderas *et al.*, 2005). The resulting plasmid, designated pSodA6, was introduced into *B. abortus* JB12 by electroporation.

Determination of SOD activity of *B. abortus* strains. The methods described by Anderson *et al.* (2009) were used to measure the manganese, copper/zinc and iron SOD activities in cell lysates from the *B. abortus* strains.

Growth characteristics of *B. abortus* strains in rich and nutrient-limited culture media. *B. abortus* strains were grown on SBA at 37 °C with 5 % CO₂ for 72 h and harvested in PBS. The resulting cell suspensions were used to inoculate 100 ml brucella broth, low iron minimal medium supplemented with 50 µM FeCl₃ or the latter medium supplemented with 0.5 mM of a mixture containing all 20 amino acids in 500 ml Erlenmeyer flasks at a final cell density of approximately 5 × 10⁵ c.f.u. ml⁻¹. Cultures were incubated at 37 °C with shaking at 165 r.p.m., and at selected time points these cultures were serially diluted in PBS and plated on SBA, followed by incubation at 37 °C under 5 % CO₂.

Disc sensitivity assays for the resistance of *B. abortus* strains to O₂⁻ generators and H₂O₂. Mid-exponential-phase cultures of either *B. abortus* 2308 or JB12 were grown in brucella broth and adjusted to OD₆₀₀ 0.15 (~10⁹ c.f.u. ml⁻¹). Aliquots of bacterial cell suspensions (100 µl) were mixed with 3 ml brucella broth containing 0.5 % agar and the mixtures were overlaid onto either SA (for the assays measuring sensitivity to H₂O₂) or SA supplemented with 1000 U bovine liver catalase ml⁻¹ (for the assays measuring sensitivity to the O₂⁻ generators paraquat, menadione and pyrogallol). A 7 mm Whatman filter paper disc was placed in the centre of each plate and impregnated with 7 µl of one of the following compounds: 30 % H₂O₂ (Sigma), 250 mM paraquat (Aldrich), 10 mM menadione (Aldrich) or 1 M pyrogallol (Aldrich). After 3 days of incubation at 37 °C with 5 % CO₂, the diameter of the clear zones surrounding each disc was measured to the nearest millimetre. Zones from five separate plates were measured and the diameters were averaged for each strain and the data presented as zones of inhibition (in mm).

Sensitivity of *B. abortus* strains to exposure to acidic pH. *B. abortus* strains containing either pMR10-Ap or pSodA6 were grown on SBA plates at 37 °C under 5 % CO₂ and harvested in PBS, and this cell suspension was then used to inoculate 10 ml brucella broth containing 25 µg ampicillin ml⁻¹ or this medium buffered at pH 4.5 with 100 mM citrate at a final inoculum of 5 × 10⁵ c.f.u. ml⁻¹ in 50 ml conical tubes. These cultures were incubated with shaking at 37 °C, and at selected times after inoculation, the number of viable brucellae in these cultures was determined by serial dilution and plating on SBA, followed by incubation at 37 °C under 5 % CO₂.

Infection of cultured murine peritoneal macrophages. Previously described procedures were used to evaluate the capacity of the *B. abortus* strains to survive and replicate in cultured resident peritoneal macrophages obtained from 6- to 8-week-old female C57BL/6J mice (Gee *et al.*, 2005). Macrophages were activated 30 min prior to infection by the addition of 25 U interferon-γ ml⁻¹ (Steele *et al.*, 2010). For inhibition of the NADPH oxidase, cultured macrophages were treated with apocynin (Sigma) at a final concentration of 500 µM per well (Hart & Simons, 1992) immediately following seeding and then once more approximately 6 h prior to the first time point after infection.

Experimental infection of mice. Brucella strains were grown on SBA and infection doses were prepared as described previously (Elzer *et al.*, 1994). Six- to 8-week-old female C57BL/6J mice were infected via the intraperitoneal route with approximately 5 × 10⁴ *B. abortus* 2308, JB12 or JB12 [pSodA6]. At 1, 3 and 5 weeks post-infection, five mice from each experimental group were sacrificed by isoflurane overdose followed by cervical dislocation. Immediately following euthanasia, spleens were harvested. Serial dilution and plating of spleen homogenates on SBA or SBA supplemented with 45 µg kanamycin µl⁻¹ were performed to determine the number of brucellae per spleen at each time point. All experiments with mice were carried out under a protocol (AUP #K1436) approved by the Animal Care and Use Committee of East Carolina University.

Fig. 1. SOD activity in *B. abortus* 2308, JB12 (*sodA*) and MEK6 (*sodC*).

Statistical analysis. All statistical analysis was performed using Student's two-tailed *t*-test. *P*-values of less than 0.05 were considered significant (Rosner, 2000).

RESULTS

The *B. abortus* *sodA* mutant lacks manganese SOD activity and is sensitive to endogenous O_2^- and H_2O_2 in *in vitro* assays

SOD activity gels verified that the *B. abortus* *sodA* mutant JB12 lacks manganese SOD activity, but retains its copper/zinc SOD activity (Fig. 1). No evidence for iron SOD activity was detected in *B. abortus* 2308 or JB12, which is consistent with an earlier report (Sriranganathan *et al.*, 1991) and also consistent with the fact that no genes predicted to encode SODs can be detected in the genome sequence of this strain other than *sodA* (BAB1_0591) and *sodC* (BAB2_0535).

The *B. abortus* *sodA* mutant JB12 displayed significantly greater susceptibility to killing by both paraquat and menadione in disc sensitivity assays compared to the parental strain 2308 (Fig. 2a, b, respectively). Paraquat and menadione are both redox cycling agents that stimulate the production of supraphysiological levels of endogenous O_2^- during aerobic respiration in bacterial cells (Farrington *et al.*, 1973; Cadenas *et al.*, 1977; Hassan & Fridovich, 1979). Notably, the *B. abortus* *sodA* mutant did not exhibit a significant difference from the parent strain in its sensitivity to pyrogallol in disc sensitivity assays (Fig. 2c). Pyrogallol undergoes a spontaneous auto-oxidation in the

Fig. 2. Resistance profiles of *B. abortus* 2308, JB12 (*sodA*), and JB12 [pSodA6] to paraquat (1.75 μ mol) (a), menadione (70 mmol) (b), pyrogallol (7 μ mol) (c) and H_2O_2 (6.5 μ mol) (d) in disc sensitivity assays. The amount indicated above each graph represents the total quantity of the reagent in the 7 μ l applied to the filter paper disc. Plate assays employing the superoxide generators menadione, paraquat and pyrogallol were performed on Schaedler agar supplemented with 1000 U bovine liver catalase ml^{-1} to detoxify H_2O_2 resulting from the spontaneous dismutation of superoxide. Bars represent the mean $\pm$ SD of five zones of inhibition per strain per condition. The results presented are means $\pm$ SD from a single experiment that is representative of multiple (≥ 3) experiments performed from which equivalent results were obtained. Significance values: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$ for comparisons of 2308 versus JB12 and JB12 [pSodA6].

presence of molecular oxygen to produce O_2^- and this compound does not require interaction with the respiratory chain, NADP or NADPH in order to generate O_2^- . Consequently, it is often used as a generator of external O_2^- in biological assays (Marklund & Marklund, 1974), and in fact, pyrogallol has been used reliably in a previous study to measure the sensitivity of brucella strains to exogenous superoxide (Gee *et al.*, 2005). In addition, JB12 displayed an increased susceptibility to killing by H_2O_2 (Fig. 2d), which is consistent with the susceptibility profiles seen with *sodA* mutants in other species (Carlioz & Touati, 1986; Poyart *et al.*, 2001). The sensitivity of *sodA* mutants to H_2O_2 has been attributed to increased damage to

Fig. 3. Growth of *B. abortus* 2308, JB12 (*sodA*) and JB12 [pSodA6] in brucella broth. The results presented are means $\pm$ SD (not visible on graph) from a single experiment that is representative of multiple (≥ 3) experiments performed from which equivalent results were obtained.

iron-sulfur centres (Fe-S), the release of free iron and the resulting damage from increased Fenton chemistry (Imlay, 2006). Thus, the phenotype displayed by JB12 is what would be expected of a bacterial strain that has lost an important cytoplasmic defence against endogenous O₂⁻, and is similar to the phenotypes reported in the literature for other bacterial *sodA* mutants (Lynch & Kuramitsu, 2000).

The *B. abortus sodA* mutant has a growth defect that can be alleviated by the addition of exogenous amino acids

During its initial characterization, it was noted that the *B. abortus sodA* mutant JB12 produces smaller colonies on SBA plates than either the parental 2308 strain or a derivative of JB12 carrying pSodA6. As shown in Fig. 3, *B. abortus* JB12 also has a much longer generation time (8.17 h) than 2308 (2.06 h) or JB12 [pSodA6] (2.12 h) during growth in a rich medium, although the *B. abortus sodA* mutant eventually reaches the same cell density at stationary phase in this medium as 2308 and JB12 [pSodA6].

E. coli sodA sodB mutants exhibit a similar aerobic growth defect that results from O₂⁻-mediated damage to Fe-S centres in enzymes involved in amino acid biosynthesis (Carlioz & Touati, 1986; Brown *et al.*, 1995; Imlay, 2003) and the tricarboxylic acid pathway (Gardner & Fridovich, 1991; Liochev & Fridovich, 1993), and this growth defect can be overcome by the addition of exogenous amino acids to the growth medium (Carlioz & Touati, 1986). To determine if a similar deficiency in amino acid biosynthesis underlies the growth defect observed for the *B. abortus sodA* mutant, 2308, JB12 and JB12 [pSodA6] were cultured

Fig. 4. Growth of *B. abortus* 2308, JB12 (*sodA*) and JB12 [pSodA6] in minimal medium (MM) (a) and minimal medium supplemented with all 20 amino acids at a final concentration of 0.5 mM (b). The results presented are means $\pm$ SD from a single experiment that is representative of multiple (≥ 3) experiments performed from which equivalent results were obtained. Significance values: * $P < 0.05$ and *** $P < 0.005$ for comparisons of 2308 vs JB12 and JB12 [pSodA6].

in a nutrient-limited medium with or without the addition of a mixture of all 20 amino acids. While the parental 2308 strain and complemented *sodA* mutant exhibited comparable levels of growth over a 72 h period in the un-supplemented minimal medium, the *sodA* mutant did not exhibit net replication over this same period (Fig. 4a). The addition of all 20 amino acids to this culture medium rescued the growth defect exhibited by the *B. abortus sodA* mutant (Fig. 4b), but the combination of the branched-chain amino acids leucine, isoleucine and valine, plus pantothenic acid (which is also synthesized from 2-oxo-isovalerate, the product of the dihydroxy acid dehydratase reaction) did not (data not shown).

The *B. abortus sodA* mutant is sensitive to acidic pH

Increased sensitivity to acidic pH is a phenotypic characteristic that has been described for other bacterial *sodA*

Fig. 5. Sensitivity of *B. abortus* 2308 [pMR10-Ap] (black bars), JB12 [pMR10-Ap] (white bars) and JB12 [pSodA6] (cross-hatched bars) to exposure to pH 4.5. The results presented are means $\pm$ SD from a single experiment that is representative of multiple (≥ 3) experiments performed from which equivalent results were obtained. Significance values: ** $P < 0.01$ and *** $P < 0.005$ for comparisons of 2308 [pMR10-Ap] versus JB12 [pMR10-Ap]; †† $P < 0.01$ and ††† $P < 0.005$ for comparisons of 2308 [pMR10-Ap] versus JB12 [pSodA6].

mutants (Kim *et al.*, 2005), and such a phenotype would potentially be relevant for the *B. abortus* *sodA* mutant because the brucellae reside in acidified compartments during the early stages of their interactions with host macrophages (Porte *et al.*, 1999). As shown in Fig. 5, *B. abortus* JB12 displayed a significant defect in its capacity to resist exposure to pH 4.5 *in vitro* compared with the parental 2308 strain, and this defect was largely alleviated when a wild-type copy of *sodA* was introduced into this strain on a plasmid.

The *B. abortus* *sodA* mutant displays modest attenuation in the mouse model of infection

Reduced numbers of intracellular *B. abortus* JB12 were recovered from cultured murine macrophages compared to strains 2308 and JB12 [pSodA6] at all experimental time points examined after infection, regardless of whether or not these phagocytes had been treated with the NADPH oxidase inhibitor apocynin (Fig. 6a, b). These results suggest that the attenuation exhibited by *B. abortus* JB12 in cultured murine macrophages is not linked to a reduced ability to resist exposure to the oxidative burst of these phagocytes. Although the *B. abortus* *sodA* mutant JB12 exhibited limited, but significant, attenuation at 1 week post-infection in C57BL/6J mice compared with the parental strain and complemented mutant (Fig. 7), the *sodA* mutant was not attenuated at 3 and 5 weeks post-infection in mice.

Fig. 6. Survival and replication of *B. abortus* 2308, JB12 (*sodA*) and JB12 [pSodA6] in cultured murine resident peritoneal macrophages activated with interferon- γ (a) and in activated macrophages treated with 500 μ M of the NADPH oxidase inhibitor apocynin (b). Error bars represent SD from triplicate platings taken from three individual wells per strain per time point. The results presented are means $\pm$ SD from a single experiment that is representative of multiple (≥ 3) experiments performed from which equivalent results were obtained. Significance values: ** $P < 0.01$ and *** $P < 0.005$ for comparisons of 2308 versus JB12 and JB12 [pSodA6].

DISCUSSION

The results presented in this paper indicate that SodA is a major cytoplasmic antioxidant that protects *B. abortus* 2308 from $O_2^{\cdot-}$ generated as a by-product of its respiratory metabolism. As has been shown with other bacteria, SodA activity appears to be particularly important for preventing $O_2^{\cdot-}$ -mediated damage to the Fe-S centres of enzymes involved in the biosynthesis of amino acids in this strain. Although the specific enzymes that are prone to $O_2^{\cdot-}$ -mediated damage cannot be determined from the studies presented here, the dihydroxy acid dehydratase that participates in the biosynthesis of the branched-chain amino acids valine, leucine and isoleucine would appear to be a logical candidate, since the Fe-S centres in this enzyme in *E. coli* have been shown to be susceptible to $O_2^{\cdot-}$ -mediated damage (Kuo *et al.*, 1987; Brown *et al.*, 1995),

Fig. 7. Spleen colonization profiles for *B. abortus* 2308, JB12 (*sodA*) and JB12 [pSodA6] in C57BL/6J mice. Error bars represent SD of the number of viable brucellae recovered from five mice per strain per experimental time point. Significance value: *** $P < 0.005$ for comparisons of 2308 versus JB12 and JB12 [pSodA6].

and branched-chain amino acid auxotrophy is a characteristic feature of *E. coli* *sodA* *sodB* mutants (Carlioz & Touati, 1986). The inability of the branched-chain amino acids alone to rescue the growth defect exhibited by the *B. abortus* *sodA* mutant strain in a minimal medium, however, indicates that damage to dihydroxy acid dehydratase is not the sole basis for this phenotype. Certain forms of the enzymes fumarase and aconitase also possess Fe-S centres that are susceptible to $O_2^{\cdot-}$ -mediated damage (Gardner & Fridovich, 1991; Liochev & Fridovich, 1993) and inactivation of these enzymes may also play a role in the growth defect observed in the *B. abortus* *sodA* mutant. Fumarase and aconitase play important roles in carbon flow through the tricarboxylic acid pathway, and a survey of the *B. abortus* 2308 genome sequence indicates the presence of genes predicted to encode both the superoxide-sensitive (FumA; BAB1_0977) and superoxide-resistant (FumC; BAB2_0186) forms of fumarase (Woods *et al.*, 1988), but only the superoxide-sensitive form of aconitase (AcnA; BAB1_0090) (Varghese *et al.*, 2003). Thus, it seems likely that SodA protects multiple key metabolic enzymes in *B. abortus* 2308, but the precise identity of these enzymes remains to be experimentally determined. Because $O_2^{\cdot-}$ -mediated release of iron from Fe-S centres leads to DNA damage through the generation of $OH^{\cdot}$ via Fenton chemistry, SodA may also play an indirect role in preventing DNA damage in this bacterium, like it does in *E. coli* (Keyser *et al.*, 1995).

The *B. abortus* *sodA* mutant exhibits significant, but limited, attenuation in both cultured murine macrophages and experimentally infected mice during the early stages of these infections. The brucella strains were opsonized with brucella-specific IgG prior to infection of the macrophages, which means that their entry into these host cells was primarily due to Fc receptor-mediated phagocytosis.

Consequently, it is doubtful that the early attenuation displayed by the *B. abortus* *sodA* mutant is due to a defect in host cell entry. It also seems unlikely that the amino acid auxotrophy displayed by this mutant plays a major role in its attenuation, because brucella strains with defined mutations in branched-chain amino biosynthetic pathways generally show much greater attenuation in macrophages and mice (Foulongne *et al.*, 2000; Köhler *et al.*, 2002; Lestrade *et al.*, 2003; Alcantara *et al.*, 2004) than that exhibited by the *sodA* mutant. It is conceivable, however, that the metabolic defects underlying the *in vitro* growth defect displayed by the *B. abortus* *sodA* mutant also result in slower growth *in vivo*, which would result in a delay in the peak of spleen colonization levels in mice, and be manifested as 'early attenuation' in this model.

Based on its cytoplasmic location, SodA would not be expected to provide *B. abortus* 2308 with direct protection against exogenous $O_2^{\cdot-}$ generated by the respiratory burst of host phagocytes, an event that occurs during the early stages of macrophage infection, and indeed inhibition of NADPH oxidase activity did not alleviate the attenuation of the *sodA* mutant in cultured murine macrophages. Increased sensitivity to acidic pH is a phenotype that has been described for some bacterial *sodA* mutants (Kim *et al.*, 2005), but the physiological basis for this phenotype is unclear. The *B. abortus* *sodA* mutant also exhibits an increased susceptibility to acidic pH compared to the parental strain, and this characteristic is notable because the phagosomal compartments within which the brucellae reside during the early stages of their interactions with host macrophages have an acidic pH (Porte *et al.*, 1999). Hence, it is possible that the acid-sensitive nature of the *sodA* mutant contributes to its attenuation in macrophages and mice. Confirming such a relationship is not straightforward experimentally, however, since the acidic pH encountered during the early stages of the intracellular infection of host macrophages serves as an important environmental stimulus for inducing the expression of the *virB* genes which encode the type IV secretion system (Boschiroli *et al.*, 2002). Effectors secreted by the type IV secretion system are required for the proper trafficking of BCVs in host macrophages (Celli *et al.*, 2003) and neutralizing the pH in BCVs during the early stages of their development prevents the intracellular brucellae from replicating (Porte *et al.*, 1999). Thus, we were unable to make a definitive link between the acid-sensitive nature of the *B. abortus* *sodA* mutant and its attenuation in cultured macrophages.

Regardless of the basis for the attenuation of the *B. abortus* *sodA* mutant, the phenotype exhibited by this strain in mice indicates that SodA is dispensable once the brucellae have established an infection in the host. Since SodA is the only cytoplasmic SOD produced by brucella strains (Sriranganathan *et al.*, 1991) and our phenotypic analysis of JB12 indicates that SodA serves as a major cytoplasmic antioxidant during routine aerobic cultivation of the parental strain *in vitro*, the phenotype exhibited by the *B. abortus* *sodA* mutant in mice may be indicative of the

physiological state of the brucellae during their residence in the host. Multiple studies have shown that microaerobic and/or anaerobic respiration is important for the virulence of brucella strains (Endley *et al.*, 2001; Köhler *et al.*, 2002; Haine *et al.*, 2006; Loisel-Meyer *et al.*, 2006; Jiménez de Bagüés *et al.*, 2007). Thus, the combination of a reduced growth rate (Roop *et al.*, 2003) and lower O₂ availability may prevent endogenous O₂^{-•} levels from reaching a point where a cytoplasmic SOD is required for their detoxification in brucella strains once they have established their intracellular residence in the host. The presence of O₂^{-•}-resistant metabolic enzymes, such as FumC, may also contribute to a baseline resistance of this strain to endogenous O₂^{-•}, but it is important to note that the attenuation exhibited by the *B. abortus* *sodA* mutant in the mouse model is much less than that displayed by an isogenic mutant that lacks both KatE and AhpC (cytoplasmic antioxidants that detoxify H₂O₂ of endogenous origin) (Steele *et al.*, 2010). Thus, it appears that there is still a lot to learn about the nature of the oxidative stresses brucella strains encounter in the host and how they protect themselves against these stresses.

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