Regulation of \textit{Brucella abortus} Catalase

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All aerobic organisms have mechanisms that protect against oxidative compounds. Catalase, peroxidase, superoxide dismutase, glutathione, and thioredoxin are widely distributed in many taxa and constitute elements of a nearly ubiquitous antioxidant metabolic strategy. Interestingly, the regulatory mechanisms that control these elements are rather different depending on the nature of the oxidative stress and the organism. Catalase is well documented to play an important role in protecting cells from oxidative stress. In particular, pathogenic bacteria seem to use this enzyme as a defensive tool against attack by the host. To investigate the significance of catalase in hostile environments, we made catalase deletion mutations in two different \textit{B. abortus} strains and used two-dimensional gel analysis, survival tests, and adaptation experiments to explore the behavior and role of catalase under several oxidative stress conditions. These studies show that \textit{B. abortus} strains that do not express catalase activity exhibit increased sensitivity to hydrogen peroxide. We also demonstrate that catalase expression is regulated in this species, and that preexposure to a sublethal concentration of hydrogen peroxide allows \textit{B. abortus} to adapt so as to survive subsequent exposure to higher concentrations of hydrogen peroxide.

Catalase activity is widely regarded as essential or nearly essential for aerobic life because oxidative metabolism produces superoxide and hydrogen peroxide as inevitable by-products (15). Pathogens face the additional challenge of oxidative intermediates produced by neutrophils and macrophages. Upon infection, these phagocytes suddenly increase oxygen consumption and produce oxygen intermediates, such as \textit{H}_2\textit{O}_2, superoxide, \textit{HOCl}, hydroxyl radical, and singlet oxygen (2–4, 33).

\textit{Brucella abortus} is an intracellular parasite that causes bovine brucellosis, a disease characterized by fever and reproductive failure due to abortion, epididymitis, and male sterility (18, 48, 52). Within hours after exposure of a host animal to \textit{B. abortus} by ingestion or via the conjunctiva, most bacteria are found in phagocytic cells. In chronic disease, the bacteria persist and multiply inside phagocytic cells (9, 21, 41, 47, 49, 75).

The genus \textit{Brucella} consists of a very closely related group of pathogens classified into six species (48). These species have a very similar genetic makeup but exhibit different host species virulence. They are estimated to differ from one another by subtle alterations in outer membrane characteristics and genetic differences between strain 19 and more virulent strains in infections in many animals including humans. The only known prolonged passage in the laboratory. Both strains cause persistent infections in many animals including humans. The only known genetic differences between strain 19 and more virulent strains are subtle alterations in outer membrane characteristics and the inactivation of the erythritol catabolic pathway in strain 19 (58). In other respects, strain 19 represents a convenient model for the entire \textit{Brucella} genus.

Generally, pathogenic bacteria possess adaptive and defensive mechanisms that allow survival in the hostile phagocyte environment (6, 13, 24, 38, 41). Those that survive inside the phagosome are thought to change their physiology by altering their protein expression patterns in response to the new environment. The physiological changes undergone by \textit{Escherichia coli} in response to oxidative stress have been extensively studied (19, 70, 74). Since superoxide and \textit{H}_2\textit{O}_2 are central to the chemistry of the oxidative burst, superoxide dismutase (SOD) and catalase are considered to be important aspects of bacterial defenses (2, 17, 20, 30, 32), but the overall responses are much more complicated than two enzymatic activities. In \textit{E. coli}, 30 proteins are induced by external \textit{H}_2\textit{O}_2 (70). \textit{E. coli} expresses two types of catalase, a periplasmic peroxidase-catalase (HPI, encoded by \textit{katG}), and a cytoplasmic catalase (HPII, encoded by \textit{katF}). \textit{E. coli} exhibits 40 superoxide-inducible proteins, some of which are also inducible by \textit{H}_2\textit{O}_2 (28, 74). \textit{E. coli} expresses three SODs: constitutive Fe-SOD, superoxide-inducible Mn-SOD (32), and periplasmic Cu-Zn SOD (5). The distinctive functions of the three enzyme types are not well understood. Heat shock proteins are induced by oxidative stress in \textit{E. coli} and may play an important role through general mechanisms that protect cellular proteins from a wide variety of stress conditions (8, 26, 53, 69, 70).

\textit{Brucella} species express a Mn-SOD in the cytoplasm (65) and a Cu-Zn SOD in the periplasm (66). The only known catalase activity is restricted to the periplasm (63). Due to their periplasmic location, Cu-Zn SOD and catalase are thought to be involved in protecting the bacteria from external sources of oxidative compounds (63). Deletion of the \textit{Brucella} Cu-Zn SOD has only a moderate effect on survival in vivo (42, 71). A direct test of the importance of catalase in \textit{B. abortus} has not been previously reported, although catalase activity has long been considered to be a virulence factor (36). In vitro studies with neutrophil extracts, showing that oxygen-dependent killing is more potent than oxygen-independent killing of \textit{B. abortus} (55), and the observation that addition of exogenous catalase protects brucellae from being killed by cultured murine peritoneal macrophages and J774A.1 cells (39) indirectly confirm the importance of catalase to this species.

In this study, we directly explored the importance of the
Materials and Methods

Bacterial strains and medium. B. abortus strain 19, which is used as a cattle vaccine, and strain 2308 were obtained in duplicates from the National Animal Disease Center, Ames, Iowa, and reconstituted as instructed. Growing brucellae were maintained on tryptose agar (Difco). For two-dimensional (2-D) gel analysis, bacteria were grown in liquid minimal medium. Minimal medium was prepared as described by Gerhardt (28), except that glutamine replaced asparagine. The Cu-Zn SOD deletion mutant, S19ΔsodC, was described previously (71).

For adaptation experiments, B. abortus was grown on tryptose agar plates for 2 days. Bacteria were scraped from the agar and resuspended in tryptose broth. The cell concentration was adjusted to 0.01 absorbance at 600 nm (A600) unit. Cultures were pretreated with 1 mM H2O2, 100 µg of chloramphenicol per ml, plus 1 mM H2O2 for 1 h or left untreated, and then different concentrations of hydrogen peroxide (10 to 100 mM) were added. After 30 min of H2O2 treatment, diluted liquid culture was spread onto tryptose agar plates.

Gene replacement. ColE1-based plasmids have been previously shown not to replicate in B. abortus (29). Plasmid pCat5 is based on pUC119, which has a ColE1 replication origin and a gene for ampicillin resistance. The catalase replacement plasmid (see Results) was introduced into B. abortus by electroporation, and double recombinants were selected for kanamycin resistance and ampicillin sensitivity. The procedure followed the method of Tatum et al. (71).

Briefly, B. abortus was prepared by washing with water and kept frozen in 10% glycerol. A 2-µg portion of plasmid was mixed with Brucella and electroporated at a setting of 25 µF and 2.5 kV, with the pulser controller set at 200 Ω, using a Gene Pulser transfection apparatus (Bio-Rad Laboratories). After electroporation, bacteria were plated on tryptose agar containing 50 µg of kanamycin per ml and replica plated on tryptose agar containing 50 µg of ampicillin per ml.

2-D protein gel analysis. The procedures for 2-D protein gel analysis were based on those of O’Farrell (51). The detailed methods are described in the Millipore manual (Millipore investigator 2-D electrophoresis system operating and maintenance manual, 1991, Millipore Corp., Bedford, Mass.). B. abortus strain 19 was grown to log phase (A600 of 0.2 to 0.5) in liquid minimal medium.

The cultures were divided into small tubes, and 50 µl of [35S]methionine was added. Then, either nothing, 10 mM H2O2, or superoxide mixture (0.04 μM of xanthine oxidase per ml, 10 mM xanthine) was added to the liquid cultures and the bacteria were incubated for 1 h. After the labeling, bacteria were harvested by centrifugation for 10 min at 5,000 × g and were then mixed with sodium dodecyl sulfate sample buffer (0.3% sodium dodecyl sulfate, 0.06 M β-mercaptoethanol, 50 mM Tris-HCl [pH 8.4], boiled for 5 min, and treated with DNase and RNase for 10 min. The mixture was precipitated with 5% trichloroacetic acid to remove unincorporated radioactivity. Pellets were extracted with acetone to remove the trichloroacetic acid and redissolved in sample buffer containing 8 M urea, 3.2% NP-40, and 1.8% ampholytes, and 100,000 cpm of each sample was loaded on the first-dimension tube gel. Isoelectric focusing was done for 17 h at 1,000 V, and the first gel was placed on a 12% polyacrylamide second-dimension gel and run for 3.5 h at 14,000 mW per gel. Each gel was fixed with 5% acetic acid solution and placed on X-ray film after treatment with 1 M sodium salicylate for 30 min (14).

A series of exposures were taken to ensure that spot densities were within the working range of the film. Each fluorograph was scanned and analyzed with Millipore Bioimage software running on a Sun workstation. This software normalizes the density of each spot to the total spot density present on the film. The entire experiment was repeated three times. Values reported are from one complete set of experiments. The other experiments yielded similar but not identical results.

Western blot analysis. Antibodies used for the Western blots were polyclonal rabbit antisera. Antibodies for this study were developed in our laboratory and described in detail previously (10, 16, 27, 29, 62). The Western blot procedure used was based on that of Towbin et al. (72). After the 2-D gel electrophoresis had been performed, the proteins were electrophoretically transferred to a polyvinylidene difluoride (Micron Separations Inc.) membrane. The membranes were blocked with 1% nonfat dry milk in phosphate-buffered saline (PBS). The blots were incubated with any of the antigens (anti-catalase, anti-Cu-Zn SOD, anti-DnAκ, or anti-GroEL) and then washed four times for 10 min with PBS containing 0.03% Tween 20. The membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (ImmuNo Select Inc.) in PBS containing 1% nonfat milk and rinsed twice with 0.03% Tween 20 in PBS and twice with PBS. The blots were visualized by the color reaction (57) of H2O2 and 4-chloro-1-naphthol (Bio-Rad Laboratories).

Catalase assay. B. abortus was grown in tryptose broth to an A600 of 0.2 to 0.5. Then 10 mM H2O2 or superoxide mixture was added, and after 1 h the culture was centrifuged and resuspended in PBS. The resuspended cells were sonicated and mixed (ii) Halo assay. B. abortus culture was spread evenly on tryptose agar plates. A 5-mm-diameter filter disc was placed on the center of the plate. Then, various concentrations of HzO2 were applied to the filter disc. The diameter of the clear zone surrounding each filter disc was measured after 3 days of incubation at 37°C. Each experiment was done in triplicate and repeated at least three times.

Results

Deletion mutants. The Cu-Zn SOD deletion mutant (S19ΔsodC) of B. abortus strain 19 used in this study was previously described (71). Catalase deletion mutants of strains 2308 and strain 19 were constructed in a similar manner by gene replacement. Plasmid pCat5 (62), which contains the entire Brucella catalase coding sequence and flanking sequences, was doubly digested with BglII and PstI to remove 759 bp of the catalase coding region. The staggered ends were polished to blunt ends by incubation with the Klenow fragment of E. coli DNA polymerase I, and the neomycin-kanamycin resistance gene from Tn5 was inserted into the plasmid, effectively replacing the 3’ half of the catalase gene in the plasmid (29).

Both the 5’ and 3’ ends of the replacement were sequenced to confirm the gene deletion. The respective mutant strains are designated S2308cat and S19Δcat. Catalase deletion mutants did not express detectable levels of catalase activity.

Response of catalase deletion mutants to oxidative stress. Survival of bacteria in response to oxidative stress is sensitive to the details of the experiment. Figure 1 compares the survival of the catalase-deficient strains when exposed to H2O2 while growing in liquid tryptose medium. H2O2 challenge in liquid culture represents an acute exposure for catalase-expressing strains since H2O2 is destroyed in a short time. Under these conditions, a functional catalase provides significant protection to strain 19 but is much less important to the survival of strain 2308.
stress and that catalase activity is regulated. The bacteria respond to this level of oxidative superoxide mixture (Table 1) in tryptose broth. This result indicates that the bacteria respond to this level of oxidative superoxide mixture (Table 1) in tryptose broth.

The halo assay provides a test of resistance to longer-term exposure to H2O2. In this test, a filter disc is placed on a lawn of newly seeded bacteria and different concentrations of hydrogen peroxide are applied to the discs. H2O2 diffuses from each disc, providing chronic exposure to different concentrations of H2O2 depending on the distance from the disc. The diameter of the clear zone surrounding each filter disc provides a measure of the bacterial sensitivity to the diffusing agent.

**FIG. 2. Halo assay comparing the hydrogen peroxide sensitivities of strains 19 and S19cat.** Liquid culture was grown to mid log phase and then spread on tryptose agar plates to form a bacterial lawn. A filter disc containing the indicated concentration of hydrogen peroxide was placed at the center of each plate. After incubation for 3 days, the clear zone surrounding each filter disc was measured and plotted against the hydrogen peroxide concentration.

The halo assay was used to test the effects of H2O2 and superoxide mixture on the survival of *B. abortus* strain 19. The survival of strain 19 and the *sodC* mutant was greater than 95% whereas the catalase deletion mutant, *S19cat*, was 100% for the wild type and 70% for *S19ΔsodC*. The survival of *B. abortus* strain 19 in minimal medium plus superoxide mixture for 1 h was rapidly killed (data not shown). The survival of the wild type and *S19Δcat* and 70% for *S19ΔsodC*.

Catalase enzyme activity increased approximately fourfold after 1 h of exposure to either 10 mM hydrogen peroxide or superoxide mixture (Table 1) in tryptose broth. This result indicates that the bacteria respond to this level of oxidative stress and that catalase activity is regulated.

To confirm the apparent regulation of catalase, we conducted 2-D protein gel analysis. This technique also allowed a comparison of catalase expression with that of other polypeptides of interest. The 2-D gel system used typically resolves about 1,000 polypeptide spots. In this study, we used specific rabbit antisera to identify DnaK heat shock protein, GroEL heat shock protein, Cu-Zn SOD, and catalase on the 2-D gel electrophoresis pattern. Identification of the catalase and SOD spots was confirmed by comparison with 2-D gels of the *S19Δcat* and *S19ΔsodC* mutants. DnaK and GroEL were both revealed as multiple spots on 2-D gels, catalase was revealed as an elongated smear, and Cu-Zn SOD was revealed as a simple but minor spot.

**TABLE 1. Induction of catalase activity in Brucella strain 19 by 10 mM H2O2 or superoxide mixture for 1 h**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Catalase activity (U/mg)</th>
<th>Increase (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>H2O2</td>
<td>2.35</td>
<td>3.6</td>
</tr>
<tr>
<td>Superoxide mixture</td>
<td>2.80</td>
<td>4.7</td>
</tr>
</tbody>
</table>

**TABLE 2. Observed polypeptide changes on 2-D gels**

<table>
<thead>
<tr>
<th>Strain and treatment</th>
<th>GroEL</th>
<th>DnaK</th>
<th>Cu-Zn SOD</th>
<th>Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>19, H2O2</td>
<td>Small</td>
<td>Small</td>
<td>2-fold</td>
<td>&gt;10-fold</td>
</tr>
<tr>
<td>19, superoxide</td>
<td>Small</td>
<td>Small</td>
<td>5-fold</td>
<td>&gt;10-fold</td>
</tr>
<tr>
<td>S19Δcat, H2O2</td>
<td>Small</td>
<td>Small</td>
<td>3-fold</td>
<td></td>
</tr>
<tr>
<td>S19Δcat, superoxide</td>
<td>Small</td>
<td>Small</td>
<td>5-fold</td>
<td></td>
</tr>
<tr>
<td>S19ΔsodC, H2O2</td>
<td>Small</td>
<td>Small</td>
<td>&gt;10-fold</td>
<td></td>
</tr>
<tr>
<td>S19ΔsodC, superoxide</td>
<td>Small</td>
<td>Small</td>
<td>&gt;10-fold</td>
<td></td>
</tr>
</tbody>
</table>

*Integrated spot density increases following treatment.*

**FIG. 2. Halo assay comparing the hydrogen peroxide sensitivities of strains 19 and S19cat.**
Adaptation to external hydrogen peroxide. Adaptation is an important strategy for bacterial survival in changing environments. To test for adaptation, log phase cultures of strain 19 growing in tryptose broth were divided into three groups of small cultures. One group received no pretreatment, one was exposed to 1 mM H$_2$O$_2$ for 1 h, and one was exposed to 1 mM H$_2$O$_2$ plus 100 μg of chloramphenicol per ml for 1 h. Immediately following pretreatment, either 0, 10, 20, 30, or 100 mM hydrogen peroxide was added and each culture was incubated for an additional 30 min, and the cultures were then plated. The results are presented in Table 3. The data show that simple pretreatment with a sublethal concentration of H$_2$O$_2$ provided dramatic protection. The amount of protection depended on the concentration of H$_2$O$_2$ in the challenge. With a 10 mM challenge, the protective effect was only 2-fold, while at 100 mM, pretreatment enhanced survival more than 25-fold. Chloramphenicol abrogated the protective effect of pretreatment, indicating that protein synthesis is required for adaptation.

### DISCUSSION

It is well established that phagocytic cells release active oxygen species which kill invading bacteria. Superoxide and hydrogen peroxide are toxic in their own right and are required for the production of more toxic molecules such as hydroxyl radical and HOCI (31, 33). Direct inactivation of superoxide and hydrogen peroxide would seem to be an effective strategy for pathogenic bacteria. Because some bacteria such as Salmonella (23) and Brucella (21) survive inside phagocytes for prolonged periods, it seems likely that these species alter their metabolism in response to the phagocytic environment. In fact, previous studies have shown that both Salmonella enterica serovar Typhimurium and B. abortus change their protein synthetic patterns when ingested by macrophages (17, 43, 54).

Pulse-labeling with $[^{35}\text{S}]$methionine followed by 2-D gel electrophoresis gives a rough measure of synthetic rates for individual polypeptides. In this study, we determined the effect of H$_2$O$_2$ and superoxide on the synthetic rate of DnaK, GroEL, Cu-Zn SOD, and catalase by this technique. In one report on B. abortus strain 2308 (54), both DnaK and GroEL exhibited decreased protein expression after a 60-min exposure to 50 mM H$_2$O$_2$. In contrast, we found that the synthetic rate of DnaK and GroEL did not change in response to oxidative stress. Both studies agree that the synthetic rates of both DnaK and GroEL respond very little to oxidative stress. Abshire and Neidhardt (1) reported that S. enterica serovar Typhimurium DnaK, GroEL, and GroES production did not increase significantly in the phagocyte. Ericsson et al. (22) showed that Francisella tularensis DnaK production was increased about fivefold but GroEL production did not increase greatly when the organism was phagocytized by macrophages. In another study of phagocytosis, virulent strains of S. enterica serovar Typhimurium increased heat shock protein synthesis but avirulent strains did not (11). Even though it is difficult to compare 2-D gel electrophoresis results from different laboratories, it is clear that macrophage-induced proteins are quite often different from those induced by chemical stimulation in vitro. For B. abortus, heat shock proteins do not seem to change synthetic rates greatly in response to intracellular conditions or to external oxidative stress.

Our results indicate that expression of Cu-Zn SOD is increased two- to fivefold in log-phase bacteria in response to oxidative stress, with the higher stimulation in response to superoxide. Log-phase synthesis of this enzyme is never very high, as judged by the intensity of the 2-D gel spot. In contrast, synthesis of catalase increases from nearly invisible to a major spot pattern in response to both H$_2$O$_2$ and superoxide. B. abortus expresses both Cu-Zn SOD and catalase in the periplasmic space. Because of the cellular location, we previously hypothesized that these two enzymes play a role in protecting the cells from external sources of oxidative damage (66). E. coli (44), Pseudomonas syringae (40), Sinorhizobium meliloti (64), and Bacillus subtilis (43a) are known to have more than one catalase, whereas Helicobacter pylori (50), Haemophilus influenzae (7), Bacteroides fragilis (56), and B. abortus (63) seem to have only one catalase. Brucella catalase exhibits sequence homology to the E. coli cytoplasmic catalase (HPII) but is regulated differently. Brucella catalase is regulated in response to external H$_2$O$_2$, in a manner similar to E. coli catalase-peroxidase (HPI) (59). H. influenzae catalase (7) and Rhizobium meliloti catalase A (34).

It has been reported that periplasmic Cu-Zn SOD protects cells from external superoxide in Caulobacter crescentus (67). Even though superoxide itself is not very toxic and in the ionized form cannot easily pass through membranes, it can react with H$_2$O$_2$ in the presence of transition metals to produce hydroxyl radical, which is very toxic to cells (3, 4, 35). Cu-Zn SOD is upregulated in response to external superoxide in C. crescentus (67). We report here that B. abortus Cu-Zn SOD is upregulated in response to oxidative agents. Under the conditions tested in this study, the increased synthesis of the enzyme was modest, no more than fivefold. This is less than the induction reported for C. crescentus (60, 67, 68) but does not rule out the possibility that regulation of Brucella Cu-Zn SOD might be more responsive under other growth conditions. For example, E. coli Cu-Zn SOD is expressed at high levels only very late in stationary phase (37). Several reports indicate that overexpression of SOD in the absence of a parallel increase in catalase expression causes cells to become more susceptible to oxidative damage (46, 61). This is probably because the product of Cu-Zn SOD activity, H$_2$O$_2$, is more toxic to cells than is superoxide. The observation that for B. abortus, both Cu-Zn SOD and catalase levels increase in response to superoxide is consistent with this principle.

Survival studies with the catalase deletion mutants (Fig. 1 and 2) clearly indicate that B. abortus catalase protects against hydrogen peroxide. Regulation of this enzyme is presumably one aspect of the adaptation process that allows the bacteria to survive under hostile conditions. During repeated experiments, we noticed that survival rates depended on cell density. This phenomenon is well documented in other species and is believed to be a consequence of the high diffusion rate of hydrogen peroxide (12, 45). In this study, the hydrogen peroxide stress condition (10 mM H$_2$O$_2$, $A_{600}$ of 0.2 to 0.5) used for 2-D gel analysis resulted in greater than 90% survival. However, for the adaptation experiments (Table 2), only 20.8% of Brucella organisms survived in 10 mM H$_2$O$_2$ when the $A_{600}$ was 0.01. The lower cell density was used for the adaptation experiment to reduce this density effect and to allow adaptation to be observed.

### TABLE 3. Adaptation to hydrogen peroxide

<table>
<thead>
<tr>
<th>H$_2$O$_2$ treatment</th>
<th>% Survival after 30 min of H$_2$O$_2$ at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mM</td>
</tr>
<tr>
<td>No pretreatment</td>
<td>20.8</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>44.6</td>
</tr>
<tr>
<td>Pretreatment plus chloramphenicol</td>
<td>33.0</td>
</tr>
</tbody>
</table>

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Adaptation may partially explain the different responses illustrated in Fig. 1 and 2. Figure 1 shows that strain 2308 is much more resistant to acute exposure to H₂O₂ whether or not catalase is present. The halo assay (Fig. 2) is based on prolonged exposure. Under these conditions, adaptation may play a more dominant role. Table 3 shows that adaptation can increase the resistance of strain 19 to H₂O₂ by 20-fold. This adaptive response is more than enough to account for the observation that strain 19 is nearly as resistant to H₂O₂ as is strain 2308 in the halo assay.

The data presented here indicate that B. abortus catalase and Cu-Zn SOD are regulated in response to oxidative stress and that under certain conditions the periplasmic catalase protects the bacteria from external hydrogen peroxide.

REFERENCES


