Abstract

The *Brucella abortus* alkyl hydroperoxide reductase complex (AhpCD) is the primary scavenger of endogenous hydrogen peroxide and the *ahpCD* operon is regulated in response to endogenous hydrogen peroxide.

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Brucella abortus is a facultative intracellular pathogen that causes abortion and infertility in cattle and relapsing fever in humans. These bacteria reside and replicate inside macrophages during infection, and the ability of *Brucella* to survive within this intracellular niche is critical for the establishment of disease in host animals. Exposure to the oxidative burst of host phagocytes is one of the environmental stresses the brucellae must deal with during their intracellular residence. Because they are aerobes, these bacteria must also detoxify reactive oxygen species (ROS) generated as a by-product of their respiratory metabolism. Production of *Brucella*'s sole catalase, KatE, enhances viability of the brucellae exposed to high levels of exogenous hydrogen peroxide (H_2O_2), but *Brucella katE* mutants are virulent in both the experimental and natural hosts. So it is currently unknown how the brucellae detoxify hydrogen peroxide produced from the macrophage and respiratory metabolism. The genes designated as BAB2_0531 and BAB2_0532 in the *B. abortus* 2308 genome sequence are predicted to encode the components (AhpC and AhpD, respectively) of an alkyl hydroperoxide reductase complex. Peroxiredoxins of the AhpC family are important enzymes that detoxify H_2O_2, organic peroxides, and

peroxynitrite in bacterial cells. The research described in this dissertation shows that AhpC is the primary detoxifier of endogenous H_2O_2 generated by aerobic metabolism. KatE, on the other hand, plays a major role in scavenging exogenous H_2O_2, although this enzyme can play a supporting role in the detoxification of H_2O_2 of endogenous origin if AhpC is absent. Our data also show that *ahpC* expression is H_2O_2-responsive, and AhpCD is part of the *Brucella* oxidative stress response. We have found three regulators—OxyR, BAB2_0530, Irr—that affect *ahpC* expression in some manner in *B. abortus* 2308, but only one, the iron-responsive regulator Irr, affects the H_2O_2-responsive expression of *ahpC*.

The *Brucella abortus* alkyl hydroperoxide reductase complex (AhpCD) is the primary scavenger of endogenous hydrogen peroxide and the *ahpCD* operon is regulated in response to endogenous hydrogen peroxide.

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Animal Brucellosis

Brucellae are facultative intracellular pathogens that infect a wide range of hosts. Hosts include cattle, goats, sheep, dogs, and marine mammals (Murray et al., 2007). Animal brucellosis causes abortion, fetal death, and genital infection and is passed from one animal to the next through the contaminating by-products shed during an abortion and milking (Corbel 2006).

The pathogenesis of brucellae within an animal is described well in a book edited by Nielsen and Duncan called Animal Brucellosis (1990). Briefly, an infection begins when the brucellae penetrate the epithelial lining of the mucosal membrane. From there, they enter the lymph nodes, where they are phagocytosized by neutrophils and macrophages which carry the brucellae throughout the reticuloendothelial system.

The primary site of infection is the lymphoid tissue, mammary gland, and the reproductive tract, but *Brucella* cells are also found in secondary sites such as bones, joints, and eyes (Nielsen and Duncan, 1990). Male animals are also infected in the testes, epididymis, and other accessory sex organs. In the female reproductive tract, the brucellae are primarily found in trophoblasts, which are cells that form the outer layer of a blastocyte, which provides nutrients to the embryo and eventually forms part of the placenta. The brucellae can damage the placenta, preventing the delivery of nutrients and oxygen to the fetus and the removal of waste products. Cortisol levels increase, inducing the delivery process prematurely and causing abortion. After the abortion, the brucellae can be found in udders where they are secreted in the milk and transmitted to other animals.

Susceptibility of an animal is variable based on age, gender, and sexually maturity, and an infection can persist for life (Nielsen and Duncan, 1990). The various animal hosts are infected in similar ways. There are 10^{10} bacteria found per gram of tissue of an aborted placenta, and cows are infected by inhaling the bacteria, getting infected through the conjunctiva, or getting infected through their udders by infected milking cups (Hoover and Friedlander, 1997; Joint FAO/WHO Expert Committee on Brucellosis 1986; Corbel 2006). Sheep and goats are infected the same way but sexual transmission can also occur (Corbel 2006). Transmission can occur more rapidly in goats and sheep because the animals are in flocks and within close contact, and because *B. melitensis* is highly contagious (Corbel 2006). Swine are primarily infected by ingesting aborted placentas but also through sexual transmission (Corbel 2006; Joint FAO/WHO Expert Committee on Brucellosis 1986).

Human Brucellosis

Incidence. Human brucellosis is considered the most widespread zoonotic disease in the world with more than 500,000 new cases each year (Joint FAO/WHO Expert Committee on Brucellosis 1986; Corbel 1997; Pappas et al., 2006b). Incidence is much lower in the United States: there are 100-200 cases per year of human brucellosis; the CDC last reported 131 cases in 2007 (Centers for Disease Control 2009; Pappas et al., 2006b).

The incidence of human brucellosis is directly proportional to the occurrence of animal brucellosis, because we are exposed to the bacteria through animal by-products. The incidence of human brucellosis is greater in countries with a higher rate of animal brucellosis. In 2004, Great Britain had four cases of bovine brucellosis, no cases of caprine/ovine brucellosis, and only nineteen cases of human brucellosis (Oie 2010). In comparison, during the same year Turkey had 117 cases of bovine brucellosis, 1,980 cases of caprine/ovine brucellosis, and over

18,000 cases of human brucellosis (Oie 2010). When the United States Eradication Program eliminated cattle infections with *B. abortus*, human infections also dramatically declined (Ragan 2002). In fact, most U.S. cases of human brucellosis are now derived from *B. melitensis*-infected dairy products ingested during international travel or imported from endemic countries (Pappas et al., 2006b).

The incidence of human brucellosis is not evenly distributed throughout the world. Brucellosis-free countries include Canada, Australia, Denmark, Finland, Austria, Belgium, Switzerland, Germany, Netherlands, Norway, Sweden, France, and the United Kingdom (Pappas et al., 2006b). There are other parts of the world where brucellosis is endemic, namely in the Mediterranean Basin and Asia. In 2004 there were 631 cases in Italy, 7,261 cases in Iraq, 18,264 cases in Turkey, and 29,580 cases in Syria (Oie 2010). It is common to have high incidences of brucellosis in economically poor countries, because these countries lack funds for controlling the disease in animals through vaccination and testing and lack a consistent infrastructure for maintaining strong public health programs such as the requirement to pasteurize milk products (Franco et al., 2007). Therefore, while some countries are winning the battle against brucellosis, in other countries the disease is still a large problem.

Transmission. Human brucellosis was first described as an occupational disease in 1964 by Boycott and Oxon (Boycott 1964). Researchers already knew at this time that farmers often got brucellosis by ingesting unpasteurized dairy products, but Boycott and Oxon noticed that veterinarians could get the disease through accidental inoculation with the vaccine, and slaughterhouse workers and butchers could get the disease through abrasions of the skin, through the conjunctiva, or by inhalation. Occupations with the highest risk are ranchers, farmers, farm laborers, shepherds, laboratory personnel, veterinarians, slaughterhouse workers, or anyone who

works with animals and their waste products (Corbel 2006; Hoover and Friedlander, 1997; Joint FAO/WHO Expert Committee on Brucellosis 1986).

Laboratory exposures are a common method of acquiring the disease in the U. S. (Corbel 2006; Yagupsky and Baron, 2005). Symptoms of brucellosis are briefly described as flu-like (Murray et al., 2007; Corbel 2006). This vague analysis can lead doctors to incorrectly diagnose the illness. Cultures of the organism are often sent to laboratory personnel to identify without warning that the organism may be *Brucella.* And since ten to one hundred organisms are sufficient to cause an infection through inhalation, laboratory personnel often do not take the necessary precautions to prevent breathing in the culture (Pappas et al., 2006a).

In developing countries brucellosis is mostly acquired by ingesting unpasteurized milk and cheese (Hoover and Friedlander, 1997). Mammary glands of cows are persistently infected, and the cows shed the brucellae in their milk. Anyone who milks the cows, handles the raw milk during transport or the pasteurization process can become infected. The milk must be pasteurized to prevent infection, but in many developing countries, pasteurization does not occur (Joint FAO/WHO Expert Committee on Brucellosis 1986; Corbel 2006). Anyone can be at risk in these countries. Meat is not often a cause of infection because it is cooked. However, it should be noted that muscle tissue can contain low concentrations of *Brucella* (Corbel 2006).

Pathogenesis and symptoms. Once a human is infected, the brucellae are engulfed by neutrophils, dendritic cells, and macrophages (Fugier et al., 2007; Franco et al., 2007). Neutrophils are the first immune phagocytes to engulf the brucellae, but neutrophils do not have a long life span and therefore do not make an ideal host. Macrophages phagocytize the brucellae and are able to kill 80-90% of the bacteria within the first couple of hours (Fugier et al.,

2007). Unfortunately, since some of the brucellae survive, the macrophage ends up being the ecological niche for the brucellae, and the macrophage carries the brucellae to the lymphoid tissue, allowing the brucellae to enter the lymph nodes, spleen, liver, mammary gland, joints, kidneys, and bone marrow (Hoover and Friedlander, 1997; Ko and Splitter, 2003; Fugier et al., 2007).

Brucellosis is a severely debilitating disease (Franco et al., 2007). Symptoms are often nonspecific: fatigue, malaise, night sweats, backache, muscle aches, weight loss, depression, and anorexia (Murray et al., 2007; Hoover and Friedlander, 1997; Joint FAO/WHO Expert Committee on Brucellosis 1986; Sauret and Vilissova, 2002; World health organization 1997). The hallmark symptom is an undulant fever. Symptoms typically last for three to six months, but can occur for a year or more. Since the brucellae infect the lymphoid tissue, many complications can occur in these organs. Ten to thirty percent of patients suffering from brucellosis get splenomegaly and ten to seventy percent get hepatomegaly (Hoover and Friedlander, 1997; Franco et al., 2007). Other complications include osteomyelitis, meningitis, spondylitis, arthritis, and neuropsychiatric symptoms such as depression, headache, and irritability (Cutler et al., 2005). Endocarditis is a rare complication, but eighty percent of endocarditis cases due to *Brucella* infections cause death (Hoover and Friedlander, 1997).

Diagnosis and treatment. Diagnosis can be tricky because human brucellosis is rare in the United States, symptoms are non-specific, and *Brucella* grows slowly in blood cultures (Franco et al., 2007). The most important diagnostic tool is a detailed patient history (Sauret and Vilissova, 2002; Hoover and Friedlander, 1997). Brucellosis should be suspected in individuals who work in laboratories with *Brucella* or with animals, who travel to endemic countries, and who ingest unpasteurized milk products (Franco et al., 2007). In 1986, the

World Health Organization recommended treating human brucellosis with doxycycline in combination with either rifampicin or streptomycin for six weeks. This is still the standard regime except that gentamicin can replace streptomycin (Ariza et al., 2007; Murray et al., 2007). Children and pregnant women receive trimethoprim-sulfamethoxazole for six weeks (Corbel 2006). The prophylaxis regimen for potentially exposed people is to serologically test the individual for *Brucella* antibodies and to administer antibiotics. The antibiotic regimen can be a full six week course of antibiotics or doxycycline for one week followed by a combination of doxycycline and rifampin for three weeks (Corbel 2006; Murray et al., 2007).

Since the brucellae reside in an intracellular niche, they can in effect hide from the immune system while in their host, because they do not come into direct contract with other phagocytes, B and T cells, and antibodies (Fugier et al., 2007). This makes the relapse rate rather high after an oral antibiotic treatment (10- 20%) (Franco et al., 2007). It is generally recommended that patients be serologically tested for at least one year after symptoms decline (Yagupsky and Baron, 2005; Hoover and Friedlander, 1997).

A Biowarfare Agent

Brucella is one of the top five bacteria that would make an effective biological warfare agent (Eitzen 1997; Robinson-Dunn 2002). Features that make a bacterium an effective biological warfare agent are the ability to cause incapacitating effects in humans, ease of dissemination as an aerosol, stability while maintaining virulence after production, and susceptibility of the intended victims to it (Robinson-Dunn 2002; Pappas et al., 2006a). The *Brucella* species have many of these features. Brucellosis is incapacitating, the bacteria can infect through inhalation with a low infectious dose, there is no human vaccine, the disease is

difficult to treat, and since brucellosis is rare in developed countries, physicians tend to misdiagnose cases of brucellosis (Pappas et al., 2006a).

Brucella suis was the first biological agent weaponized by the U. S. (Pappas et al., 2006a; Smart 1997). In 1945, Camp Detrick near Frederick, Maryland inoculated embryonic eggs with *Brucella suis* for test studies. Also in 1945, Pine Bluff laboratories in Arkansas were producing and filling bombs with *Brucella suis* (Franz et al., 1997). In the 1950's, Dugway Proving Ground, Utah standardized the M33 bomb, which held over 34 liters of *Brucella suis* culture (Smart 1997). By 1964, research programs began to change to focus on biological agents that would incapacitate but not kill people. This meant that more research went to studying how *Brucella* could be used as a biological warfare agent (Franz et al., 1997). In 1969, President Nixon disbanded the biological weapons program and limited funding to defensive research only. So for the time being—in the United States—the *Brucella* species are not being developed as a biological weapon. However, there is plenty of evidence that suggests Russia, North Korea, China, and Iraq are developing biological weapons (Caudle 1997). And so, it is still a legitimate concern that the *Brucella* spp. will be used as a biological weapon to incapacitate unvaccinated U. S. soldiers and citizens.

Oxidative killing of the *Brucella* spp.

Oxidative burst of the macrophage

When the brucellae enter host macrophages through an opsonic route, they activate the oxidative burst. Below is a description of how the oxidative burst occurs, and the susceptibility of the brucellae to the reactive oxygen species that the burst produces.

NADPH oxidase. A macrophage is activated by cytokines (IFN-γ and TNF-α), or the binding of bacterial cell components (LPS and peptidoglycan), naturally occurring sugars and proteins (zymosan and phospholipase C), and some compounds used in research (phorbol myristate acetate) to macrophage receptors (Pick 1986; James et al., 1998; Ding and Nathan, 1987; Goldberg et al., 1990; Ding et al., 1988; Murray et al., 1985). Once activated, the macrophage responds in 30 – 60 seconds by passively diffusing molecular oxygen from the cytosol to the phagosome through a membrane flavocytochrome called NADPH oxidase (Babior 1978; Pick 1986; Flannagan et al., 2009; Gabig et al., 1978; Root and Cohen, 1981; Clark 1990; Sbarra and Karnovsky, 1959). The NADPH oxidase is an enzyme that catalyzes the production of superoxide ($O_2^{\bullet -}$) by transferring an electron from NADPH to oxygen and releasing $O_2^{\bullet -}$ into the phagosomal lumen (reaction 1) (Gabig et al., 1978; Flannagan et al., 2009).

Reaction 1. NADPH oxidase reaction

$2\ O_2 + NADPH \rightarrow 2\ O_2^{\bullet -} + NADP^+ + H^+$

Damage caused by reactive oxygen species (ROS). $O_2^{\bullet -}$ damage may be limited, because its negative charge prevents it from crossing membranes (Root and Cohen, 1981). Nevertheless, $O_2^{\bullet -}$ can react with unsaturated lipids in the membranes and produce lipid hydroperoxide radicals (Missall et al., 2004). $O_2^{\bullet -}$ can also release iron from iron-sulfur clusters and oxidize thiol groups on cysteine and methionine amino acids causing disulfide bridges and methionine sulfoxides (Missall et al., 2004; Dubbs and Mongkolsuk, 2007).

$O_2^{\bullet -}$ can also readily react with other compounds forming more reactive and toxic species. Superoxide and nitric oxide react to form peroxynitrite (reaction 2); superoxide accepts protons to form hydrogen peroxide (reaction 3) (Vazquez-Torres et al.,

Reaction 2. Production of peroxynitrite

$NO\bullet + O_2\bullet^- \rightarrow ONOO^-$

Reaction 3. The dismutation of superoxide

$2\ O_2^{\bullet -} + 2\ H^+ \rightarrow O_2 + H_2O_2$

2000; Babior 2004; Hassett and Cohen, 1989; Dubbs and Mongkolsuk, 2007; Root and Cohen, 1981). Superoxide can also form hydroxyl radical by reacting with hydrogen peroxide (reaction 4) and organic molecules (reaction 5) (Babior 2004; Tauber and Babior, 1977; Hassett and Cohen, 1989; Dubbs and Mongkolsuk, 2007; Root and Cohen, 1981). Therefore, although $O_2^{\bullet -}$ is not considered a highly stable ROS, it can form downstream oxidizing agents that have lethal effects (Hassett and Cohen, 1989).

Reaction 4. The Haber-Weiss reaction

$O_2^{\bullet -} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + O_2$

Reaction 5. Production of hydroxyl radical from organic peroxides and superoxide

$ROOH + O_2^{\bullet -} \rightarrow OH^{\bullet} + RO^- + O_2$

Hydrogen peroxide (H_2O_2) is generated by either spontaneous or enzymatic dismutation of $O_2^{\bullet -}$ (reaction 3, Pick 1986). This reaction is so favorable that eighty percent of the superoxide formed from NADPH oxidase becomes hydrogen peroxide (Root and Metcalf, 1977). The resulting estimate is that 100 nM H_2O_2 accumulates inside the phagosome (Jang and Imlay, 2007).

H_2O_2 can cause cellular damage in a variety of ways. H_2O_2 will directly oxidize iron-sulfur clusters and thus inactivate any enzyme requiring the iron cofactor for activity. An example is H_2O_2 inactivating the iron-sulfur cluster of isopropylmalate isomerase, an enzyme found in the leucine biosynthesis pathway (Jang and Imlay, 2007). Other dehydratases with iron-sulfur clusters—such as fumarases A and B and 6-phosphogluconate dehydratase—are also sensitive to H_2O_2. Additionaly, hydrogen peroxide will oxidize methionine and cysteine residues (Jang and Imlay, 2007).

Hydrogen peroxide reacts with ferrous iron to form hydroxyl radical (OH•) in what is known as the Fenton Reaction (reaction 6). OH• is a highly unstable compound that reacts instantly with organic molecules (Babior 1978; Hassett and Cohen, 1989). OH• causes single-strand DNA breaks and oxidizes lipids, carbohydrates, and proteins (Hassett and Cohen, 1989). Because of its high redox potential (+2.38V), OH• damage occurs wherever it is formed (Dubbs and Mongkolsuk, 2007), and this ROS is especially dangerous to a bacterium, because there are no enzymatic antioxidants that can remove it before damage occurs.

Reaction 6. The Fenton Reaction
$Fe^{+2} + H_2O_2 \rightarrow OH\bullet + OH^- + Fe^{+3}$

Susceptibility of the *Brucella* spp. to oxidative killing by the macrophage. The *Brucella* spp. are susceptible to the oxidative burst of host macrophages, which produces an exogenous source of reactive oxygen species (Jiang et al., 1993). If methylene blue, an electron carrier that produces intracellular superoxide, is added to *Brucella*-infected J774A.1 macrophages, the killing of the brucellae is enhanced by one log. However, if superoxide dismutase or catalase (detoxifies superoxide and hydrogen peroxide, respectively) is added to the macrophages, then recovery of the brucellae is enhanced by one log. If mannitol, a hydroxyl radical scavenger, is added to the *Brucella*-infected macrophages, then there is a greater recovery at 24 hours post infection (Jiang et al., 1993). These data suggest that the *Brucella* spp. are susceptible to superoxide, hydrogen peroxide, and hydroxyl radical, and that these ROS contribute to the brucellacidal activity of the macrophage.

Oxidative killing of the brucellae within the macrophage is further enhanced if the macrophages are activated by IFN-γ or the brucellae are opsonized with IgG (Jiang et al., 1993; Sun et al., 2002; Liautard et al., 1996; Harmon and Adams, 1987; Roop et al., 2004). One study compared the survival of *B. abortus* in two different macrophage-like cell lines (Sun et al., 2002).

One cell line, J774.16, produces superoxide when activated with IFN-γ; the other cell line, J774.D9, lacks the gene that encodes the $gp91^{PHOX}$ subunit of the NADPH oxidase and therefore cannot produce superoxide. Without IFN-γ treatment, the same amount of *Brucella* survived in each macrophage cell line. However, the addition of IFN-γ greatly enhanced killing in the J774.16 cell line. So, *B. abortus* is susceptible to IFN-γ activation of NADPH oxidase inside the macrophage.

Oxidative killing due to aerobic metabolism

The *Brucella* spp. have to produce antioxidants to survive the oxidative burst produced by the macrophage. But the brucellae also require these antioxidants to scavenge the endogenous ROS the bacteria produce during aerobic metabolism.

Where $O_2•^-$ and H_2O_2 are produced in the bacterial cell. Respiring bacteria produce superoxide as a by-product of aerobic metabolism. This production occurs on the cytoplasmic side of the inner membrane and is generated primarily from the respiratory chain (75%) (Imlay and Fridovich, 1991). NADH dehydrogenase II produces $O_2•^-$ by directly transferring an electron from NADH to molecular oxygen (Messner and Imlay, 1999). It is estimated that individual *E. coli* cells produce 4-5 μM/s $O_2•^-$ when growing in glucose-containing rich medium and 3 μM/s $O_2•^-$ in a minimal medium (Imlay and Fridovich, 1991). Since toxicity occurs when $O_2•^-$ levels reach 1 nM, it is important for *E. coli* to have antioxidants that can rapidly detoxify superoxide (Gort and Imlay, 1998). Superoxide dismutase activity restricts $O_2•^-$ accumulation to 200 pM in *E. coli* (Imlay and Fridovich, 1991).

The fact that *E. coli* produces a periplasmic superoxide dismutase suggests that superoxide is made, at least to a small degree, in the periplasm. This theory is derived from the

fact that superoxide made from the respiratory chain is produced on the cytoplasmic membrane entering into the cytoplasm, and because of its anionic charge, it cannot cross the inner membrane into the periplasm. Periplasmic superoxide levels are estimated to be small, because only ten percent of endogenous H_2O_2 is made in the periplasm (Seaver and Imlay, 2004). Currently, researchers do not know the source of superoxide in the periplasm of bacterial cells.

Hydrogen peroxide is also directly made from the respiratory chain by NADH dehydrogenase II (González-Flecha and Demple, 1995; Messner and Imlay, 1999). The flavin cofactor has the ability to transfer two electrons to molecular oxygen and thus form H_2O_2 (Messner and Imlay, 1999). H_2O_2 can also be formed by the dismutation of superoxide (Seaver and Imlay, 2004). *E. coli* cells grown in rich media form an estimated 15 µM/s H_2O_2 (Imlay 2008). Since DNA mutagenesis occurs when the bacterial cell is exposed to 1 µM H_2O_2, it is important for *E. coli* to have antioxidants that can rapidly remove H_2O_2 from the bacterial cell (Imlay 2008).

Brucella enzymes that produce H_2O_2 and $O_2^{\bullet-}$. *B. abortus* produces homologs of the proteins that generate intracellular $O_2^{\bullet-}$ and H_2O_2 in *E. coli*. *B. abortus* 2308 produces the components of the NADH dehydrogenase II complex (also known as succinate dehydrogenase) SdhB, SdhA, and SdhC (encoded by BAB1_1900, BAB1_1901, and BAB1_1902 respectively) (Chain et al., 2005). Since NADH dehydrogenase II is the primary source of endogenous $O_2^{\bullet-}$ and H_2O_2 in *E. coli* (Messner and Imlay, 1999), this may be the primary source of endogenous ROS in *B. abortus*. $O_2^{\bullet-}$ is also generated from fumarate reductase in *E. coli* (Messner and Imlay, 1999), but this enzyme is not present in *B. abortus* 2308. H_2O_2 is generated from sulfite reductase, fumarate reductase, and NadB in *E. coli* (Messner and Imlay, 1999; Korshunov and Imlay, 2010). There are two genes in the *B. abortus* 2308 genome sequence that are predicted

to encode a sulfite reductase, BAB1_0181 and BAB1_1304, so H_2O_2 could be produced from these enzymes.

B. abortus 2308 also produce enzymes that are vulnerable to oxidative damage caused by H_2O_2 and $O_2^{\bullet -}$. In *E. coli,* $O_2^{\bullet -}$ has been shown to damage dihydroxyacid dehydratase, and H_2O_2 has been shown to damage isopropylmalate isomerase, fumarases A and B, and 6-phosphogluconate dehydratase (Jang and Imlay, 2007). *B. abortus* 2308 produces dihydroxyacid dehydratase (BAB2_0294, BAB1_0096), fumarase C (BAB2_0186), isopropylmalate isomerase subunit genes *leuC* (BAB1_1905) and *leuD* (BAB2_0353), and 6-phosphogluconate dehydratase subunit genes BAB2_0109 and BAB1_0969. It is hard to predict whether the *Brucella* spp. would be more sensitive to endogenous ROS than *E. coli* would. Any flavin protein can be oxidized by $O_2^{\bullet -}$ and H_2O_2 (Jang and Imlay, 2007), so it is difficult to estimate how many proteins produced by the brucellae are vulnerable to oxidative damage. To this author's knowledge, no studies have been published that measure how much $O_2^{\bullet -}$ and H_2O_2 the brucellae can be exposed to before DNA mutagenesis occurs. Therefore, we do not know yet if the *Brucella* spp. are more or less susceptible than *E. coli* to oxidative DNA damage.

Known *Brucella* H_2O_2 detoxifiers

Analysis of the *Brucella abortus* 2308 genome reveals the presence of five genes that are predicted to encode antioxidants that can detoxify H_2O_2 (Table 1.1): catalase, alkyl hydroperoxide reductase, bacterioferritin comigratory protein, thiol peroxidase, and rubrerythrin.

The following discussion will review the literature on two of these, catalase and alkyl hydroperoxide reductase.

Catalases

Properties of catalase enzymes. Catalases are unifunctional; their only function is to catalyze the degradation of peroxides into water and oxygen (reaction 7) (Loewen 1997). The kinetics of these enzymes make catalases efficient scavengers of H_2O_2, and so they are ubiquitous throughout the microbial world. Catalases have been purified from Gram-negative bacteria, Gram-positive bacteria, and archaea. They are also well characterized in yeast and other eukaryotic cells (Loewen 1997).

Reaction 7. The catalase reaction

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

There are three types of catalases: monofunctional catalases, catalase-peroxidases, and non-heme, Mn-containing catalases. Monofunctional catalases only degrade hydrogen peroxide, while catalase-peroxidases have the additional activity of detoxifying organic peroxides (Loewen 1997). Both monofunctional catalases and catalase-peroxidases have the same two stage enzymatic reaction for degrading hydrogen peroxide into water and oxygen (Reaction 8). H_2O_2 directly oxidizes the iron molecule in the heme in catalase and produces water and an oxidized catalase intermediate. Then a second H_2O_2 reduces the catalase intermediate and produces oxygen and another water molecule.

Reaction 8. The two-part enzymatic reaction catalyzed by monofunctional catalases and catalase-peroxidases (Switala and Loewen, 2002).

(1) Catalase-Fe^{+3} + H_2O_2 → Catalase-Fe^{+4}=O + H_2O

(2) Catalase-Fe^{+4}=O + H_2O_2 → Catalase-Fe^{+3} + H_2O + O_2

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

Mn-containing catalases are the rarest of the three types and are only found in bacteria. These catalases do not contain heme molecules; instead they contain dimanganese in the catalytic center. The two-part enzymatic reaction is different from the other types of catalases, but the overall reaction is the same: the enzyme degrades two hydrogen peroxide molecules and produces two waters and one molecular oxygen (Reaction 9).

Reaction 9. The two-part enzymatic reaction catalyzed by Mn-containing catalases (Chelikani et al., 2004).

(1) Catalase-Mn^{II}-Mn^{II} (2 H^+) + H_2O_2 → Catalase-Mn^{III}-Mn^{III} + 2 H_2O

(2) Catalase-Mn^{III}-Mn^{III} + H_2O_2 → Catalase-Mn^{II}-Mn^{II} (2 H^+) + O_2

2 H_2O_2 → 2 H_2O + O_2

Catalase enzymes have impressive kinetics for detoxifying H_2O_2. They only follow Michaelis-Menten kinetics when H_2O_2 concentrations are low (Chelikani et al., 2004; Switala and Loewen, 2002). Otherwise, catalases never become saturated by biologically relevant levels of H_2O_2, because hydrogen peroxide acts as an oxidant and a reductant for catalases (Reactions 8 and 9) (Switala and Loewen, 2002). Thus, the higher the concentration of H_2O_2, the better the enzyme works.

The *Brucella* catalase. *B. abortus* produces one catalase, KatE. KatE is a monofunctional catalase and functions as a 55 kDa tetramer (Sha et al., 1994). Despite being exported into the periplasm, KatE does not have an amino-terminal signal sequence (Sha et al., 1994). The kinetic potential of *B. abortus* KatE is the same as other catalases: it does not follow Michaelis-Menton kinetics and does not reach saturation (Richardson and Huddleson, 1953). The concentration of H_2O_2 needed for the *B. abortus* KatE to reach 50% of its full activity (the "apparent" Km) is 174 mM (Switala and Loewen, 2002). This is relatively high

compared to the *E. coli* KatE (64 mM) and to one of the *X. campestris* catalases (77 mM), but not as high as other catalases (the Km of *B. fragilis* KatB is 279 mM) (Switala and Loewen, 2002). This means that the *B. abortus* KatE protein can detoxify supraphysiologic concentrations of H_2O_2. Indeed, *B. abortus katE* mutants are hypersensitive to millimolar levels of H_2O_2 compared to the parental strain (Kim et al., 2000; Gee et al., 2004).

The addition of exogenous catalase enhances brucellae's survival within macrophages (Jiang et al., 1993). Since catalase only degrades H_2O_2, these data show that H_2O_2 decreases brucellae survival within its host. KatE, however, is not required for survival of these bacteria within the animal host. *B. abortus katE* mutants are as virulent as wild-type in mice, and *B. melitensis katE* mutants are fully virulent in goats (Sangari and Agüero, 1996; Gee et al., 2004). Therefore, the *Brucella* spp. can still survive exposure to H_2O_2 from the respiratory chain and from the macrophage without its sole catalase, which suggest *B. abortus* have other mechanisms of detoxifying H_2O_2.

Alkyl hydroperoxide reductase

AhpC enzymatic mechanism and structure. Peroxiredoxins are enzymes that reduce peroxides into alcohols or water (Reaction 10) (Hofmann et al., 2002; Flohé et al., 2003; Wood et al., 2003). These proteins do not use cofactors like Fe^{+2} or Mn^{+2} or prosthetic groups like heme. Instead, peroxiredoxins use redox-active cysteine residues to reduce peroxide molecules (Hofmann et al., 2002; Flohé et al., 2003). Peroxiredoxins are ubiquitous: they are found in yeast, plant cells, mammalian cells, archaea, and bacteria (Wood et al., 2003). Mostly, peroxiredoxins function in

Reaction 10. The peroxidase-catalyzed reaction (Imlay 2008)

$RH_2 + H_2O_2 \rightarrow R + 2\ H_2O$

the cytosol of eukaryotic and prokaryotic cells, but they can also be found in the mitochondria, chloroplast, and peroxisomes of eukaryotic cells (Wood et al., 2003).

The bacterial alkyl hydroperoxide reductase, AhpC, is a ubiquitous enzyme described as a typical 2-Cys peroxiredoxin (Dubbs and Mongkolsuk, 2007). Typical 2-Cys peroxiredoxins function as homodimers arranged in a head-to-tail arrangement. They contain a cysteine in their amino-terminus that is called the "peroxidatic cysteine." This cysteine is conserved in a V-C-P motif and becomes oxidized to a cysteine sulfenic acid directly by the peroxide molecule (Hofmann et al., 2002; Wood et al., 2003). The second cysteine is called the "resolving cysteine," and it forms an intramolecular disulfide bond with the peroxidatic cysteine sulfenic acid. Figure 1.1 illustrates the enzymatic reaction for AhpC in more detail. In this example hydrogen peroxide is the substrate. H_2O_2 reacts with the peroxidatic cysteine in the amino-terminus of AhpC to form a sulfenic acid. The resolving cysteine of a second monomer reacts with the sulfenic acid in the first monomer to form an intermolecular disulfide bond. Two water molecules are released. Next, a second substrate, a reductase, interacts with AhpC to reduce the disulfide bond and regenerate AhpC to the reduced form (Dubbs and Mongkolsuk, 2007).

In some bacteria, AhpC is reduced by AhpD. AhpD functions as a trimer in both its reduced and oxidized states (Nunn et al., 2002; Bryk et al., 2002). Each subunit has two redox active cysteine residues that make up a C-H-S-C motif (Koshkin et al., 2004). These cysteine residues reduce the disulfide bond in oxidized AhpC. When AhpD oligomerizes into the trimer, the cysteines are located in a central cavity where they are easily accessible to AhpC (Nunn et al., 2002).

Reduction of the *M. tuberculosis* AhpC by AhpD uses two other proteins, both of which belong to the α-ketoacid dehydrogenase complex. These proteins are Lpd (dihydrolipoamide dehydrogenase) and SucB (dihydrolipoamide succinyltransferase). Reaction 11 illustrates that electrons are passed from NADH to Lpd to SucB and then to AhpD. When AhpD reduces the disulfide bond in oxidized AhpC, AhpD becomes oxidized, having its own disulfide bond between its cysteine residues. AhpD requires Lpd and SucB for reduction so it can be reset to reduce more AhpC dimers (Bryk et al., 2002).

Reaction 11. The electron transfer procession necessary to reduce AhpC dimers.

NADH → Lpd → SucB → AhpD → AhpC → $ROOH/H_2O_2$ (Bryk et al., 2002)

<u>*B. abortus* AhpCD</u>. The genes designated as BAB2_0531 and BAB2_0532 in the *B. abortus* 2308 genome sequence are annotated as *ahpC* and *ahpD*, respectively. The putative *Brucella* AhpC shares 47% amino acid identity with the *Mycobacterium tuberculosis* AhpC, and the C61, C174 and C176 residues that have been shown to be important for activity in the latter protein (Hillas et al., 2000; Chauhan and Mande, 2002) are conserved as C57, C171 and C173 in the *Brucella* AhpC ortholog. Likewise, the *Brucella* AhpD displays 44% amino acid identity with its *Mycobacterium tuberculosis* counterpart, and amino acid sequence alignment indicates that the C131 and C134 residues in this protein are equivalent to the C130 and C133 that are required for the activity of the mycobacterial AhpD (Bryk et al., 2000; Hillas et al., 2000). The *Brucella ahpC* and *ahpD* are encoded in an operon and both genes are transcribed from the same promoter (Seleem et al., 2008). Currently, there is little information known about the *Brucella* AhpCD.

Bacterial regulators of *ahpC* and *kat* genes

OxyR

OxyR properties. OxyR was first discovered in 1985 when *E. coli* and *S. typhimurium* researchers discovered that exponentially growing cells can adapt to small amounts of H_2O_2 (60 µM) and later resist killing by a lethal amount of H_2O_2 (10 mM). Adaptation does not occur when the bacterial cells are treated with chloramphenicol to block protein synthesis. These data suggest that the bacteria are responding to the low doses of H_2O_2 by synthesizing proteins capable of detoxifying lethal amounts of H_2O_2 (Christman et al., 1985). Two dimensional protein gel electrophoresis revealed that the production of thirty proteins is elevated by the pretreatment, and the genes of nine of these are activated by a protein named OxyR (Christman et al., 1985). The *E. coli* and *S. typhimurium* OxyR proteins (both 34.4 kDa) differ by only one amino acid, V234 (Christman et al., 1989), and both *oxyR* mutants are more sensitive than wild-type to hydrogen peroxide, cumene hydroperoxide, tert-butyl hydroperoxide, menadione, and heat exposure (Christman et al., 1985). The *oxyR* mutant also has increased DNA mutagenesis during aerobic growth because of an increase in oxidative damage (Storz et al., 1987).

OxyR belongs to the LysR family of transcriptional regulators. These types of regulators share similar structures and have similar properties (Schell 1993). There are four characteristics that most LysR-type regulators share (Schell 1993). The first characteristic is that the protein is responsive to a coinducer. The second is that the LysR protein will bind DNA whether or not it binds to the coinducer. The third characteristic is that the gene encoding the LysR-type regulator is divergently transcribed from one of its target genes. The promoters

of the gene encoding the LysR-type regulator and of the target gene are either very close or overlapping. The final characteristic is that most LysR-type regulators negatively autoregulate their own transcription (Schell 1993).

OxyR has been studied in many bacterial species, but the majority of the OxyR research has been done in *S. typhimurium* and *E. coli* (Christman et al., 1985). These OxyR homologs have the structural properties of LysR-type regulators: the helix-turn-helix motif (HTH) is in the amino terminus, and the response domain is in the middle of the protein and the carboxy terminus. Five amino acid residues in the HTH have been identified as critical for OxyR binding to DNA (R4, Y8, A22, P30, and S33) (Paget and Buttner, 2003). C199 and C208 are essential for oxidation and activation of OxyR (Kullik et al., 1995; Zheng et al., 1998). D142 and T238 are necessary for the ability of OxyR to activate transcription by binding to RNA polymerase (Paget and Buttner, 2003).

The *E. coli* OxyR binding site was determined as four ATAG motifs each separated by seven base pairs, and each motif is on a major groove in the DNA helix (Eisenstark et al., 1996). At first, researchers had trouble identifying the OxyR binding site on DNA. Using competition assays, OxyR was shown to bind with equal and high affinity to the different promoters of its known target genes despite there being no apparent consensus sequence (Tartaglia et al., 1989; Tartaglia et al., 1992). The only similarity between OxyR binding sites seemed to be that OxyR binds directly upstream of the -35 sites of various target gene promoters (Figure 1.2) (Tartaglia et al., 1989; Tartaglia et al., 1992). There must be some specificity, though, because OxyR does not bind to the promoters of nontarget genes (Tartaglia et al., 1992). Finally, the researchers realized that OxyR did not have a consensus nucleotide sequence for its binding site, but rather a “pattern” motif. OxyR binds to the four ATAG motifs (Table 1.2) in most bacteria.

The one exception is in the *Mycobacterium* spp. In these bacteria, the OxyR binding site is a palindrome sequence, cTTATCggc-N_3-gccGATAAg (Dhandayuthapani et al., 1997).

OxyR activation. OxyR function correlates to its oxidation state. True to the LysR characteristics, though, OxyR binds to DNA no matter what conformation its structure is in (Storz et al., 1990). When OxyR is in a reduced form, it is in an inactivated state (Storz et al., 1990). This reduced form will bind to two major grooves in the DNA, directly upstream of the -35 site in the promoter of its target genes, but it cannot bind to RNA polymerase and therefore transcription will not be induced (Tartaglia et al., 1989; Tartaglia et al., 1992).

OxyR is primarily activated by hydrogen peroxide (Åslund et al., 1999; Zheng et al., 2001), but it can also be activated by nitrosylation, although to a lesser degree (Hausladen et al., 1996). OxyR can be activated in the absence of oxidants when cellular thiol levels increase, which suggests that OxyR can respond to the redox state of the bacterial cytoplasm (Åslund et al., 1999; Pomposiello and Demple, 2001). OxyR is activated through amino acid residue cysteine 199. Some believe that this is all the modification required to activate OxyR (Kim et al., 2002). However, most researchers believe that this is just an intermediate form of OxyR that causes a necessary conformational change so C199 can form an intramolecular disulfide bond with C208 (Imlay 2008; Zheng et al., 1998; Mongkolsuk and Helmann, 2002). This disulfide bond, which has been seen in crystallization models, causes another significant structural change in OxyR, allowing OxyR to bind to four major grooves in the DNA and letting OxyR bind to the α-subunit of RNA polymerase (Toledano et al., 1994; Tao et al., 1993). After the oxidative stress subsides, OxyR is then re-reduced by glutaredoxin or thioredoxin (Zheng et al., 1998).

It is important to note that oxidative stress does not activate expression of *oxyR* or induce protein synthesis of OxyR. Induction of the OxyR regulon is purely due to modification of preexisting OxyR (Storz et al., 1990). So in the absence of oxidative stress, *E. coli* growing cells produce OxyR, but it remains in the inactivated, reduced state (Pomposiello and Demple, 2001).

OxyR regulation. The many genes that *E. coli* OxyR regulates can be divided into subcategories: genes whose products detoxify ROS, genes whose products are involved with re-reducing OxyR, and genes whose products control iron metabolism to prevent Fenton chemistry. *E. coli* OxyR activates expression of *ahpC* and *katG*; the gene products of both detoxify hydrogen peroxide (Christman et al., 1985). *E. coli* OxyR also activates *gorA* and *grxA*, which encode for glutathione reductase and glutaredoxin, respectively. GorA and GrxA re-reduce OxyR, thus creating an autoregulatory feedback loop (Christman et al., 1985; Zheng et al., 2001). Also OxyR will repress its own transcription creating another negative feedback loop (Christman et al., 1989).

Many gene products of the *E. coli* OxyR regulon are involved with preventing Fenton chemistry. OxyR activates *dps*, which encodes a DNA- and iron-binding protein that protects DNA by sequestering iron and preventing it from reacting with H_2O_2 (Altuvia et al., 1994). OxyR also activates the *suf* operon (Zheng et al., 2001). Suf proteins are involved with iron-sulfur cluster assembly and repair and thus will repair oxidatively damaged proteins. OxyR induces *hemH*, whose gene product incorporates iron into a protoporphyrin ring to make heme. Since the iron is being used, it is unavailable to react with H_2O_2 in Fenton chemistry (Zheng et al., 2001). OxyR also activates *fur* (Zheng et al., 1999). Fur represses iron uptake genes when cellular iron levels are high. Since Fur functions when bound to iron, the iron is

again not available to react with H_2O_2. *E. coli* OxyR also activates *mntH* during H_2O_2 exposure (Anjem et al., 2009). MntH is the major manganese transporter in *E. coli* and is required for survival during peroxide stress. Manganese can substitute for iron as a cofactor in some metalloenzymes and this reduces the iron requirement and limits iron availability for Fenton chemistry (Anjem et al., 2009). And so, the *E. coli* OxyR not only activates ROS scavengers, but also activates genes whose gene products are involved with preventing and repairing oxidative cellular damage.

Iron responsive regulators

Zheng et al. (1999) wrote, "…iron metabolism is coordinately regulated with the oxidative stress defenses [in *E. coli*]." This coordinated regulation occurs in many bacteria, because iron contributes to oxygen toxicity. Fenton chemistry is defined as the reaction of ferrous iron and hydrogen peroxide to form hydroxyl radical. Hydroxyl radical instantaneously reacts with all cellular macromolecules, causing protein, lipid, and DNA damage (Babior 1978; Hassett and Cohen, 1989). In fact—as one will see—the production of hydroxyl radical is so damaging to bacterial cells, that bacteria have regulatory defenses to keep iron levels and hydrogen peroxide levels simultaneously low.

Bacteria need to keep cellular iron levels low to prevent Fenton chemistry and oxidative damage, but they require iron as cofactors for some antioxidants. Monofunctional catalases and catalase-peroxidases require heme as a cofactor (Loewen 1997). Heme contains iron. SodB, the Fe^{+2}-containing superoxide dismutase, obviously requires iron for activity. Cellular iron levels also impact SodA, the Mn^{+2}-containing superoxide dismutase, because Mn^{+2} becomes the cofactor for many enzymes when iron levels are low (Anjem et al., 2009).

Many bacteria use Fur, the ferric-uptake regulator, to keep iron concentrations at an appropriate level. If iron levels are high, Fur represses genes whose gene products are involved with iron uptake because the bacterium does not need any more iron. If iron levels are low, Fur becomes inactive, and the Fur repression on these genes is relieved (Touati 2000). One way that the oxidative stress and iron regulatory mechanisms are intertwined is through Fur. Fur affects the expression of many antioxidants. The *Agrobacterium tumefaciens* Fur activates *katA* expression causing a 30% increase in catalase activity, but we do not know if Fur directly activates *katA* expression (Kitphati et al., 2007). Fur also affects which SOD is used in *E. coli*. When iron is available to bind and activate Fur, Fur will indirectly activate the expression of *sodB* (FeSOD) through the small RNA *ryhB* and directly repress expression of *sodA* (MnSOD) (Touati 2000; Touati et al., 1995; Varghese et al., 2007).

The *fur* gene is often part of an oxidative stress regulon. In *E. coli*, OxyR activates *fur* in response to H_2O_2 (Zheng et al., 1999). So in *E. coli*, if H_2O_2 levels are high, the bacterium responds by increasing Fur levels, so that if iron levels are also high, the cell can immediate respond through Fur to decrease iron-uptake and prevent iron availability for Fenton chemistry.

The Fur-like protein, PerR, is a regulator that responds concomitantly to high iron and high H_2O_2 levels. PerR exists in two forms: one that binds Fe^{+2} and one that binds Mn^{+2} (Lee and Helmann, 2006). PerR-Fe is obviously responsive to cellular iron levels, but PerR can still function as a repressor even when iron levels are low within the bacterial cell, because it can function when bound to Mn^{+2}. The iron-bound form of PerR is inactivated by H_2O_2 (Herbig and Helmann, 2001). Since PerR activity is affected by both iron and H_2O_2, it is well-suited to control the expression of genes whose gene products are involved with iron metabolism and oxidative stress. In *Bacillus subtilis*, the PerR regulon consists of oxidative defense genes such

as *katA* and *ahpC* and the iron metabolism genes including *hemAXCDBL* (heme biosynthesis), *fur, perR,* and *mrgA* (iron-binding *dps* homolog) (Helmann et al., 2003). In *Staphylococcus aureus*, PerR controls expression of the oxidative defense genes *katA, ahpC, bcp* (bacterioferritin comigratory protein), and *trxB* (thioredoxin B). PerR also controls the expression of the iron metabolism genes *ftn* (ferritin), *mrgA*, and *perR* (Horsburgh et al., 2001). PerR controls *sodA* in *Streptococcus pyogenes* and *ahpC* and *katA* in *Campylobacter jejuni* (Ricci et al., 2002; van Vliet et al., 1999). And so, the iron-responsive regulator PerR controls the expression of oxidative stress genes in many bacteria.

Another Fur-like regulator found in the α-proteobacteria is Irr. Irr is defined as an iron responsive regulator that regulates genes whose gene products are involved in iron metabolism (Hamza et al., 1998). Active heme biosynthesis causes Irr degradation, because the heme bound to ferrochelatase deactivates Irr (Qi et al., 1999). Since iron is in heme, Irr is described as being iron-responsive indirectly through heme biosynthesis (Yang et al., 2006; Qi et al., 1999). Irr is also responsive to H_2O_2 exposure. In *B. japonicum,* H_2O_2 will oxidize the carboxy-terminus of Irr in the presence of heme and oxygen and cause Irr degradation (Yang et al., 2006). Thus Irr degrades in response to high levels of H_2O_2 and iron, both of which are the reactants of Fenton chemistry (reaction 6).

In summary, iron contribures to oxygen toxicity through Fenton chemistry. To prevent this, bacteria use iron-responsive regulators and oxidative stress regulators concomitantly to keep iron and H_2O_2 cellular levels low enough to minimize hydroxyl radical formation. Therefore, when examining the regulation of iron metabolism genes, researchers should include OxyR as a potential regulator. Likewise, when examining the regulation of antioxidant genes, researchers should study the effects of Fur and Fur homologs such as PerR and Irr.

Potential *B. abortus* oxidative stress regulators

The putative *Brucella* antioxidants KatE, AhpCD, Bcp, Rbr (rubrerythrin) and Tpx (thiol peroxidase) may all degrade hydrogen peroxide. Therefore, they may all be members of the same oxidative stress regulon in *B. abortus*. Currently, the only known oxidative stress regulator in *B. abortus* is OxyR, but evidence suggests this is not the primary oxidative stress regulator (see below; Kim and Mayfield, 2000). There are five regulators that affect antioxidant gene expression in other bacteria that are encoded in the *B. abortus* genome sequence. These five are OxyR, PerR, NolR, the Fur-homolog Irr, and the OxyR-like regulator BAB2_0530. With the exception of OxyR, none of these regulators have been tested to regulate the expression of a *B. abortus* antioxidant gene.

B. abortus OxyR

Kim and Mayfield (2000) published evidence that suggested *Brucella abortus* S19 has two oxidative stress regulators. Both regulators are activated by H_2O_2 exposure. However one regulator, which is currently unidentified, protects bacterial cells immediately after exposure, while the second regulator, OxyR, protects bacterial cells after a prolonged exposure to H_2O_2.

B. abortus OxyR is a 35 kDa protein that contains all of the conserved OxyR domains. It has a HTH motif within amino acid residues 19 - 37 and two conserved cysteines at positions 200 and 209 that correspond to C199 and C208 in the *E. coli* OxyR. OxyR is hypothesized to be the primary regulator of *B. abortus katE*. This is for many reasons: 1) the *oxyR* gene is divergently transcribed from *katE* and they share a common promoter region of 169 bp, 2) the *oxyR* mutant is more sensitive than wild-type to H_2O_2, 3) both *katE* and *oxyR* mutants are defective in adapting to H_2O_2, 4) there is a predicted OxyR binding site for both *katE* and *oxyR*

(Table 1.2) in their shared promoter region, and 5) a 35 kDa protein binds to the *katE-oxyR* promoter region in EMSA (Kim and Mayfield, 2000). Because of these reasons, OxyR is hypothesized to be the primary candidate for controlling *katE* expression in *B. abortus*. However to confirm this hypothesis, purified OxyR must be shown to bind to the *katE-oxyR* promoter region, and *katE* inducible expression must be shown to differ between wild-type and an *oxyR* mutant.

There are no data in the literature that support OxyR's importance for the survival of *B. abortus in vivo*. Furthermore, an OxyR regulon has not been defined in the *Brucella* spp. And so, the contributions of OxyR to the survival of *B. abortus in vivo* and *in vitro* in response to ROS produced from the respiratory chain and from the macrophage are currently unknown.

Other potential oxidative stress regulators

A *B. melitensis nolR* mutant is attenuated in HeLa cells, J774 macrophage-like cells, and in BALB/c mice by one week post-infection (Haine et al., 2005). Therefore, NolR seems to be critical for *Brucella* survival within the experimental host. The *B. melitensis* NolR has 63% amino acid identity to the *Sinorhizobium meliloti* global regulator NolR, and thus because NolR is so important *in vivo*, NolR may be a global regulator in the *Brucella* spp. as well. NolR is critical for nodulation and metabolism in *Sinorhizobium,* and NolR represses *ahpC* expression by 1.5-2.5 fold in *S. meliloti* (Chen et al., 2000). Therefore it is possible that NolR regulates *ahpC* in *B. abortus* too.

Currently, there are no reports characterizing the *Brucella* PerR in the literature, but data from our laboratory show that a *B. abortus perR* mutant is significantly more resistant to H_2O_2 than wild-type (Anderson and Roop, unppublished*)*. Resistance to H_2O_2 is indicative of an

oxidative stress regulator that represses an antioxidant gene when cellular H_2O_2 levels are low and repression is relieved when H_2O_2 levels increase. Since PerR has a role in the regulation of *ahpC* and *kat* in other bacteria, it is an obvious candidate to examine in *B. abortus*.

Irr proteins are only found in the *Rhizobiales* and *Rhodobacterales* families (Johnston et al., 2007) and have been best characterized in *Bradyrhizobium japonicum*. Irr is an iron sensor in *B. abortus* and can directly affect the expression of genes in response to heme biosynthesis. When cellular iron levels are low, *B. abortus* Irr has a two-fold repressive effect on its own expression and increases *dhbCEBA* (encodes a siderophore) expression two-fold (Rudolph et al., 2006; Martinez et al., 2006). When cellular iron levels increase, the iron is incorporated into heme and the heme binds to Irr leading to its degradation.

Irr can also act as a H_2O_2 sensor. In *B. japonicum*, H_2O_2 reacts with the carboxy-terminus of Irr and causes Irr to degrade (Yang et al., 2006). This may also occur in *B. abortus*, because data suggest that Irr may be regulating one or more antioxidants in response to H_2O_2. *B. abortus irr* mutants are more resistant to H_2O_2 than wild-type in iron-deficient media (Martinez et al., 2006), which suggests that Irr regulates an antioxidant gene.

Divergently transcribed from the *B. abortus ahpCD* operon is a LysR-type transcriptional regulator designated in the *B. abortus* genome as BAB2_0530. BAB2_0530 most likely functions as an OxyR homolog, because the *B. abortus* BAB2_0530 amino acid sequence contains most of the critical residues for OxyR activity (Table 1.3). The *B. abortus oxyR* is divergently transcribed from *katE*, which OxyR regulates, and it is interesting that the *B. abortus* BAB2_0530 gene is divergently transcribed from *ahpCD*. Because of the LysR properties and

BAB2_0530's homology to OxyR, BAB2_0530 makes an excellent candidate to be an oxidative stress regulator for *B. abortus ahpCD*.

Table 1.1. List of genes encoding antioxidants and their potential regulators in *B. abortus* 2308.

	Gene name	Gene designation in *Brucella abortus* 2308 genome
Antioxidants	*katE*	BAB2_0848
	ahpCD	BAB2_0531-0532
	bcp	BAB1_0941
	tpx	BAB1_0504
	rbr	BAB1_1691
Regulators	*irr*	BAB1_2175
	BAB2_0530	BAB2_0530
	oxyR	BAB2_0849
	perR	BAB1_0393
	nolR	BAB1_1605

The B. abortus 2308 genome is listed in GenBank as Brucella melitensis biovar abortus 2308.

Figure 1.1. The AhpC enzymatic reaction. Hydrogen peroxide or organic peroxide molecules oxidize thiol groups to sulfenic acid groups in a reduced AhpC protein (A). An intermolecular disulfide bridge forms between two AhpC monomers to form an oxidized dimer (B). AhpD or AhpF reduce the AhpC dimer (C).

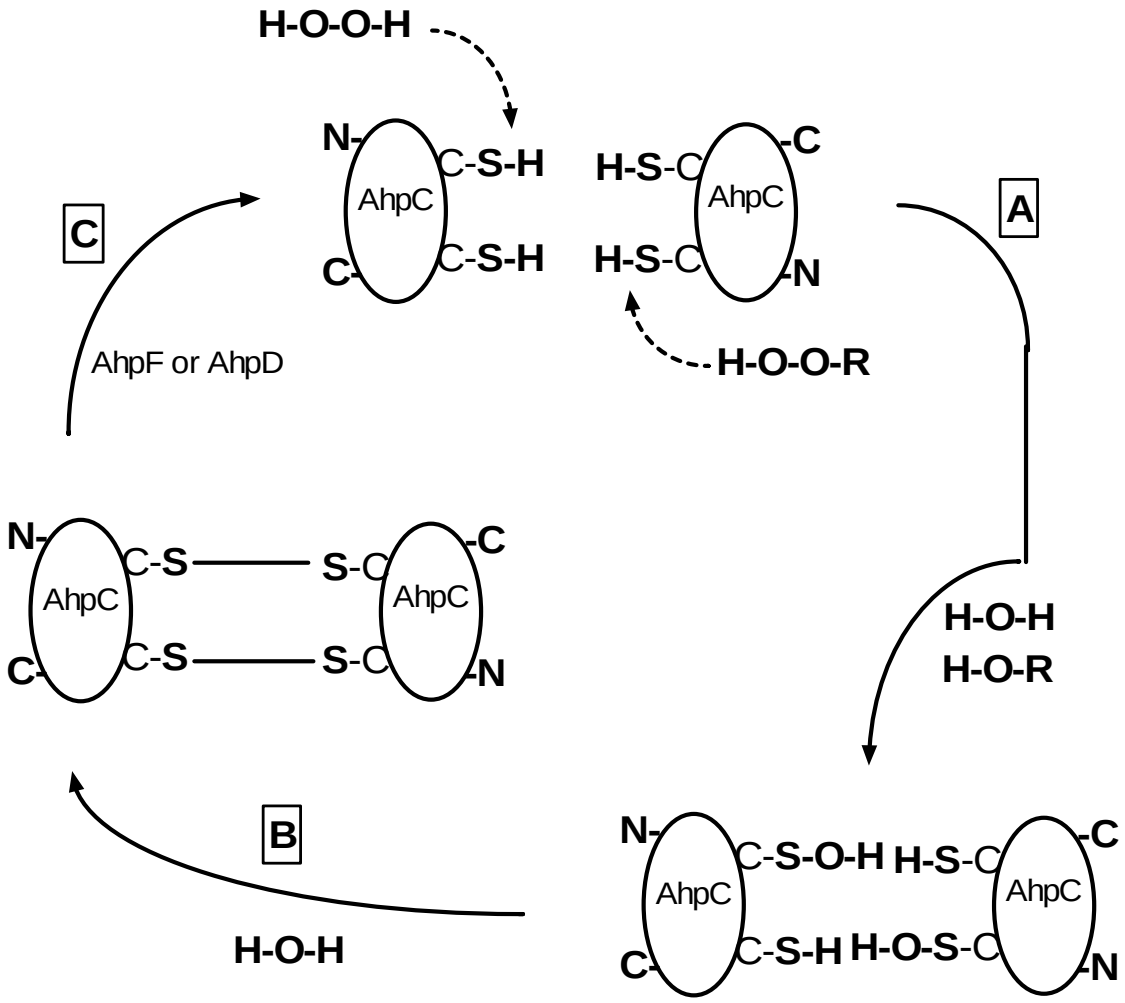

Figure 1.2. *E. coli* OxyR binds directly upstream of the -35 site in the promoter of its target genes (Tartaglia et al., 1992).

Table 1.2. The OxyR binding site is a pattern motif (Ochsner et al., 2000, Ohara et al., 2006).

Organism	**Promoter target**	**OxyR binding site**
Pseudomonas aeruginosa	*katB*	ATTG-N_7-TAAT-N_7-GTGA-N_7-CAAT
Pseudomonas aeruginosa	*ahpB*	ATAG-N_7-CTAT-N_7-ATTG-N_7-ACAT
Pseudomonas aeruginosa	*ahpC*	ATAG-N_7-TAAT-N_7-ATGG-N_7-CAAT
Escherichia coli	Consensus	ATAG-N_7-CTAT-N_7-ATAG-N_7-CTAT
Bacteroides fragilis	*sod*	ATAA-N_7-CTGT-N_7-CTAT-N_7-CAAA
Porphyromonas gingivalis	*ahpC*	ATAG-N_7-CAAT-N_6-ATAT-N_7-CTGT
Porphyromonas gingivalis	*sod*	CTAT-N_7-TCAT-N_7-ATCG-N_7-CGAC
Brucella abortus	*katE*	ATAG-N_7-TTAT-N_7-AACA-N_7-CAAT
Brucella abortus	*oxyR*	ATTG-N_7-TGTT-N_7-ATAA-N_7-CTAT

Table 1.3. BAB2_0530 and the *Brucella* OxyR have most of the *E. coli* and *S. typhimurium* amino acid residues necessary for OxyR activity.

E. coli OxyR residue	Function residue is critical for	*B. abortus* OxyR corresponding residue	*B. abortus* BAB2_0530 corresponding residue	Reference
D142	Binding to RNA polymerase	D143	D145	Paget and Buttner, 2003
T238	Binding to RNA polymerase	T239	T241	Paget and Buttner, 2003
R4	DNA binding	R5	R7	Paget and Buttner, 2003
Y8	DNA binding	Y9	Y11	Paget and Buttner, 2003
A22	DNA binding	A22	A25	Paget and Buttner, 2003
P30	DNA binding	P30	--	Paget and Buttner, 2003
S33	DNA binding	S33	S36	Paget and Buttner, 2003
C199	OxyR activation	C200	C202	Imlay 2008; Zheng et al., 1998; Mongkolsuk and Helmann, 2002
C208	OxyR activation	C209	--	Imlay 2008; Zheng et al., 1998; Mongkolsuk and Helmann, 2002

Double dashes indicate the absence of the corresponding amino acid residue

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Statement of the Problem

Brucella abortus resides and replicates inside macrophages during infection, and the ability of *Brucella* to survive within this intracellular niche is critical for the establishment of disease in host animals. Exposure to the oxidative burst of host phagocytes is one of the environmental stresses the brucellae must deal with during their intracellular residence. Also, since the *Brucella* spp. are aerobes, these bacteria must detoxify reactive oxygen species (ROS) generated as a by-product of their respiratory metabolism. *B. abortus* uses KatE to detoxify hydrogen peroxide, but *B. abortus katE* mutants are virulent in mice and *B. melitensis katE* mutants are virulent in goats. So it is still undetermined what antioxidants are required by the brucellae to detoxify hydrogen peroxide produced from aerobic metabolism and from the macrophage's oxidative burst. Furthermore, the primary oxidative stress regulator in the *Brucella* spp. is unknown. The genes designated as BAB2_0531 and 0532 in the *B. abortus* 2308 genome sequence are predicted to encode the components (AhpC and AhpD) of an alkyl hydroperoxide reductase complex. Peroxiredoxins of the AhpC family are important enzymes that detoxify H_2O_2, organic peroxides and peroxynitrite in bacterial cells. The research described in this dissertation evaluates the role of *B. abortus* AhpCD in the degradation of H_2O_2 produced endogenously from aerobic metabolism and the degradation of H_2O_2 produced from the macrophage's oxidative burst. Also, this dissertation evaluates the expression levels of *ahpCD* in response to increased oxidative stress and examines candidate regulators that may be involved.

Chapter 2

A comparative study of the roles of AhpC and KatE as respiratory antioxidants in *Brucella abortus* **2308** (Steele et al., 2010)

Abstract

Brucella strains are exposed to potentially toxic levels of H_2O_2 both as a consequence of their aerobic metabolism and through the respiratory burst of host phagocytes. To evaluate the relative contributions of *Brucella*'s sole catalase KatE and the peroxiredoxin AhpC produced by these strains in defense against H_2O_2 mediated toxicity, isogenic *katE, ahpC* and *katE ahpC* mutants were constructed. The phenotypic properties of these mutants were 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_2O_2 generated by aerobic metabolism. KatE, on the other hand, plays a major role in scavenging exogenous and supraphysiologic levels of H_2O_2, although this enzyme can play a supporting role in the detoxification of H_2O_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 the generation of endogenous H_2O_2 represents a relevant environmental stress that *B. abortus* 2308 must cope with during its residence in the host, and that AhpC and KatE perform compensatory roles in detoxifying metabolic H_2O_2.

Introduction

Brucella abortus, a facultative intracellular pathogen, causes abortion and infertility in cattle. Humans can also be infected by ingesting contaminated dairy products, through inhalation of infectious aerosols, or via direct contact with an infected fetus (Nicoletti, 1989). Human brucellosis causes flu-like symptoms with a relapsing fever, and this debilitating disease can persist for months or years without appropriate treatment. Although human brucellosis remains a significant zoonotic disease worldwide (Pappas et al., 2006) and a potential bioterrorism threat (Valderas and Roop, 2006), there is currently no vaccine to prevent human infection, and antibiotic treatment of these infections remains problematic (Ariza et al., 2007).

Prolonged survival and replication in host macrophages have a critical role in the virulence of the *Brucella* spp. (Kohler et al., 2003; Roop et al., 2009). Experimental evidence indicates that reactive oxygen species (ROS) such as superoxide ($O_2\bullet^-$) and hydrogen peroxide (H_2O_2) are important contributors to the brucellacidal activity of phagocytes (Jiang et al., 1993). Because brucellae rely on respiratory metabolism for their energy production (Rest and Robertson, 1975), these bacteria must also deal with endogenous ROS generated as a by-product of aerobic metabolism (Imlay, 2008). Several enzymes that directly detoxify $O_2\bullet^-$ and H_2O_2 have been identified in *Brucella*. SodC is a periplasmic Cu-Zn superoxide dismutase (Beck et al., 1990), and phenotypic evaluation of an isogenic *sodC* mutant indicates that this enzyme protects *B. abortus* 2308 from $O_2\bullet^-$ generated by the oxidative burst of host macrophages (Gee et al., 2005). *Brucella* strains also produce a periplasmic catalase (Sha et al., 1994), and *B. abortus* and *B. melitensis katE* mutants exhibit increased sensitivity to H_2O_2 compared to their parental strains in *in vitro* assays (Kim et al., 2000; Gee et al., 2004). *B. melitensis katE* mutants retain

virulence in experimentally infected goats (Gee et al., 2004), however, and the *B. abortus katE* mutant displays wild type virulence in the mouse model (Sangari and Agüero, 1996). This suggests that KatE plays a dispensable role in protecting the brucellae from oxidative killing by host phagocytes. A gene (BAB1_0591) encoding a Mn superoxide dismutase (SodA) has also been identified in *B. abortus* 2308. SodA activity increases in a *B. abortus sodC* mutant suggesting that SodA works in concert with SodC to protect *B. abortus* 2308 from oxidative damage (Sriranganathan et al., 1991), but the precise role that SodA plays in resistance to oxidative stress in this bacterium remains to be determined experimentally.

The genes designated BAB2_0531 and 0532 in the *B. abortus* 2308 genome sequence are predicted to encode the components of an alkyl hydroperoxide reductase complex - AhpC and AhpD, respectively. Peroxiredoxins of the AhpC family detoxify H_2O_2, organic peroxides and peroxynitrite ($ONOO^-$) (Bryk et al., 2000; Parsonage et al., 2008). AhpD and AhpF are peroxiredoxin reductases that use reducing equivalents generated by cellular metabolism to restore the enzymatic activity of AhpC (Poole, 1996; Bryk et al., 2002). Studies performed with multiple bacteria indicate that the AhpCD and AhpCF complexes serve as important antioxidants (Storz et al., 1989; Bsat et al., 1996; Mongkolsuk et al., 2000; Olczak et al., 2002; Baillon et al., 1999; Croxen et al., 2007; LeBlanc et al., 2006; La Carbona et al., 2007; Cosgrove et al., 2007; Brenot et al., 2005; Rocha and Smith, 1999), and experiments conducted in *E. coli* suggest that AhpC is the major scavenger of H_2O_2 generated in the cytoplasm as a by-product of aerobic metabolism (Seaver and Imlay, 2001a). AhpC has also been shown to play a role in the virulence of several bacterial pathogens including *Helicobacter pylori* (Olczak et al., 2003), *Mycobacterium bovis* (Wilson et al., 1998) and *Staphylococcus aureus* (Cosgrove et al., 2007), but does not appear to be required for the virulence of *Salmonella typhimurium* (Taylor et al.,

1998), *Mycobacterium tuberculosis* (Springer et al., 2001), *Legionella pneumophila* (Rankin et al., 2002) or *Porphyromonas gingivalis* (Johnson et al., 2004) in experimental models.

In this report, we present evidence that AhpC is the primary antioxidant used by *B. abortus* 2308 to detoxify endogenous H_2O_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 H_2O_2, although this enzyme can play a supporting role in the detoxification of H_2O_2 of endogenous origin if AhpC is absent. Interestingly, AhpC and KatE appear to play complementary roles in protecting *B. abortus* 2308 from H_2O_2 of metabolic origin during murine infection, 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 2.1) were cultivated on Schaedler agar (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 (Sambrook et al., 1989) or on LB agar at 37°C. Chloramphenicol (15 μg/ml for *Brucella* strains; 30 μg/ml for *E. coli* strains) (Sigma-Aldrich), kanamycin sulfate (45 μg/ml) (Invitrogen), hygromycin (25 μg/ml for *Brucella* strains; 200 μg/ml for *E. coli* strains) (A.G. Scientific) and ampicillin or carbenicillin (25 μg/ml for *Brucella* strains; 100 μg/ml for *E. coli* strains) (Sigma-Aldrich) were added to culture media as necessary for selection of bacterial strains carrying antibiotic resistance markers.

Recombinant DNA techniques. Standard methods were employed for the manipulation of recombinant DNA molecules and amplification of DNA by polymerase chain reaction (PCR) (Sambrook et al., 1989; Ausubel et al., 2000). Plasmid DNA was introduced into *Brucella* strains by electroporation (Elzer et al., 1994).

Construction of *B. abortus* mutants. A previously described gene replacement strategy (Elzer et al., 1994) was used to introduce defined mutations into the genome of *B. abortus* 2308. ColE1-based plasmids containing *cat*-disrupted versions of the *ahpCD* (pKHS3) and *katE* loci (pMEK7-9c) and a *bla*-disrupted *ahpCD* locus (pKHS5) (Table 2.1) were independently

introduced into *B. abortus* 2308 by electroporation and transformants were selected on SBA containing chloramphenicol or ampicillin. Putative *ahpCD-cat* (designated KH16), *ahpCD-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 using *ahpCD*-, *katE*-, *cat*-, and *bla*- specific primer sets as appropriate and Southern blot analysis with probes for *ahpCD*-, *cat*-, and *bla*-.

Plasmid pMEK7-9k, which contains an *aph3a*-disrupted version of *katE* (Gee et al., 2004), was introduced into the *B. abortus ahpCD* mutant KH16 by electroporation, and transformants were selected on SBA supplemented with kanamycin. A putative *B. abortus ahpCD katE* double mutant (designated as 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 ahpCD 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 pKHS5 was then used to introduce a *bla*-disrupted version of the *ahpCD* locus into MEK6 resulting in the construction of the *B. abortus ahpCD katE* double mutant KK9. The genotypes of *B. abortus* strains KK21 and KK9 were confirmed by PCR analysis of genomic DNA from these strains using *ahpCD*-, *katE*-, *cat*-, *bla*-, and *aph3a*- specific primer sets as appropriate and Southern blot analysis with probes for *ahpCD*-, *cat*-, *bla*- and *aph3a*.

Crystal violet exclusion was used to verify that the *B. abortus ahpCD* and *katE* mutants and *ahpCD katE* double mutants retain their smooth lipopolysacharide phenotypes (Alton and

Jones, 1988). A solution of 3% H_2O_2 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 × 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 (OD_{600}) of 1.0. A commercial version of the xylenol orange/ferrous iron-based hydrogen peroxide assay originally described by Wolff (1994) (National Diagnostics) was used to measure the amount of peroxides in the cell suspensions following the manufacturer's directions. Briefly, 100 µl of bacterial cell suspension was added to 900 µl of the assay reagent, the reaction mixtures incubated at room temperature for 30 minutes and the absorbance of the reaction mixtures measured in a spectrophotometer at 560 nm. Cell-free H_2O_2 standards (0 µM, 1 µM, 2 µM, 4 µM, and 8 µM) were used to construct a standard curve, and the levels of peroxides in the cell suspensions were determined by comparison to the standard curve.

This assay was also used to measure the degradation of H_2O_2 by *B. abortus* strains after an exogenous exposure. *B. abortus* strains grown on SBA supplemented with appropriate antibiotics for 48 h were inoculated into 5 ml brucella broth in 17 × 100 mm tubes and incubated at 37°C with shaking at 165 rpm. Following overnight incubation, the bacterial cells were harvested by centrifugation (12,100 × *g*, 10 min, RT) and resuspended in PBS to an OD_{600} of 1.0

in three 17 × 100 mm tubes designated as no exposure, immediately-after-exposure, time-after-exposure. For the immediately-after-exposure tubes, cultures were vortexed for 5 seconds after the addition of hydrogen peroxide, and 100 µl of bacterial cell suspension was removed and immediately added to 900 µl of the assay reagent. For the time-after-exposure tubes, cultures were vortexed for 5 seconds after the addition of H_2O_2 and allowed to incubate at 37°C with shaking at 165 rpm for the appropriate time. Following incubation, 100 µl of the bacterial cell suspension was removed and added to 900 µl of the assay reagent.

Disk sensitivity assay for measuring the sensitivity of *B. abortus* strains to paraquat and H_2O_2. *B. abortus* strains were grown on SBA supplemented with the appropriate antibiotics for two days, harvested into brucella broth and adjusted to a cell density of 10^9 CFU per ml ($OD_{600\ nm}$ = 0.15). 600 µl aliquots of each cell suspension were added to 18 ml prewarmed (55°C) brucella broth supplemented with 0.7% agar, and 3 ml portions of the resulting cell suspensions plated onto three Schaedler agar (SA) plates and three SA plates containing 7,800 U/ml bovine catalase (Sigma). A sterile 7-mm Whatman no. 3 filter paper disk was placed in the center of each plate and 10 µL of a fresh 0.5 M solution of paraquat (PQ; Acrōs Organics) was added to each disk. Plates were incubated for three days and the zones of inhibition surrounding each disk were measured in millimeters.

This same assay was also used to measure to sensitivity of *B. abortus* strains to H_2O_2 with the exceptions that 10 µl of a 30% solution of H_2O_2 was added to the filter disks instead of PQ, 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 × 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 10^3 CFU per ml or 500 ml flasks containing 100 ml Gerhardt's minimal media (GMM) (Gerhardt 1958) at a cell density of 10^8 CFU per ml and the flasks incubated at 37°C while shaking at 165 rpm. The number of viable brucellae in these cultures was determined at selected times after 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% CO_2 for 48 h. Bacterial cells were harvested and resuspended to a cell density of 10^8 in 1 ml PBS in 17 × 100 mm culture tubes. The peroxynitrite generator SIN-1 [3-morpholinosydnonimine HCl (Sigma Aldrich)] at a final concentration of 15 mM and 1000U/ml of bovine catalase were added to the cell suspensions and the mixtures incubated for 1 h at 165 rpm at 37°C. The number of viable brucellae in these cultures and in parallel cultures that were not exposed to SIN-1 was 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. (2005) 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 x 10^5 cells per well in sterile 96-well plates in DMEM-FCS [Dulbecco's Modified Eagle's

Medium (ATCC) with fetal calf serum] containing 20 µg/ml gentamicin. After an overnight incubation, 100U/ml IFN-γ (Peprotech) was added to cultured macrophages for 30 minutes before macrophages were washed and co-incubated with the *Brucella* strains (MOI 50:1) that were previously opsonized with a sub-agglutinating concentration (1:1,000) of hyperimmune mouse serum. After allowing phagocytosis to occur for 2 hours, extracellular bacteria were removed by incubating the macrophage monolayer with DMEM-FCS containing 50 µg/ml gentamicin for one hour. The cell culture medium was then replaced with DMEM-FCS containing 20 µg/ml gentamicin and this medium was replaced at 24 hour intervals for incubation times that extended to 48 hours. At 2, 24, and 48 hours post-infection, the phagocyte monolayer was washed with PBS-FCS, lysed with 0.1% deoxycholate, and the number of viable intracellular brucellae was determined by serial dilution and plating on SBA. Triplicate wells of phagocytes infected with each strain were evaluated at every time point.

Experiments were also performed to evaluate the effects of treatment of the cultured macrophages with the NADPH oxidase inhibitor apocynin (Sigma) (Muijsers et al., 2000) and the inducible nitric oxide synthase inhibitor N^G-methyl L arginine (L-NMMA; Fisher) (Ding et al., 1988). The protocol described above was used except that 500 µM of apocynin, L-NMMA, or both were included in the DMEM-FCS throughout the experiment. Microscopic analysis of nitroblue tetrazolium reduction was used to monitor the oxidative burst capacity of the cultured macrophages (Bowe F 1994), and control experiments were performed to ensure that apocynin and/or L-NMMA at this concentration in DMEM were not toxic for the *Brucella* strains.

Experimental infection of mice. The procedures previously described by Robertson and Roop (1999) were used to evaluate the spleen colonization profiles of the *B. abortus* strains in C57BL/6 and BALB/c mice from Harlan Laboratories. These methods were also used to compare the virulence of *B. abortus* strains in C57BL/6J mice and derivatives of these mice that lack a functional phagocyte NADPH oxidase (B6.129S6-*Cybb*^{tm1Din}/J) or inducible NO synthase (B6.129P2-*Nos2*^{tm1lau}/J) obtained from Jackson Laboratories. Briefly, mice were infected with 5 $\times$ 10^4 brucellae via intraperitoneal injection and at each sampling point post infection the mice were euthanized, their spleens aseptically removed, and spleen homogenates serially diluted and plated on SBA to determine the number of viable brucellae present.

Results

Identification of an alkyl hydroperoxide reductase complex (AhpCD) in *B. abortus* 2308. The genes designated as 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 encode the 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 peroxynitrite (Hillas et al., 2000; Bryk et al., 2000; Seaver and Imlay, 2001a). AhpD is a peroxiredoxin reductase that uses reducing equivalents generated by cellular metabolism to recycle the enzymatic activity of AhpC (Hillas et al., 2000; Bryk et al., 2002). 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 (Hillas et al., 2000; Chauhan and Mande, 2002) are conserved as Cys-57, Cys-171 and Cys-173 in the *Brucella* AhpC ortholog. Likewise, the *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 that are required for the peroxiredoxin reductase activity of the mycobacterial AhpD (Bryk et al., 2002; Hillas et al., 2000). Reverse transcriptase PCR analysis indicates that the *ahpC* and *ahpD* genes in *B. abortus* 2308 are co-transcribed 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 (Hillas et al., 2000; Bryk et al., 2002).

A *B. abortus ahpCD* mutant exhibits higher levels of endogenous cellular peroxides than the parental strain. Studies in *E. coli* indicate that AhpC plays a major role in removing the H_2O_2 that is generated in the cytoplasm of this bacterium as a by-product of aerobic metabolism (Seaver and Imlay, 2001a). Phenotypic analysis of the *B. abortus* mutant *ahpCD* KH16 suggests that the *Brucella* AhpC performs a similar function. Significantly higher levels of endogenous peroxides are detected in KH16 compared to the parental 2308 strain following aerobic growth (Figure 2.1), 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* (Figure 2.1). Because monofunctional catalases such as the *Brucella* KatE detoxify H_2O_2 but not organic peroxides (Loewen, 1997; Chelikani et al., 2004), these findings indicate that the elevated levels of endogenous peroxides detected in the *ahpC* mutant are predominantly made up of H_2O_2.

The biochemical properties of catalases allow these enzymes to degrade H_2O_2 across a broad range of concentrations (Switala and Loewen, 2002). Accordingly, catalases often provide bacteria with a second line of defense against the build up of endogenous H_2O_2 of metabolic origin when primary detoxifiers such as AhpC are absent (Seaver and Imlay, 2001a; Cosgrove et al., 2007). 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 that 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 H_2O_2 during routine aerobic cultivation.

AhpCD is required for the wild-type resistance of *B. abortus* 2308 to endogenous H_2O_2 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 O_2 and the generation of $O_2\bullet^-$ in these cells (Hassan and Fridovich, 1979). This $O_2\bullet^-$ then serves as a substrate for cytoplasmic superoxide dismutases such as SodA which can convert this ROS to H_2O_2 and O_2 (Fridovich, 1995). Spontaneous non-enzymatic dismutation of $O_2\bullet^-$ to H_2O_2 and O_2 also occurs under physiologic conditions (Imlay, 2008). Thus, one of the consequences of treating respiring bacterial cells with PQ is the generation of increased intracellular levels of H_2O_2. The *B. abortus ahpCD* mutant KH16 and *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 (Figure 2.2A). 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.

H_2O_2 is an uncharged ROS and can readily cross cellular membranes by diffusion. This allows extracellular catalase to serve as an efficient detoxifier of intracellular H_2O_2 (Seaver and Imlay, 2001b). 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 PQ is due to the increased intracellular accumulation of H_2O_2. As shown in Figure 2.2B, the addition of exogenous catalase to the test medium reduces the zone of inhibition around disks containing PQ 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 PQ is due to the increased intracellular accumulation of H_2O_2 and not a differential sensitivity of these mutants to $O_2^{\bullet -}$. More importantly, these experimental findings further support the contention that AhpC serves as a primary detoxifier of endogenous H_2O_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 rich medium (brucella broth) (Figure 2.3A). 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 (Figure 2.3B). 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 (Figure 2.3C), 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_2O_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_2O_2 in *B. abortus* 2308, the results presented in Figure 2.4 indicate that KatE is the major detoxifier of exogenous H_2O_2 in this strain. Even at levels of exogenous H_2O_2 as low as 5 μM, the *B. abortus katE* and *ahpCD katE* mutants exhibited a marked defect in their capacity to degrade exogenous H_2O_2 compared to their parental strains (Figure 2.4A), and these defects are much more dramatic when these strains are exposed to 50 and 100 μM H_2O_2 (Figure 2.4B-E). The role of KatE in the degradation of exogenous H_2O_2 is further reflected in the differences in the sensitivities to H_2O_2 exhibited by the *B. abortus ahpC* and *katE* mutants and the *ahpCD katE* double mutant in a disk sensitivity assay (Table 2.3), where the strains lacking KatE display a much more pronounced phenotype than the *ahpC* mutant.

The *B. abortus ahpCD* mutant KH16 displays increased sensitivity to peroxynitrite. Biochemical studies have shown that in addition to H_2O_2, AhpC can also detoxify organic peroxides such as t-butyl hydroperoxide (t-BOOH) (Hillas et al. 2000), cumene hydroperoxide (CHP) (Jacobson et al., 1989; Poole and Ellis, 1996), and peroxynitrite (ONOO-) *in vitro* (Bryk et al., 2000); and genetic studies have shown that this peroxiredoxin provides bacterial cells with

an important defense against environmental exposure to these compounds (Storz et al., 1989; Chen et al., 1998; Master et al., 2002). The results shown in Figure 2.5 suggest that AhpC plays an important role in protecting this bacterium from exposure to $ONOO^-$. Compared to the parental strain, the *ahpC* mutant KH16 displays an increased sensitivity to the $ONOO^-$ generator SIN-1 in an *in vitro* assay (Figure 2.5), 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 presently unclear. The *B. abortus ahpCD* mutant KH16 exhibits variable and inconsistent sensitivity to t-BOOH and CHP in standard *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 (Roop et al., 2009). To determine to what extent AhpC and KatE protect *B. abortus* 2308 from H_2O_2 of exogenous and endogenous origin in the host, the virulence properties of *B. abortus* 2308 and isogenic *ahpCD*, *katE* and *ahpCD katE* mutants were evaluated in cultured murine macrophages and experimentally infected mice. Only the *B. abortus ahpCD katE* mutant KK9 exhibited significant and stable attenuation compared to the parental 2308 strain in cultured murine macrophages (Figure 2.6) and this attenuation was consistently observed only when these

phagocytes were stimulated with IFN-γ. 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 (Figure 2.6). These experimental findings suggest that neither AhpC nor KatE is playing a role in protecting *B. abortus* 2308 from exogenous H_2O_2 produced as a result of 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.

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 (Figure 2.7A) or BALB/c mice (Figure 2.7B), and the severe attenuation exhibited by *B. abortus* KK9 in C57BL/6J mice was not alleviated in congenic NADPH oxidase- and iNOS-knockout mice (Figures 2.7C and D). These data provide further evidence that neither AhpC nor KatE plays a direct role in protecting *B. abortus* 2308 from the oxidative or nitrosative bursts of host phagocytes. Instead, the data shown herein support the contention that the build-up of endogenous H_2O_2 is a biologically relevant environmental stress encountered by *B. abortus* 2308 during its residence in the murine host (Roop et al., 2009). Moreover, this study demonstrates 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_2O_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 is consistent with the reported biochemical properties of this class of peroxiredoxins which work most efficiently on low levels of H_2O_2 (Seaver and Imlay 2001a). The capacity of AhpC to scavenge metabolic H_2O_2 appears to be especially important to *B. abortus* 2308 for maintenance of stationary phase viability when 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 (Roop et al., 2003; Schurig-Briccio et al., 2009; Wasim et al., 2009). The basis for the H_2O_2-responsive 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 biosynthetic enzymes in *B. abortus* 2308 may be particularly susceptible to H_2O_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. For instance, H_2O_2-mediated damage of the [4Fe-4S] clusters in isopropylmalate isomerase, a key enzyme in the leucine biosynthetic pathway, leads to growth arrest in *E. coli katG katE ahpC* mutants (Jang and Imlay, 2007).

In contrast to 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_2O_2 of metabolic origin during routine aerobic

cultivation *in vitro*. These results are intriguing considering the compensatory roles that AhpC and catalases have been reported to perform in scavenging cytoplasmic H_2O_2 of metabolic origin in *ahpC* mutants in other bacteria (Seaver and Imlay, 2001a; Cosgrove et al., 2007). 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 affect growth. 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_2O_2 in *Bradyrhizobium japonicum* (Panek and O'Brian 2004), 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_2O_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 enhance this mutant's stationary phase viability defect during aerobic culture in a minimal medium. Furthermore, the *B. abortus ahpCD katE* mutant can still degrade 50 μM H_2O_2, suggesting that other antioxidants are present to remove the H_2O_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) (Jeong et al., 2000) and BAB1_0504 is predicted to encode an AhpC/TSA type peroxiredoxin that has sequence similarity to the PrxV type peroxidiredoxins that protect mammalian mitochondria from H_2O_2 damage (Banmeyer et al., 2005). 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_2O_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_2O_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_2O_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_2O_2. This strongly suggests that H_2O_2 toxicity plays a key role in the attenuation exhibited by the *B. abortus ahpCD katE* mutant. Secondly, 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_2O_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 the bacteria from the metabolic H_2O_2 generated endogenously during replication in the host. This is perhaps to be expected of a bacterium that must cope with exposure to ROS of both endogenous origin as well as those generated by the NADPH oxidase and iNOS activity of host phagocytes (Roop et al., 2009) during residence in this environment.

The increased sensitivity of the *B. abortus ahpC* mutant to the $ONOO^-$ generator SIN-1 in *in vitro* assays suggests that the *Brucella* AhpC, like its counterparts in *S. typhimurium*, *M. tuberculosis* and *H. pylori*, has peroxynitrite reductase activity (Bryk et al., 2000). This

enzymatic activity has been proposed be important as a bacterial defense against $ONOO^-$ production by host macrophages (Master et al., 2002), but the results obtained in this study with the *B. abortus ahpCD katE* 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 the ability to detoxify H_2O_2 and $ONOO^-$, bacterial AhpC proteins have also been shown to be able to degrade organic peroxides. Indeed, the name alkyl hydroperoxide reductase reflects the fact that degradation of organic peroxides was the first property identified for many members of this class of bacterial enzymes (Jacobsen et al., 1989). 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 (Mongkolsuk et al., 1998). 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 required to determine whether or not AhpC can detoxify organic peroxides in *B. abortus* 2308.

Acknowledgments

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Table 2.1. Bacterial strains used in this study

Strain or plasmid	*Genotype or description*	*Reference or source*
Strains		
Escherichia coli DH5α	F- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_{k-}, m_{k+}) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-	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::aph3A*; Kn^r	This study
KK9	2308 *ahpCD::bla, katE::aph3A*; Ap^r; Kn^r	This study
KK21	2308 *ahpCD::cat, katE::aph3A*; Cm^r; Kn^r	This study
Plasmids		
pGEM®-T Easy	ColE1-based cloning vector; Ap^r	Promega
pBC KS+	ColE1-based cloning vector; Cm^r	Stratagene
pBBR1MCS-4	pBBR-based broad host range cloning vector; moderate copy number (10-14 copies per cell); Ap^r	Kovach et al., 1995
pMR10-Ap	RK2-based broad host range cloning vector; low copy number (2-4 copies per cell); Ap^r	Valderas et al., 2005
pMR10	RK2-based broad host range cloning vector; low copy number (2-4 copies per cell); Kn^r	Gee et al., 2005
pBlue-CM2	656 bp *cat* gene from pBC cloned into the EcoRV site of pBluescript KS+	Robertson et al., 2000
pKS + Kan	794 bp *aph3A* gene from Tn*phoA* cloned into SalI-HindIII digested pBluescript II KS+	Kovach et al., 1995

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 pBBR1MCS-4 carrying the *katE* gene from *B. abortus* S19	Gee et al., 2004
pMWV77	2,094 bp *Not*I 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 pBBR1MCS-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 pBlue-CM2	This study
pKHS4	2,534 bp fragment from pKHS2 containing *ahpCD* cloned into pBC KS+	This study
pKHS5	Derivative of pKHS4 in which a 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
pMEK7-9	1,917 bp genomic DNA fragment from *B. abortus* S19 containing *katE* cloned into the PvuII site of pUC18	Gee et al., 2004
pMEK7-9c	Derivative of pMEK7-9 in which a 1 kb PflMI/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 PflMI/EcoRI fragment internal to the *katE* coding region was replaced with the *aph3A* gene from pKS + Kan	Gee et al. 2004

Table 2.2. Oligonucleotide primers used for PCR in this study.

Designation	Sequence
ahpCD-1F	5’-GCCAGAACCAGCGAACGGAA-3’
ahpCD-1R	5’-TGGGCTGATGGGCATGACCT-3’
ahpCD-2F	5’-CCAGTGCGAGAAAATAGTGAAGCTG-3’
ahpCD-2R	5’-GATCAAAACGGATCGCTTATTCAGT-3’
ahpCD-3F	5’-GGCAGAACCTTGGGCAGAAG-3’
ahpCD-3R	5’-CATCGTCACCGTGCTGATCG-3’

Table 2.3. Sensitivity of *B. abortus* 2308, KH16 (2308 *ahpCD*), KH2 (2308 *katE*) and KK21 (2308 *ahpCD katE*) to H_2O_2.

Strain	2308	KH16	KH2	KK21
	24 ± 0.58[a]	27 ± 0.58*	41 ± 1.5**	41 ± 0.0**

[a]Zone of inhibition in mm around disks containing 10 μl of a 30% solution of H_2O_2.

Significance - * = $P \leq 0.05$ and ** = $P \leq 0.005$ for comparisons of 2308 vs. KH16, KH2 or KK21.

Figure 2.1. *B. abortus ahpC* mutants exhibit increased levels of endogenous peroxides. The levels of peroxides present in *B. abortus* 2308, KH16 (2308 *ahpCD*), KH2 (2308 *katE*), KK21 (2308 *ahpCD katE*), KH16 [pMWV77] and KH16 [pMEK21] cell suspensions following aerobic cultivation were determined using a xylenol orange/ferrous iron-based hydrogen peroxide assay (Wolff 1994). The data presented are means and standard deviations for triplicate determinations for a single strain in a single experiment. The data presented here are representative of multiple ($\geq$ 6) experiments performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test for the comparison of 2308 versus the other strains is represented by an asterisk (*).

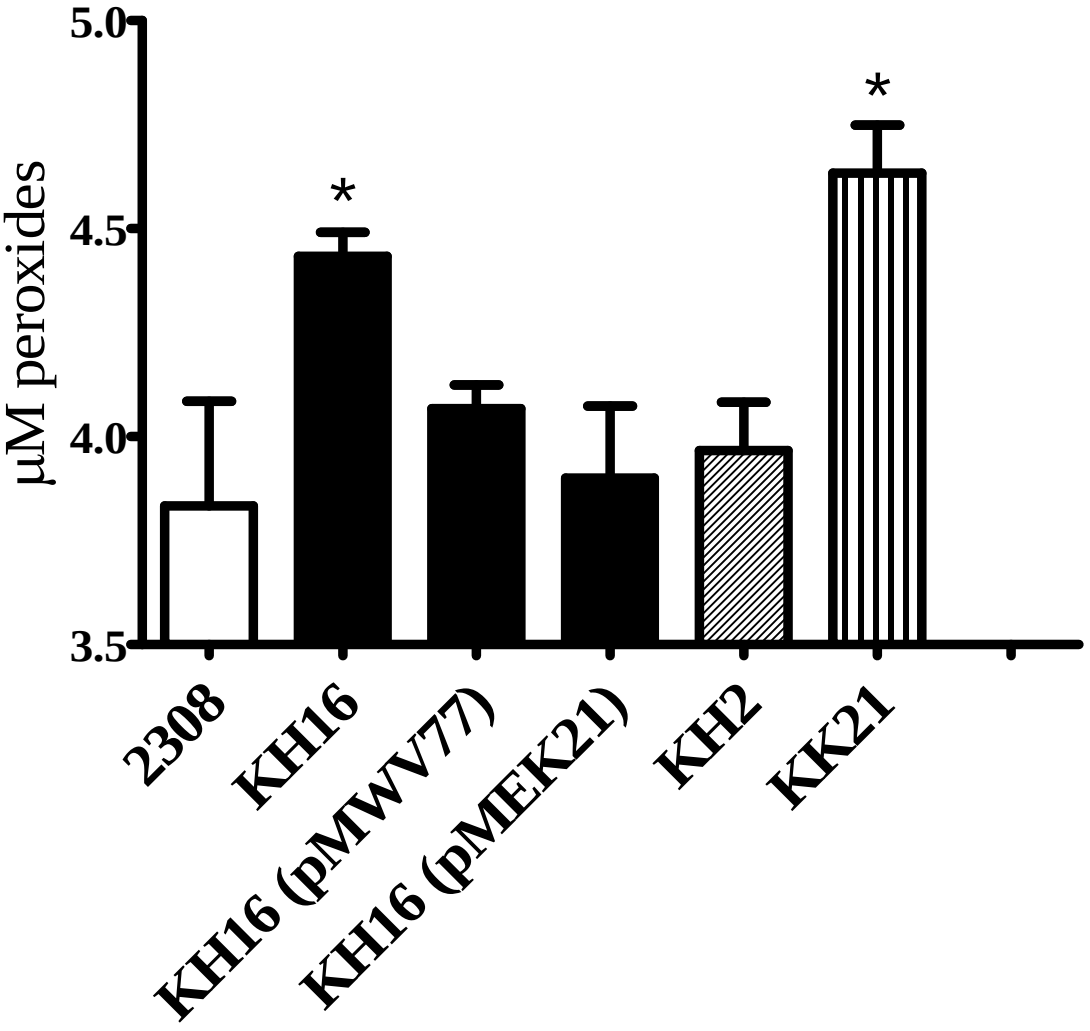

5.0
4.5
4.0
3.5
µM peroxides
*
*
2308
KH16
KH16 (pMWV77)
KH16 (pMEK21)
KH2
KK21

Figure 2.1

Figure 2.2. *B. abortus ahpCD* mutants display an increased sensitivity to endogenous H_2O_2 produced by the redox cycling activity of paraquat. Zones of inhibition for *B. abortus* 2308, KH2 (2308 *katE*), KH16 (2308 *ahpCD*), KK21 (2308 *ahpCD katE*), KH16 [pKHS6] and KK21 [pKHS6] around disks containing 0.5 M paraquat on (A) Schaedler agar or (B) Schaedler agar supplemented with 7,800 U/ml bovine catalase. The data presented are means and standard deviations for triplicate determinations for each strain in a single experiment. The data presented here are representative of multiple (≥ 4) experiments performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test for comparisons of 2308 versus the other strains is represented by an asterisk (*).

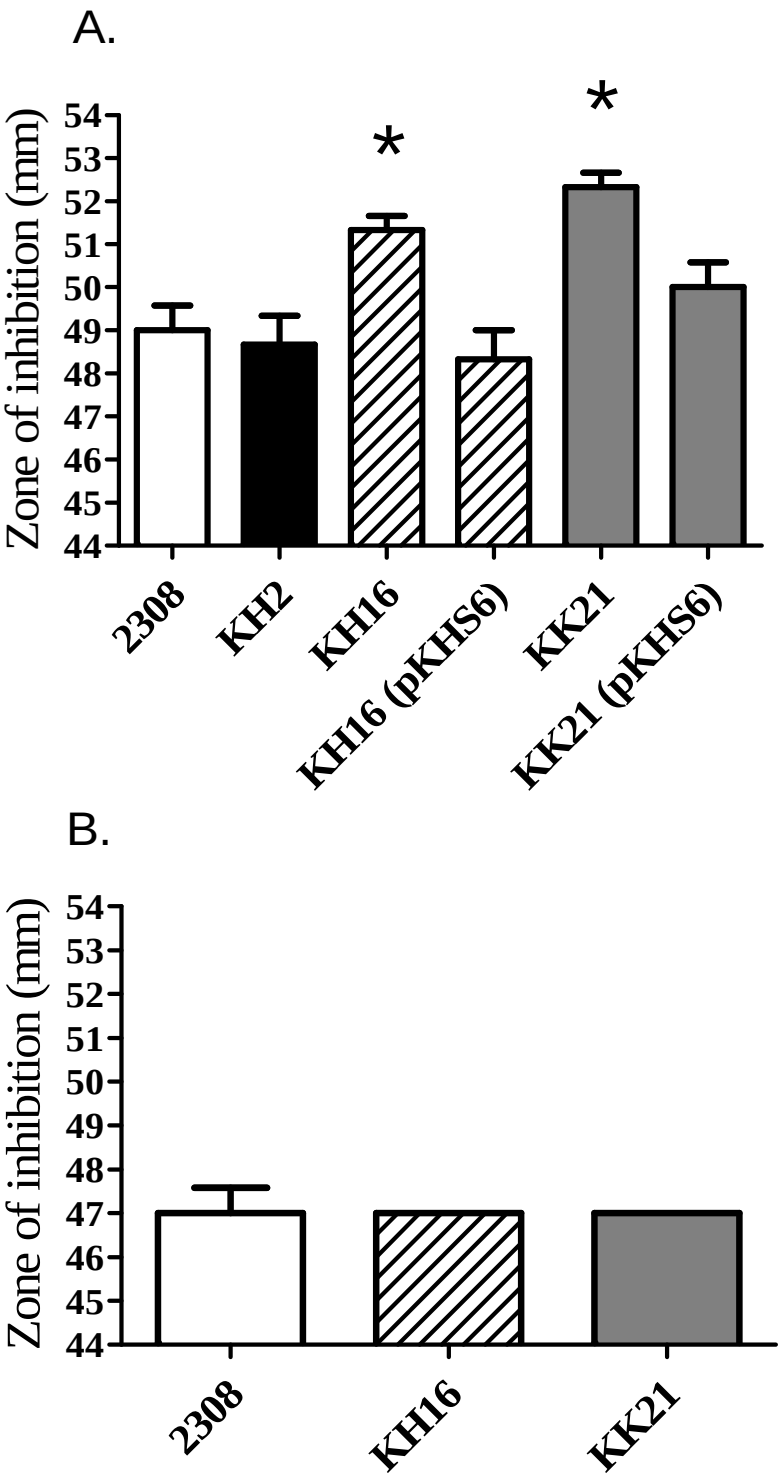

A.
Zone of inhibition (mm)
54
53
52
51
50
49
48
47
46
45
44
*
*
2308
KH2
KH16
KH16 (pKHS6)
KK21
KK21 (pKHS6)
B.
Zone of inhibition (mm)
54
53
52
51
50
49
48
47
46
45
44
2308
KH16
KK21

Figure 2.2

Figure 2.3. *B. abortus ahpCD* mutants show loss of stationary phase viability during cultivation in Gerhardt's minimal medium and this phenotype can be rescued by either AhpC or KatE. Growth of *B. abortus* 2308 (●), KH16 (2308 *ahpCD*) (■), KH2 (2308 *katE*) (▲), and KK21 (2308 *ahpCD katE*) (▼) in brucella broth (A) and Gerhardt's minimal medium [GMM] (B). Part C of this figure shows the viability of *B. abortus* 2308, KH16, KK21, KH16 [pKHS6], KH16 [pMEK21] and KK21 [pMEK21] following 240 and 288 hours cultivation in GMM. The data presented are means and standard deviations for triplicate determinations for each strain in a single experiment. The data presented here are representative of multiple (≥ 3) experiments performed from which equivalent results and statistical trends were obtained. In Figure 3B, statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test is represented by an asterisk (*) for the comparison of 2308 versus KH16 and a dagger (†) for the comparison of 2308 versus KK21. For Figure 3C, statistical significance ($P \leq 0.001$) for the comparisons of KH16 vs. KH16 [pKHS6], KH16 vs. KH16 [pMEK21] and KK21 vs. KK21 [pMEK21] is indicated by an asterisk (*).

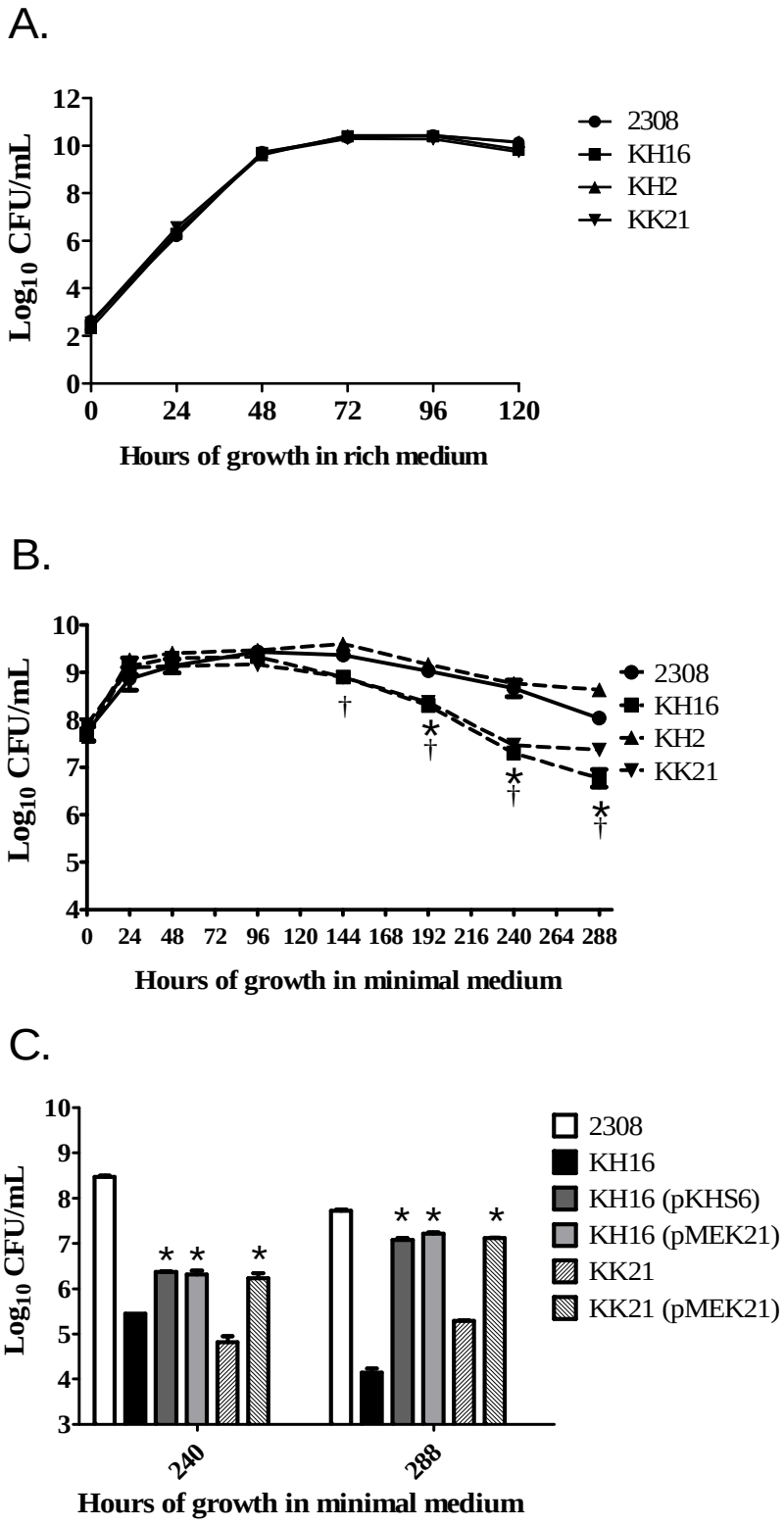

A.
Log10 CFU/mL
12
10
8
6
4
2
0
0
24
48
72
96
120
Hours of growth in rich medium
2308
KH16
KH2
KK21
B.
Log10 CFU/mL
10
9
8
7
6
5
4
0 24 48 72 96 120 144 168 192 216 240 264 288
Hours of growth in minimal medium
2308
KH16
KH2
KK21
C.
Log10 CFU/mL
10
9
8
7
6
5
4
3
240
288
Hours of growth in minimal medium
2308
KH16
KH16 (pKHS6)
KH16 (pMEK21)
KK21
KK21 (pMEK21)

Figure 2.3

Figure 2.4. *B. abortus katE* mutants degrade exogenous H_2O_2 more slowly than their parental strains. Figure 4A shows the levels of peroxides measured using the xylenol orange/ferrous iron assay in *B. abortus* 2308 (●), KH16 (2308 *ahpCD*) (■), KH2 (2308 *katE*) (▲), and KK21 (2308 *ahpCD katE*) (▼) cell suspensions at selected times after the addition of 5 μM H_2O_2. Figures 4B through E show the levels of peroxides present in *B. abortus* 2308, KH16, KH2 and KK21 cell suspensions, respectively, at selected times following the addition of 50 μM (solid lines) and 100 μM (dashed lines) H_2O_2. Figure 4F shows levels of H_2O_2 detected in cell free test medium at selected times following the addition of 50 μM (solid lines) and 100 μM (dashed lines) H_2O_2. The data presented here are representative of multiple (≥ 3) experiments performed from which equivalent results were obtained.

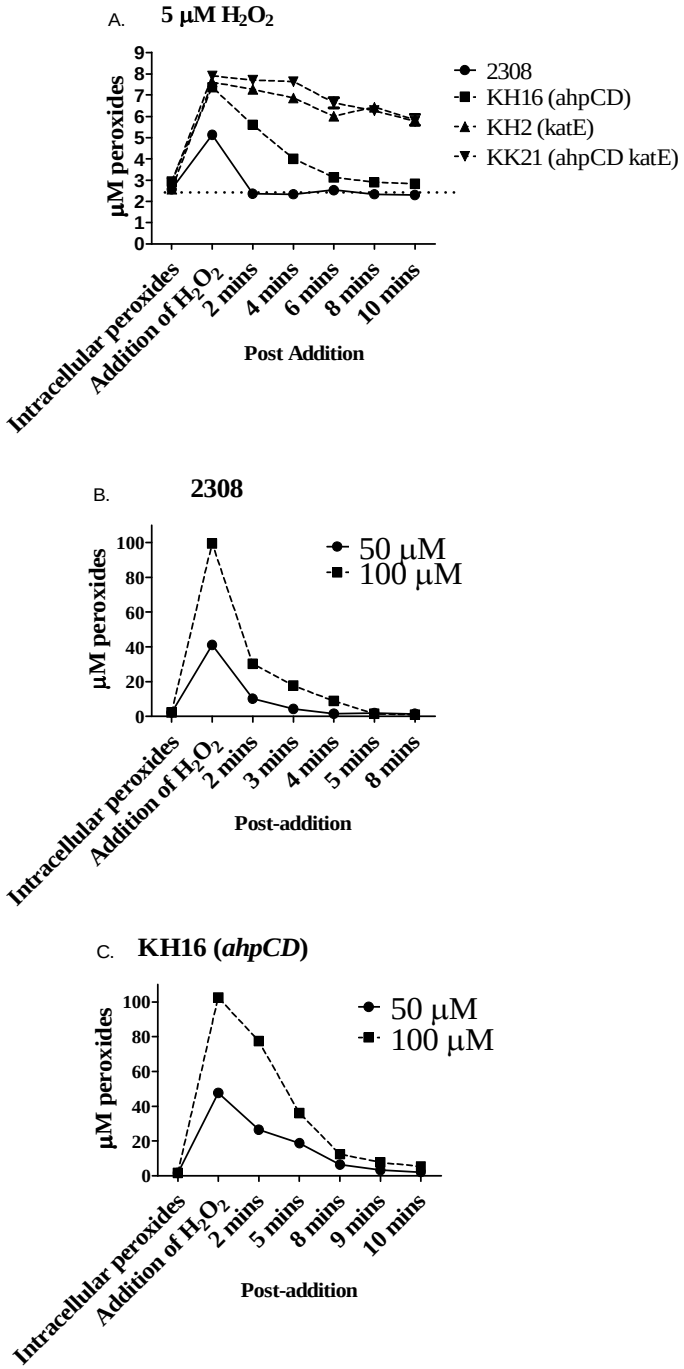
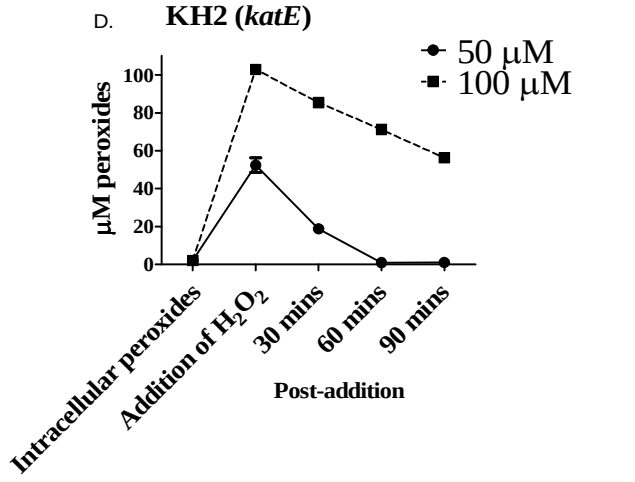
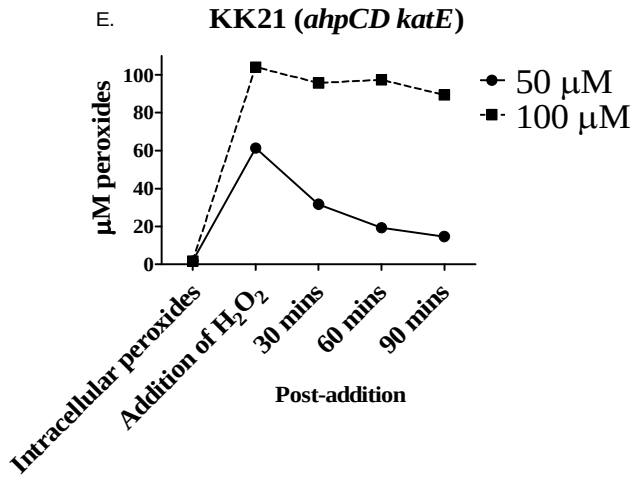

A.
5 μM H2O2
μM peroxides
2308
KH16 (ahpCD)
KH2 (katE)
KK21 (ahpCD katE)
Intracellular peroxides
Addition of H2O2
2 mins
4 mins
6 mins
8 mins
10 mins
Post Addition
B.
2308
μM peroxides
50 μM
100 μM
Intracellular peroxides
Addition of H2O2
2 mins
3 mins
4 mins
5 mins
8 mins
Post-addition
C.
KH16 (ahpCD)
μM peroxides
50 μM
100 μM
Intracellular peroxides
Addition of H2O2
2 mins
5 mins
8 mins
9 mins
10 mins
Post-addition

Figure 2.4





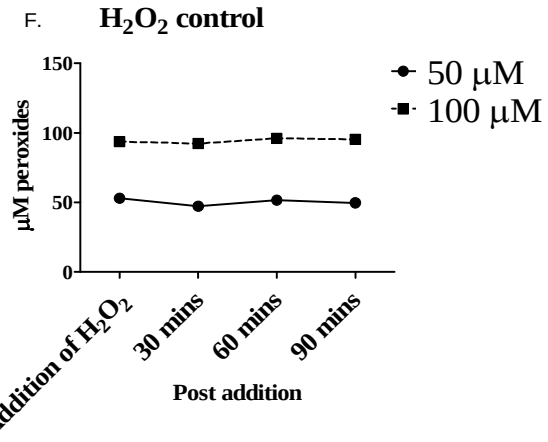


Figure 2.4 (continued)

Figure 2.5. The *B. abortus ahpCD* mutant KH16 exhibits an increased sensitivity to the peroxynitrite generator SIN-1. Viability of *B. abortus* 2308 (white), KH16 (2308 *ahpCD*) (black) and KH16 [pMWV77] (gray) before and after a 60 min exposure to SIN-1. The data presented are means and standard deviations for triplicate determinations for each strain in a single experiment. The data presented here are representative of multiple ($\geq$ 3) experiments performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test for comparison of 2308 vs. the other strains is represented by an asterisk (*).

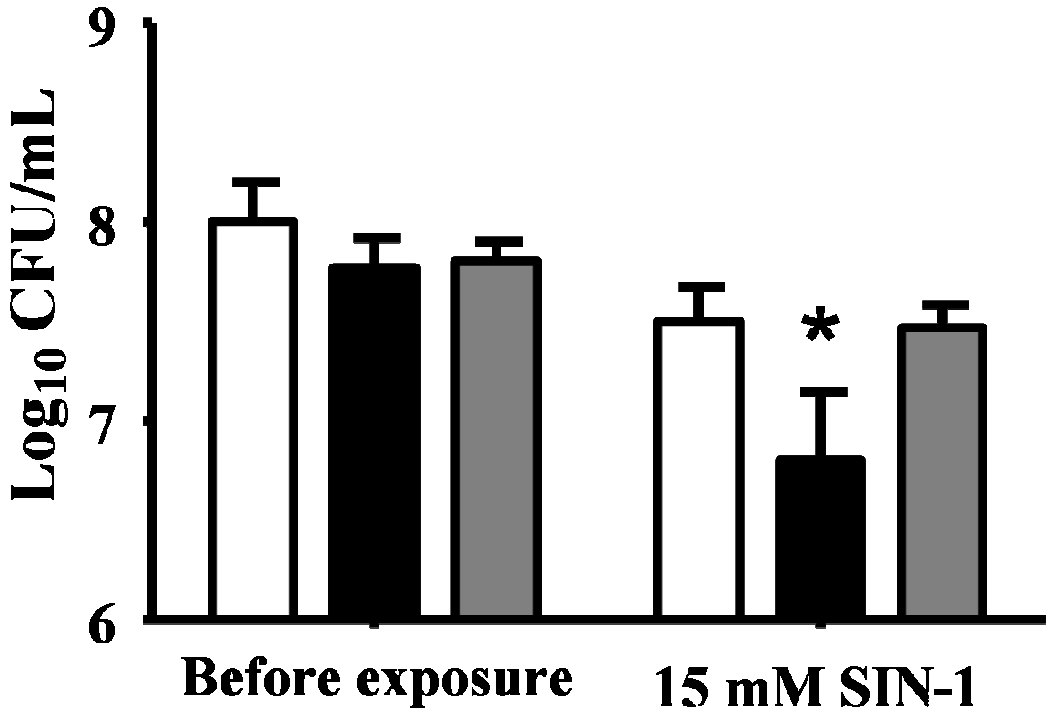


Figure 2.5

Figure 2.6. The *B. abortus ahpCD katE* mutant KK9 exhibits attenuation in IFN-γ-stimulated cultured murine peritoneal macrophages and this attenuation is not alleviated by the addition of inhibitors of the oxidative and nitrosative bursts of the host phagocytes. The intracellular survival and replication patterns of *B. abortus* 2308 and the isogenic *ahpCD katE* double mutant KK9 in IFN-γ-treated cultured resident peritoneal macrophages from BALB/c mice with or without the addition of the NADPH oxidase inhibitor apocynin and iNOS inhibitor L-NMMA are shown. The data presented are means and standard deviations obtained for each bacterial strain from three separate wells of cultured macrophages at each experimental time point in a single experiment. The data presented here are representative of multiple (≥ 3) experiments performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test for the comparison of 2308 vs. KK9 is represented by an asterisk (*).

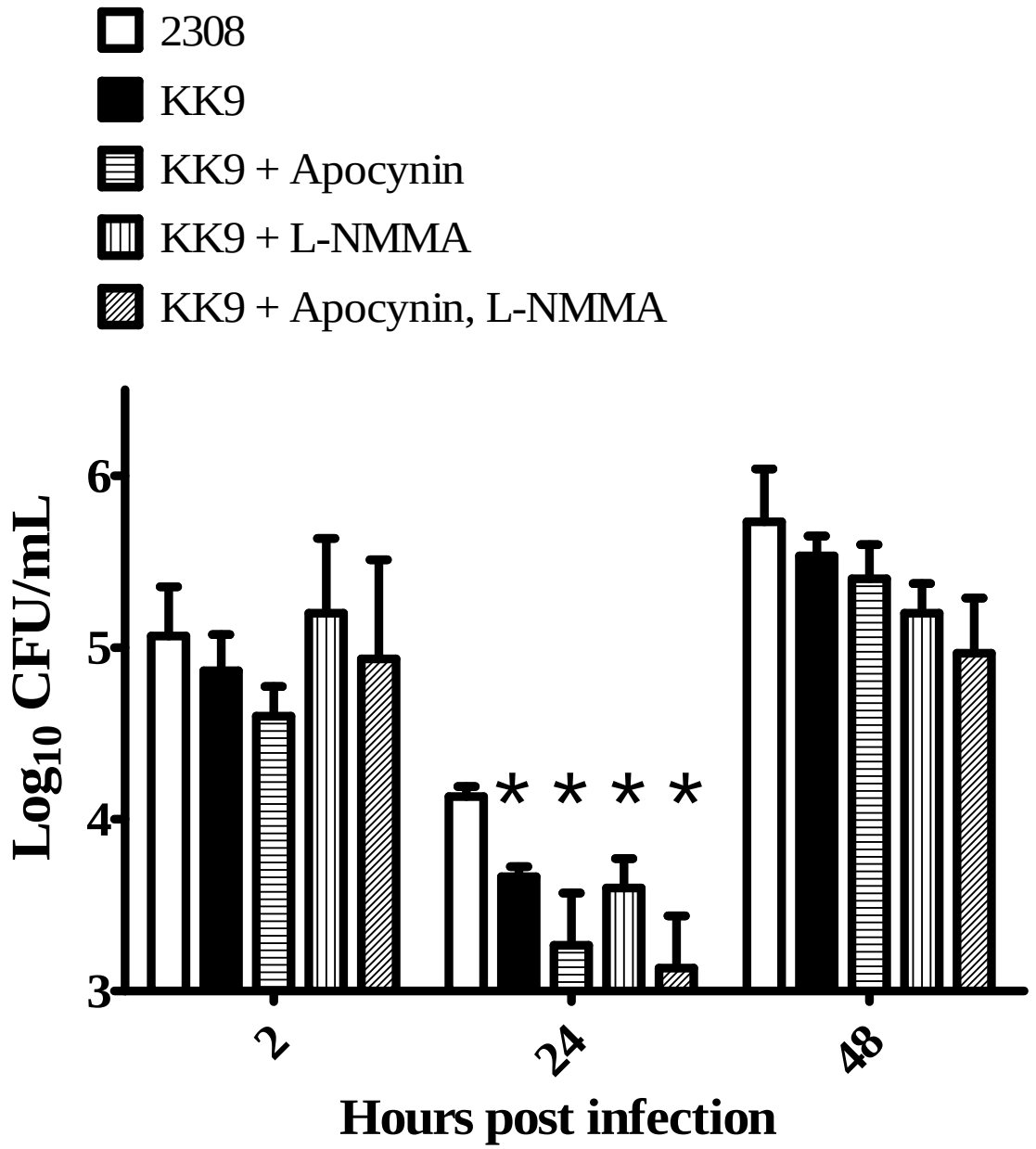

2308
KK9
KK9 + Apocynin
KK9 + L-NMMA
KK9 + Apocynin, L-NMMA
Log10 CFU/mL
6
5
4
3
* * * *
2
24
48
Hours post infection

Figure 2.6

Figure 2.7. Spleen colonization profiles of *B. abortus* 2308, MEK6 (2308 *katE*), KK9 (2308 *ahpCD katE*) and KH40 (*ahpCD*) in C57BL/6J mice (A, C and D), BALB/c mice (B) and NADPH oxidase-deficient (cybb-) and iNOS-deficient (Nos2-) knockout mice in the C57BL/6J background (C and D). The data presented are means and standard deviations for the number of brucellae recovered from the spleens of five mice infected with each strain at each experimental time point in a single experiment. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test from comparison of 2308 vs. the other strains is represented by an asterisk (*).

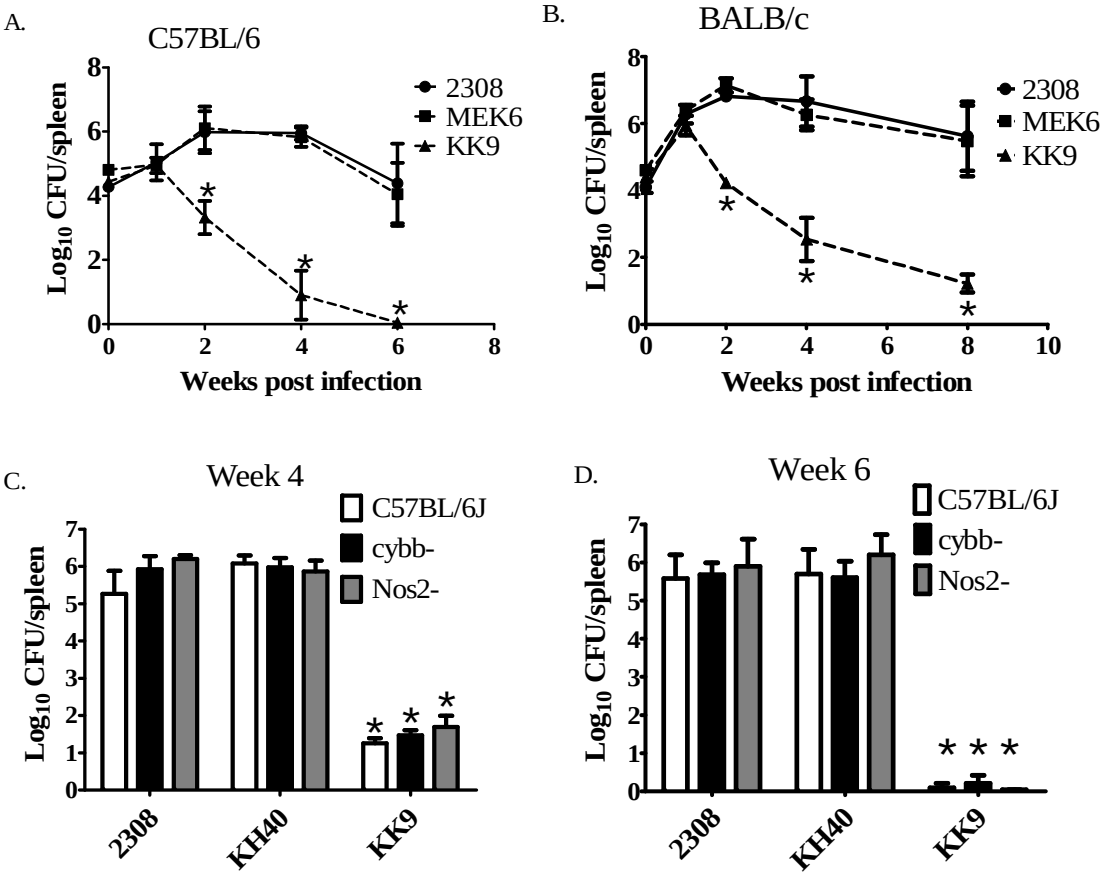

A.
C57BL/6
2308
MEK6
KK9
Log10 CFU/spleen
Weeks post infection
B.
BALB/c
2308
MEK6
KK9
Log10 CFU/spleen
Weeks post infection
C.
Week 4
C57BL/6J
cybb-
Nos2-
Log10 CFU/spleen
2308
KH40
KK9
D.
Week 6
C57BL/6J
cybb-
Nos2-
Log10 CFU/spleen
2308
KH40
KK9

Figure 2.7

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Chapter Three

Brucella abortus ahpCD expression is responsive to endogenous hydrogen peroxide through the iron-responsive regulator Irr

Abstract

Expression of an *ahpC-lacZ* fusion in *B. abortus* 2308 is responsive to endogenous H_2O_2 levels. Mutational analysis indicates that this H_2O_2-responsiveness is not mediated by OxyR or the LysR-type transcriptional regulator encoded by the gene (BAB2_0530) that lies immediately upstream of *ahpC*. Instead, the expression of *B. abortus ahpC* in response to H_2O_2 is indirectly affected by the iron-responsive regulator Irr.

Introduction

Oxygen is essential for aerobic bacteria to produce energy; on the other hand, oxygen can accept single electrons and become toxic to bacterial cells (Touati 2000; Imlay 2006). Upon univalent reduction, oxygen can be converted to superoxide and subsequently to hydrogen peroxide, both of which damage DNA, proteins, and lipids (Messner and Imlay, 1999; Imlay and Linn, 1988; Imlay 2006). Aerobic bacteria avoid this toxicity by producing antioxidants such as superoxide dismutase (SOD), catalase, and alkyl hydroperoxide reductase to remove these reactive oxygen species (ROS) (Imlay 2008). In order to respond quickly to an increase in ROS, bacteria coordinate the expression of the genes that encode these antioxidants using oxidative stress regulators. One important oxidative stress regulator in Gram-negative bacteria is OxyR (Imlay 2008). OxyR is a DNA-binding protein that is oxidized by hydrogen peroxide (H_2O_2) and activates the expression of *sod, kat,* and *ahpCF* or *ahpCD* in multiple bacteria (Åslund et al., 1999; Zheng et al., 2001; Christman et al., 1985; Pagán-Ramos et al., 2006; Mongkolsuk et al., 1998; Ochsner et al., 2000; Rocha et al., 2000; Ohara et al., 2006). Bacteria also prevent Fenton chemistry by coordinating the expression of antioxidant genes via iron-dependent regulators (Zheng et al., 1999). Fenton chemistry is the reaction of ferrous iron and H_2O_2 to form hydroxyl radical and ferric iron. Hydroxyl radicals damage proteins, lipids, and DNA (Babior 1978; Hassett and Cohen, 1989; Dubbs and Mongkolsuk, 2007). Fur is a regulatory protein that represses iron uptake genes when cellular iron levels are high (Zheng et al., 1999). Fur also activates the expression of *katA* in *Agrobacterium tumefaciens* and in *Escherichia coli*, Fur activates *sodB* and represses *sodA* (Kitphati et al., 2007; Touati 2000; Touati et al., 1995; Varghese et al., 2007).

The alkyl hydroperoxide reductase complex (AhpCD) is the primary antioxidant used to detoxify H_2O_2 produced by respiratory metabolism in *B. abortus* 2308 (Chapter 2). It is currently unknown though if the expression of *B. abortus ahpCD* is responsive to increased levels of H_2O_2 within the bacterial cell, or if the expression of these genes is constitutively high. Furthermore, our current knowledge of oxidative stress regulators in *B. abortus* is limited. OxyR regulates the expression of catalase and *ahpC* genes in many bacteria including *E. coli* (Christman et al., 1985), *Mycobacterium leprae* (Dhandayuthapani et al., 1997), *Xanthomonas campestris* (Loprasert et al., 1997; Loprasert et al., 2000), *Neisseria gonorrhoeae* (Seib et al., 2007), *Pseudomonas aeruginosa* (Ochsner et al., 2000), *Sinorhizobium meliloti* (Luo et al., 2005), and *Porphyromonas gingivalis* (Ohara et al., 2006). The *Brucella* OxyR has been characterized in the literature as a H_2O_2-responsive regulator that controls *katE* expression (Kim and Mayfield, 2000). However, evidence suggests that this OxyR homolog is only needed to protect the brucellae from prolonged exposure to high concentrations of H_2O_2 (e.g. mM) (Kim and Mayfield, 2000). Besides OxyR, there is another LysR-type transcriptional regulator, BAB2_0530, whose gene shares a promoter region with the *ahpCD* operon. This putative LysR-type transcriptional regulator possesses significant amino acid homology with bacterial OxyR homologs including the presence of key amino acids necessary for DNA binding, binding to RNA polymerase, and H_2O_2-responsiveness (Figure 3.1) (Paget and Buttner, 2003; Imlay 2008; Zheng et al., 1998; Mongkolsuk and Helmann, 2002). Since LysR transcriptional regulators often affect the expression of genes divergently transcribed from their own gene, BAB2_0530 is an excellent candidate to affect *ahpCD* expression in response to H_2O_2 exposure in *B. abortus* (Schell 1993).

Irr is an iron responsive regulator that regulates genes whose products are involved in iron metabolism (Hamza et al., 1998). In *Brucella abortus,* Irr represses the expression of its

own gene and activates expression of the siderophore biosynthesis operon *dhbCEBA* (Rudolph et al., 2006; Martinez et al., 2006). Iron and H_2O_2 work in concert to degrade Irr in *Bradyrhizobium japonicum*, and experimental evidence suggests that in addition to its role in regulating iron metabolism genes, Irr also plays a role in the regulation of bacterial genes involved in oxidative defense (Yang et al., 2006a). Martinez et al. (2006), for example, showed that a *B. abortus irr* mutant has increased catalase activity, degrades H_2O_2 faster than wild-type, and is more resistant to H_2O_2 than wild-type in iron-deficient media. In *B. japonicum*, Irr directly represses the rubrerythrin gene (*rbr*), whose gene product detoxifies H_2O_2 (Rudolph et al., 2006; Sangwan et al., 2008). Since H_2O_2 affects the protein stability of Irr, Irr may be a H_2O_2--responsive regulator that affects *ahpCD* expression in *B. abortus*.

In this report, we show that *ahpCD* expression in *B. abortus* 2308 is responsive to changes in endogenous H_2O_2 levels. This H_2O_2-responsiveness is not mediated by OxyR or the OxyR-like regulator encoded by BAB2_0530, but instead appears to be dependent upon the iron-responsive regulator Irr.

Materials and Methods

Bacterial strains and growth conditions. *Brucella abortus* 2308 and derivatives of this strain (Table 3.1) were cultivated on Schaedler agar (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* DH5α strains were grown in Luria Bertani (LB) broth (Sambrook et al., 1989) or on LB agar at 37°C. Zeocin (30 μg/mL) (Invitrogen), chloramphenicol (15 μg/ml) (Sigma-Aldrich), kanamycin sulfate (45 μg/ml) (Invitrogen), and ampicillin or carbenicillin (25 μg/ml for *Brucella* strains; 100 μg/ml for *E. coli* strains) (Sigma-Aldrich) were added to culture media as necessary for selection of bacterial strains carrying antibiotic resistance markers.

Recombinant DNA techniques. Standard methods were employed for the manipulation of recombinant DNA molecules and amplification of DNA by polymerase chain reaction (PCR) (Sambrook et al., 1989; Ausubel et al., 2000). Plasmid DNA was introduced into *Brucella* strains by electroporation (Elzer et al., 1994).

Construction of *B. abortus* mutants. A previously described gene replacement strategy (Elzer et al., 1994) was used to introduce defined mutations onto the genome of *B. abortus* 2308. A ColE1-based plasmid containing a *cat*-disrupted version of the *katE* locus (pMEK7-9c) (Chapter 2) was introduced into the *B. abortus ahpCD* mutant KH40 (Chapter 2) by electroporation and transformants were selected on SBA containing chloramphenicol. A putative *katE ahpCD* double mutant (designated KK30) was selected for further evaluation based on its lack of

catalase activity and resistance to chloramphenicol and carbenicillin. The genotype of KK30 was confirmed by PCR analysis of genomic DNA from this strain using *ahpC-, katE-, cat-*, and *bla-* specific primer sets as appropriate.

ColE1-based plasmids containing *cat*-disrupted versions of the BAB2_0530 locus (pKHS13), *oxyR* locus (pKHS12), and *irr* locus (pEA2) (Table 3.1) were independently introduced into *B. abortus* 2308, and transformants were selected on SBA containing chloramphenicol. Putative BAB2_0530 (designated KH112), *oxyR* (designated KH231), and *irr* (designated BEA2) mutants were selected for further evaluation based on their failure to grow on SBA supplemented with ampicillin. The genotypes of KH112, KH231, and BEA2 were confirmed by PCR analysis of genomic DNA from these strains using BAB2_0530-, *oxyR-*,*irr-*, *cat-*, and *bla-* specific primer sets as appropriate. Southern blot analysis with probes for *oxyR-*, *irr-*, *cat-*, and *bla-* was also done to further confirm the genotypes of KH231 and BEA2.

Production of recombinant *Brucella* BAB2_0530, OxyR, and Irr proteins. The *oxyR* and BAB2_0530 open reading frames from *B. abortus* 2308 were directionally cloned into pMAL-C2X digested with XmnI and SmaI (Table 3.1) to create *malE-oxyR* and *malE*-BAB2_0530 gene fusions (MBP). The resulting plasmids (pKHS14 and pKHS45; Table 3.1) were introduced into *E. coli* DH5α cells by chemical transformation. The MalE-OxyR and MalE-BAB2_0530 fusion proteins were purified from recombinant *E. coli* cultures via affinity binding to an amylose resin and elution with maltose following the manufacturer's directions.

The *irr* open reading frame from *B. abortus* 2308 was directionally cloned into pASK-IBA6 digested with EcoRI and EcoRV (IBA BioTAGnology) (Table 3.1) to create a gene

fusion of *irr* with nucleotides that encode an eight amino acid tag capable of binding to the biotin binding pocket of streptavidin. The resulting plasmid (pASK-irr; Table 3.1) was introduced into *E. coli* DH5α cells by chemical transformation. The tagged Irr protein was purified from recombinant *E. coli* cultures via affinity binding to a streptavidin derivative (Strep-Tactin) resin and elution with desthiobiotin, a biotin analog, following the manufacturer's directions.

β-galactosidase production by *B. abortus* strains carrying an *ahpC-lacZ* fusion in response to cellular H_2O_2 levels. A DNA fragment representing the intergenic region between BAB2_0530 and *ahpC* was amplified from the *B. abortus* 2308 chromosome using polymerase chain reaction (primer set intergenic-F/intergenic-R) and introduced into the SmaI site upstream of the promoterless *lacZ* gene in pMR15, a low copy number plasmid (Table 3.1). The *ahpC* promoter orientation with respect to *lacZ* was confirmed by PCR analysis. The resulting plasmid (designated pKHS15) was introduced by electroporation into *B. abortus* 2308, KH2 (*katE*), KH16 (*ahpCD*), KK30 (*ahpCD katE*), KH112 (BAB2_0530), KH231 (*oxyR*), BEA2 (*irr*), and derivatives of *B. abortus* 2308, KH112, KH231, and BEA2 harboring pBBRMCS4 or pMEK21. Bacterial colonies containing pKHS15 were selected by their resistance to kanamycin.

To measure the effect of lowering endogenous H_2O_2 levels on *ahpC* expression in *B. abortus* 2308, derivatives of this strain containing the *katE* bearing plasmid pMEK21 or the base vector pBBRMCS-4 in combination with the *ahpC-lacZ* bearing plasmid pKHS15 were grown on SBA for 48 h, and inoculated into 3 ml of brucella broth in 17 × 100 mm tubes followed by incubation at 37°C with shaking at 165 rpm. After overnight incubation, the bacterial cells were inoculated into 500 ml flasks containing 100 ml of brucella broth at a cell density of 10^4

CFU per ml and the flasks incubated at 37°C while shaking at 165 rpm for 30 h. Bacterial cells were harvested by centrifugation (12,100 × *g*, 10 mins, room temperature) and resuspended in PBS to an OD_{600} of 0.8. One hundred microliters of this bacterial cell suspension was removed and the levels of endogenous H_2O_2 present determined using the xylenol/orange ferrous iron based assay described previously (Wolff 1994; Chapter 2). β-galactosidase production by these cultures was measured using the procedures described by Miller (1972).

Previous work has shown that *B. abortus* AhpC is a major scavenger of metabolic H_2O_2 (Chapter 2). To evaluate the effect of increasing endogenous H_2O_2 levels on *ahpC* expression in *B. abortus* 2308, the methods described in the previous paragraph were used to compare β-galactosidase production from the *ahpC-lacZ* fusion in *B. abortus* 2308 and the isogenic *ahpC* mutant KH16.

Electrophoretic mobility shift assays. Purified recombinant OxyR-MBP and BAB2_0530-MBP fusion proteins were dialyzed in 1× binding buffer (4 mM Tris-HCl, 40 mM NaCl, 4 mM $MgCl_2$, 4% glycerol, H_2O) and adjusted to a protein concentration of 1 μg/μL using the Bradford assay (Bradford 1976) Using PCR, DNA fragments representing a 180 bp intergenic region between *katE* (BAB2_0848) and *oxyR* (BAB2_0849) [primer set pkatE-F/pkatE-R; Table 3.1], a 147 bp intergenic region between the BAB2_0530 gene and *ahpC* (BAB2_0531) [primer set pahpC-F/pahpC-R; Table 3.1], and a 169 bp DNA region in the *serA* (BAB1_0005) open reading frame [primer set p0005-F/p0005-R; Table 3.1] were amplified from *B. abortus* 2308 chromosomal DNA and radiolabeled with [α^{32}P]dATP using the following procedure previously described by Anderson et al. (2008). Briefly, 14 μL of purified

PCR fragment (10-20 ng/μL) was added to 2 μL *Taq* polymerase 10× reaction buffer (200 mM Tris pH 8.4, 500 mM KCl), 50 mM $MgCl_2$, 20 μCi [α^{32}P]dATP, and 5 units of *Taq* DNA polymerase. This mixture was incubated at 70°C for 30 min, unincorporated radiolabeled nucleotide was removed from the fragment using the QIAquick nucleotide removal kit (Qiagen), and the fragment was suspended in 30 μL elution buffer. Binding of the MalE-OxyR and MalE-BAB2_0530 fusion proteins to the radiolabeled DNA fragments was measured by mixing the corresponding purified protein with 2 μL radiolabeled promoter fragment, 10 μg sonicated salmon sperm DNA (Strategene), 2 μL 5× binding buffer, and sterile water. This mixture was incubated for 60 min at room temperature and then loaded onto a 6% non-denaturing acrylamide gel and subjected to electrophoresis at 150 V. The contents of the gel were visualized by autoradiography. To evaluate the specificity of the interactions between the MalE-OxyR fusion protein and the *katE* radiolabeled DNA fragment, an unlabeled version of the *katE* fragment was used as a specific inhibitor. The *serA* fragment was used as a nonspecific inhibitor.

A similar procedure was used to determine if Irr binds to the *ahpC* and *rbr* promoters. A 398 bp DNA fragment encompassing the *ahpC* promoter region [primer set pahpC-F2/pahpC-R; Table 3.1] and a 276 bp DNA fragment encompassing the region upstream of the transcriptional start site for *rbr* (BAB1_1691) [primer set prbr-F/prbr-R; Table 3.1] were amplified from *B. abortus* 2308 genomic DNA. The same protocols were used as described in the previous paragraph except purified recombinant Irr was dialyzed in 1× TB buffer (0.89 M Tris-Borate solution) and adjusted to a protein concentration of 1 ng/μL using the Bradford assay. Accordingly, 1× TB buffer was also the gel buffer used. We added 100 μM $MnCl_2$ to the gel buffer, acrylamide gel, and binding reactions because Sangwan et al. (2008) showed that the addition of $MnCl_2$ increases Irr binding to DNA.

Results

Genetic organization of the *B. abortus ahpCD* locus and surrounding regions. The genes designated as 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 encode the components of the alkyl hydroperoxide reductase complex AhpCD. Reverse transcriptase PCR analysis indicates that the *ahpC* and *ahpD* genes in *B. abortus* 2308 are co-transcribed as an operon (data not shown). As shown in Figure 3.2, directly upstream of the *ahpC* gene is a gene (BAB2_0530) predicted to encode a LysR-transcriptional regulator with significant homology to OxyR (Figure 3.1).

Primer extension analysis (data not shown) reveals one transcriptional start site for the *B. abortus ahpCD* operon located 60 bp upstream of the *ahpC* start codon. Within the intergenic region between *ahpC* and BAB2_0530 are two identical 17 bp direct repeats (Figure 3.2). The second 17 bp repeat overlaps the -35 region relative to the transcriptional start site for the *ahpCD* promoter. Interestingly, a predicted OxyR binding site with an AATC sequence that repeats at regular intervals overlaps both repeats. There is also a predicted Irr binding site with 71% homology to the predicted *Brucella* Irr binding motif (Rodionov et al., 2006) that overlaps the second repeat and -35 region of the *ahpCD* promoter. Since both the OxyR and Irr boxes overlap the -35 region of the *ahpCD* promoter, both regulators are presumed to function as activators if they regulate these genes.

***ahpC* expression is responsive to changes in endogenous H_2O_2 levels in *B. abortus* 2308.** *B. abortus* AhpCD is the primary detoxifier of endogenously produced H_2O_2 (Chapter 2). We

have shown that *B. abortus ahpCD* mutants have higher levels of intracellular H_2O_2 than wild-type, and that the build-up of this H_2O_2 reduces viability of *B. abortus ahpCD* mutants in late stationary phase when grown in minimal medium (Chapter 2). To learn if *ahpC* is constitutively expressed to remove H_2O_2 created from respiratory metabolism or if *ahpC* expression is responsive to increases in intracellular H_2O_2 concentrations, we measured the activity of an *ahpC-lacZ* fusion in response to both the removal of intracellular H_2O_2 and to an increase in intracellular H_2O_2.

pBBR1MCS-based plasmids replicate in *Brucella* strains with a copy number of approximately ten per bacterial genome (Elzer et al., 1995). Consequently, we used a derivative of pBBR1MCS4 carrying the *Brucella katE* gene (pMEK21) to reduce the levels of endogenous H_2O_2 present in *B. abortus* 2308 (Chapter 2). As seen in Figure 3.3, significantly reduced levels of endogenous H_2O_2 (Figure 3.3A) and β-galactosidase production from the *ahpC-lacZ* fusion (Figure 3.3B) were observed in the *B. abortus* 2308 derivative carrying pMEK21 compared to the isogenic derivative carrying the base vector pBBR1MCS4.

AhpC has previously been shown to be a primary scavenger of metabolic H_2O_2 in *B. abortus* 2308 (Chapter 2), and *ahpC* mutants exhibit elevated levels of endogenous H_2O_2 compared to the parent strain. As seen in Figure 3.4, significantly increased β-galactosidase production is observed from the *ahpC-lacZ* fusion in the *B. abortus ahpC* mutant KH16 compared to that observed in 2308, and this elevated *ahpC* expression correlates with an increased level of endogenous H_2O_2 in *B. abortus* KH16 compared to 2308. These experimental results indicate that *ahpC* expression in *B. abortus* 2308 is responsive to endogenous H_2O_2 levels.

Overproduction of KatE does not repress *ahpC* expression in a *B. abortus irr* mutant. Elevated expression of *katE* results in a significant decrease in β-galactosidase production from the *ahpC-lacZ* fusion in the *B. abortus* BAB2_0530 mutant KH112 (Figure 3.5A) and the *oxyR* mutant KH231 (Figure 3.5B) in the same manner as it does in the parental 2308 strain. In contrast, elevated expression of *katE* does not repress *ahpC* expression in the *irr* mutant BEA2 (Figure 3.5C). These experimental findings suggest that Irr has a role in the H_2O_2-responsive expression of *ahpC*.

Recombinant Irr, OxyR, and BAB2_0530 do not bind to the *ahpC* promoter region in electrophoretic mobility shift assays. As shown in Figure 3.6, recombinant *Brucella* Irr does not bind in a specific manner to a 398 bp DNA fragment representing the *ahpC* promoter region. These findings suggest that Irr plays an indirect role in regulating *ahpC* expression in *B. abortus* 2308. Similarly, no specific interactions between the OxyR-MBP (Figure 3.7A) and BAB2_0530-MBP (Figure 3.7B) fusion proteins and the *ahpC* promoter region could be detected in these assays. The OxyR-MBP fusion protein did exhibit a specific interaction in EMSA with the *katE* promoter region as expected based on the previously demonstrated role of OxyR in regulating *katE* expression in *Brucella* strains (Kim and Mayfield, 2000).

Discussion

In this report we show that β-galactosidase production from an *ahpC-lacZ* transcriptional fusion directly correlates with endogenous H_2O_2 levels in *B. abortus* 2308: *ahpC* expression decreases in response to decreased levels of endogenous H_2O_2, and *ahpC* expression increases in response to increased levels of endogenous H_2O_2. These data suggest that *ahpC* expression is regulated by one or more H_2O_2-responsive transcriptional regulators, and that AhpC is part of a *Brucella* oxidative stress response.

One of the regulators we considered was Irr. Irr is an iron responsive regulator that regulates genes involved in heme biosynthesis and iron metabolism in *Brucella* and other α-proteobacteria (Rudolph et al., 2006; Martinez et al., 2006; Martinez et al., 2005; Sangwan et al., 2008; Qi et al., 1999; Yang et al., 2006b), but this regulator has also been linked to oxidative defense in *Bradyrhizobium japonicum* and *Brucella abortus*. In *B. abortus*, an *irr* mutant degrades H_2O_2 faster than wild-type in iron-deficient media (Martinez et al., 2006). In *B. japonicum*, Irr directly represses *rbr*, whose gene product detoxifies H_2O_2 (Rudolph et al., 2006; Sangwan et al., 2008). When endogenous H_2O_2 levels increase, H_2O_2 interacts with the carboxy-terminus and destabilizes Irr (Yang et al., 2006a), increasing *rbr* expression so its gene product can then degrade the H_2O_2 (Rudolph et al., 2006; Sangwan et al., 2008). Thus, Irr is a regulator that can sense increases in cellular H_2O_2 levels and affect gene expression accordingly. Since Irr detects changes in endogenous H_2O_2 levels in *B. japonicum*, Irr would be a good regulator for sensing endogenous H_2O_2 levels in *B. abortus* and affecting *ahpC* expression.

Our data originally suggested that Irr directly represses *ahpC* expression at low endogenous H_2O_2 levels, but as these levels increase, Irr is degraded (Figure 3.8). The

electrophoretic mobility shift assays, however, showed no interaction between Irr and the *ahpC* promoter under the conditions examined. This suggests that Irr may be having an indirect effect on *ahpC* expression. One possible explanation is that Irr controls expression of the regulator that directly affects *ahpC* expression (Figure 3.9). For example, when cellular H_2O_2 levels are low, Irr activates *ahpR* (alkyl hydroperoxide repressor), whose gene product represses *ahpCD*; when cellular H_2O_2 levels increase, the H_2O_2 interacts with Irr leading to Irr's degradation, preventing *ahpR* gene activation, and keeping *ahpCD* expression high. Since Irr degrades when cellular iron levels are high (Hamza et al., 1998), elevated iron levels would also prevent *ahpR* gene activation and keep *ahpCD* expression high. Another possible explanation (Figure 3.9) is that the direct regulator that affects *ahpC* expression is a repressor and requires iron or iron-sulfur clusters for its regulatory ability. Irr potentially regulates genes (*sufBCDSIX*) encoding proteins involved in iron-sulfur cluster assembly in *B. abortus* (Rodionov et al., 2006), and since there are low cellular iron levels in an *irr* mutant (Martinez et al., 2006), the repressor would be inactive causing *ahpC* expression to increase independently of cellular H_2O_2 levels. In either scenario, *ahpC* expression is affected by cellular iron levels, which suggests that *Brucella* coordinates its oxidative stress response with iron metabolism to prevent Fenton chemistry and cellular damage caused by hydroxyl radical. Further experiments must be done to determine if *ahpCD* expression is iron-responsive, and what the precise role of Irr is in regulating *ahpC* expression.

ahpC is part of the OxyR regulon in several bacteria including *Escherichia coli* (Christman et al., 1985), *Mycobacterium leprae* (Dhandayuthapani et al., 1997), *Xanthomonas campestris* (Loprasert et al., 2000), *Pseudomonas aeruginosa* (Ochsner et al., 2000), and *Porphyromonas gingivalis* (Ohara et al., 2006). This does not appear to be the case in *B.*

abortus 2308 though, because the responsiveness of *ahpC* to endogenous H_2O_2 levels is not affected by an *oxyR* mutation. Furthermore, the recombinant OxyR-MBP fusion protein does not bind to the *ahpC* promoter region. This was unexpected because there is a predicted OxyR binding motif spaced at regular intervals in the *ahpC* promoter region that overlaps the -35 region. It is also unusual that OxyR would have no effect on *ahpC* expression considering that increasing endogenous H_2O_2 levels induces the OxyR regulon in other bacteria. In *P. aeruginosa* for example, OxyR induces *ahpB* 90-fold in response to increased endogenous $O_2\bullet^-$ and H_2O_2 levels generated from the redox cycler paraquat (Ochsner et al., 2000). In *X. campestris*, inactivation of *katA* or *ahpC* increases endogenous H_2O_2 levels, and the entire OxyR regulon is activated (Charoenlap et al., 2005).

The putative LysR-type regulator encoded by BAB2_0530 also does not appear to be playing a role in the regulation of *ahpC* in *B. abortus* 2308 in response to endogenous H_2O_2 levels. This is surprising because BAB2_0530 has OxyR-like properties. BAB2_0530 contains key amino acids that are required for OxyR to bind to the RNA polymerase and to bind DNA (Paget and Buttner, 2003). *B. abortus* BAB2_0530 also contains C202 which is involved in H_2O_2 sensing, but not C211, the second cysteine residue involved H_2O_2 sensing in *E. coli* (Imlay 2008; Zheng et al., 1998). Therefore BAB2_0530 would be classified as a 1-Cys OxyR (Chen et al., 2008) similar to the 1-Cys OxyR in *Deinococcus radiodurans* that activates *katE* expression and represses *dps* and *mntH* expression in response to H_2O_2 (Chen et al., 2008). It is also unexpected that BAB2_0530 does not regulate *ahpC* in response to endogenous H_2O_2 because of the proximity of the BAB2_0530 gene to the *ahpCD* operon. One of the properties of LysR-transcriptional regulators is that they often affect the expression of genes divergently transcribed from their own genes (Schell 1993). There is a two-fold increase in the basal level

of *ahpC* expression in BAB2_0530 mutants compared to *B. abortus* 2308, but changes in intracellular H_2O_2 levels have the same effect on *ahpC* expression in the wild-type strain as they do in the BAB2_0530 mutant. We cannot presently explain how BAB2_0530 affects *ahpC* transcription, because the EMSAs show that BAB2_0530 does not bind to the *ahpC* promoter. It must be noted though that we do not know if the EMSA conditions are correct for optimal BAB2_0530 binding, because we do not have a positive control for this experiment. Further examination of BAB2_0530 will be necessary to clarify its potential role as a regulator of *ahpC*.

Although we have found three regulators that affect *ahpC* expression in some manner in *B. abortus* 2308, neither Irr, OxyR (see Chapter 4), nor BAB2_0530 directly activate *ahpC* expression in response to increased levels of endogenous H_2O_2. But there are other candidate regulators to examine. PerR, a member of the Fur superfamily of metalloregulators (Lee and Helmann, 2006), is a major oxidative stress regulator in Gram-positive bacteria (Mongkolsuk and Helmann, 2002). PerR directly senses H_2O_2 and intracellular Fe^{+2} and Mn^{+2} levels. Data from our laboratory show that a *B. abortus perR* mutant is significantly more resistant to H_2O_2 than wild-type (E. Anderson unpublished), and since PerR has a role in the regulation of *ahpC* and *kat* in other bacteria (Bsat et al., 1998; Imlay 2008; Horsburgh et al., 2001), it is an obvious candidate to examine in *B. abortus* for being the H_2O_2-responsive regulator we are trying to identify.

NolR is global regulator in *Sinorhizobium meliloti*, and studies showed that *ahpC* is a primary target of NolR (Chen et al., 2000). Since *S. meliloti* is a close phylogenic relative of *B. abortus*, it is possible that NolR affects *ahpC* expression in our bacterium too. *B. melitensis nolR* mutants are attenuated in HeLa cells, J774 macrophages, and in BALB/c mice by one week

post-infection (Haine et al., 2005), so NolR is critical for survival of this bacterium within the experimental host.

The *Rhizobium* group of bacteria uses NifA, an oxygen-responsive regulator, to activate genes whose products are involved in nitrogen fixation (Hauser et al., 2007). In *Bradyrhizobium japonicum*, σ^{54} (RpoN) binding to RNA polymerase is required for transcription of the NifA target genes, and microarray analysis indicates that *ahpC* is regulated by NifA and RpoN (Hauser et al., 2007). Analysis of the published *B. abortus* 2308 genome shows that *Brucella* does not possess a *nifA* homolog, but the *B. abortus* RpoN can restore normal growth and formation of functional nodules in a *S. meliloti rpoN* mutant (Iannino et al., 2008). Furthermore, *B. abortus rpoN* mutants are similar to *B. abortus ahpCD* mutants (Chapter 2) because they have a stationary phase growth defect and are fully virulent in host macrophages and BALB/c mice (Iannino et al., 2008). Because of the phenotypic similarities between *B. abortus rpoN* and *ahpCD* mutants and the fact that RpoN affects *ahpC* expression in a close phylogenic relative to *B. abortus*, it is plausible that RpoN is involved in *ahpC* expression, but probably through an oxygen-responsive or H_2O_2-responsive regulator. It should be noted though that the predicted RpoN promoter sequence for *B. melitensis* ($AAGCAAN_2TTN_4CCAN_2TT$; Dombrecht et al., 2002) and for *B. japonicum* [(T/C)GG(C/T)(A/C)(C/T)(G/A)N_4(T/A)TGC(T/A); Hauser et al., 2007] is not found in the *B. abortus ahpC* promoter DNA region.

The *B. japonicum* RegSR (also known as RegBA in *Rhodobacter capsulatus*, ActSR in *S. meliloti*, and RoxSR in *P. aeruginosa*) is a two-component regulatory system that activates *ahpC* expression indirectly by activating *nifA* expression (Bauer et al., 1998; Lindemann et al., 2007). BAB1_0136 and BAB1_0132 in *B. abortus* 2308 have 71% and 40% amino acid identity with

RegA and RegB, respectively, of *Rhodobacter sphaeroides*. *R. capsulatus* RegBA responds to the redox state of the bacterial cell, and its activity is dependent on aerobic respiration. The expression of RegBA target genes is higher in cytochrome cbb_3 mutants (Elsen et al., 2004). Since *B. abortus ahpC* is responsive to increased levels of H_2O_2 generated from aerobic metabolism, it would be plausible that the H_2O_2-responsive expression of *ahpC* is through a redox-sensing system like RegBA.

In summary, we have now identified AhpCD as a member of the *Brucella* oxidative stress response to H_2O_2. We have shown that *ahpC* expression is responsive to changes in endogenous H_2O_2 levels, and our data indicate that *ahpC* expression may be iron-responsive as well. If this is the case, then *B. abortus* coordinates its oxidative stress response with iron metabolism, which demonstrates the importance for the brucellae to prevent hydroxyl radical formation. At this time, we have failed to identify a regulator that directly affects *ahpC* expression. To complete this study, we are currently constructing *ahpC irr*, *ahpC oxyR*, and *ahpC* BAB2_0530 double mutants to determine if one of these regulators is responsible for activating *ahpC* in response to elevated cellular H_2O_2 levels. If this is the case, then *ahpC* expression will not increase in the corresponding strain. At present, our KatE overproduction studies suggest that neither of the *B. abortus* two OxyR homologs regulate *ahpC* in response to endogenous H_2O_2. OxyR is a H_2O_2-responsive regulator that is critical to the oxidative stress defense in many Gram-negative bacteria (Christman et al., 1985; Storz et al., 1987), so it is difficult to explain why *B. abortus* would use a different regulator to sense increased endogenous H_2O_2 levels and to activate antioxidant gene expression. We further ask what roles do OxyR and BAB2_0530 have in *B. abortus*? Earlier data suggested that OxyR's role is to activate *katE* expression beyond basal levels in response to high levels of H_2O_2 (e.g. mM) (Kim and Mayfield,

2000). Since KatE does not compensate for the loss of AhpC in *in vitro* cultivation experiments (Chapter 2) and has a minor role in removing endogenous H_2O_2, it is plausible that OxyR and KatE are part of a secondary line of oxidative stress defense. For example, the first line of defense in response to increased levels of H_2O_2 is to activate *ahpC* expression through our unidentified regulator. If H_2O_2 levels continue to increase, then the second line of defense uses OxyR to activate *katE*. At this time, it is hard to explain why *B.abortus* has two levels of oxidative stress defense.

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Table 3.1 Bacterial strains, plasmids, and primers used in this study

Strain or plasmid	*Genotype or description*	*Reference or source*
Strains		
Escherichia coli DH5α	F- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_{k-}, m_{k+}) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-	Invitrogen
Brucella abortus		
2308	Virulent challenge strain	Laboratory stock
KH16	2308 *ahpCD::cat*; Cm^r	Chapter 2
KH2	2308 *katE::cat*; Cm^r	Chapter 2
KK30	2308 *ahpCD::bla, katE::cat*; Ap^r; Cm^r	This study
KH112	2308 BAB2_0530*::cat;* Cm^r	This study
KH231	2308 *oxyR::cat;* Cm^r	This study
BEA2	2308 *irr::cat*; Cm^r	This study
Plasmids		
pGEM®-T Easy	ColE1-based cloning vector; Ap^r	Promega
pBlue-CM2	656 bp *cat* gene from pBC cloned into the EcoRV site of pBluescript KS+	Robertson et al., 2000
pKHS17	2,749 bp genomic DNA fragment from *B. abortus* 2308 containing BAB2_0530 [PCR primers lysR8-F/lysR8-R] cloned into pGEM®-T Easy	This study
pKHS13	Derivative of pKHS17 in which a 259 bp BsiWI/Eco0109I fragment internal to the BAB2_0530 coding region was replaced with the *cat* gene from pBlue-CM2	This study

pKHS11	2,836 bp genomic DNA fragment from *B. abortus* 2308 containing *oxyR* [PCR primers oxyR-F/oxyR-R] cloned into pGEM®-T Easy	This study
pKHS12	Derivative of pKHS11 in which a 703 bp AfeI fragment internal to the *oxyR* coding region was replaced with the *cat* gene from pBlue-CM2	This study
pBluescript KS+	ColE1-based cloning vector; Ap^r	Strategene
pMF4	577 bp genomic DNA fragment from *B. abortus* 2308 containing *irr* [PCR primers irr-F/irr-R] cloned into pBluescript KS+	This study
pMF4delta	Derivative of pMF4 in which the *cat* gene from pBlue-CM2 was inserted into a NruI site internal to the *irr* coding region	This study
pKHS2	2,499 bp genomic DNA fragment from *B. abortus* 2308 containing *ahpCD* cloned into pGEM®-T Easy	Chapter 2
pKSZeo1	463 bp *ble* gene from pZEO19 cloned into the EcoRV site of pBluescript KS+	This study
pMR15	RK2-based broad host range transcriptional fusion vector; contains the *E. coli lacZ* gene from pRSZ3 subcloned into pRK290; low copy number (2-4 copies per cell); Kn^r	Gober and Shapiro 1992
pKHS15	Derivative of pMR15 carrying an 185 bp fragment [PCR primers intergenic-F/intergenic-R] containing the *ahpC* promoter region from *B. abortus* 2308	This study
pBBR1MCS-4	pBBR-based broad host range cloning vector; moderate copy number (10-14 copies per cell); Ap^r	Kovach et al., 1995
pMEK21	Derivative of pBBR1MCS-4 carrying the *katE* gene from *B. abortus* S19; Ap^r	Gee et al., 2004
pMAL-C2X	pBR322-based protein fusion vector; contains *malE* gene which encodes maltose-binding protein; Ap^r	New England BioLabs
pASK-IBA6	pUC-based, Strep-Tactin tag protein purification vector; Ap^r	IBA BioTAGnology

pKHS45	953 bp genomic DNA fragment from *B. abortus* 2308 containing *oxyR* [PCR primers oxyR-F2/oxyR-R2] cloned into pMAL-C2X	This study
pKHS14	900 bp genomic DNA fragment from *B. abortus* 2308 containing BAB2_0530 [PCR primers lysR8-F2/lysR8-R2] cloned into pMAL-C2X	This study
pASK-irr	435 bp genomic DNA fragment from *B. abortus* 2308 containing *irr* [PCR primers irr-F2/irr-R2] cloned into pASK-IBA6	This study

Primers

Designation	Sequence
lysR8-F	5’-CTCCATGTGGGTCGAGGGTGAACTG-3’
lysR8-R	5’-CAAACAGGCCATATTTCTGCTGCGG-3’
oxyR-F	5’-CATGATCTGAACCTGCACCT-3’
oxyR-R	5’-GAAGTTTGCAGCCGAGATAA-3’
irr-F	5’-GCGGCCGCATGAAAACTCTT-3’
irr-R	3’-ACCGGCTTTCGGATCAAGGC-3’
intergenic-F	5’-TAAAACCCGAATAATCGCTT-3’
intergenic-R	5’-CCTTGAAAGGGGGAAGCTTG-3’
pahpC-F	5’-TAAAACCCGAATAATCGCTT-3’
pahpC-R	5’-CCTTGAAAGGGGGAAGCTTG-3’
pahpC-F2	5’-CACGCGCCAGAAAGTCCTCA-3’
pkatE-F	5’-GATAGGAAAATCTTATCAATAATAACAAA-3’
pkatE-R	5’-GCGATCTGTCATGGAATAAT-3’
p0005-F	5’-AGATTGACATTGATGCCGTCCA-3’
p0005-R	5’-CAAAGCATCTGCTTGCAGCC-3’
prbr-F	5’-GGAAATCCTGCCGGTTAAGC-3’
prbr-R	5’-ACGGAAGAATCGGCTGAACA-3’

oxyR-F2	5’-ATGTTTACAGTCCGTCAAATACG-3’
oxyR-R2	5’-TTAAGAATATGAAGGGGTAT-3’
lysR8-F2	5’-ATGCTCAATATTAGTGTCAGGCAGC-3’
lysR8-R2	5’-GTCCTGCAAGCAATTGCTGC-3’
irr-F2	5’-GTGAATATGCATTCTTCACATACCCACTC-3’
irr-R2	5’-TCAGCGGGCCTGACGGCGCAGACGCACAATGATATCCACAT-3’

Figure 3.1. The *Brucella* OxyR and BAB2_0530 proteins share biologically relevant conserved domains with the OxyR protein of *Escherichia coli.* Indicated are the five amino acids necessary for *E. coli* OxyR to bind to DNA (*) (Paget and Buttner, 2003), the two amino acids necessary for *E. coli* OxyR to interact with RNA polymerase (•) (Paget and Buttner, 2003), and the two conserved cysteine residues that become oxidized and allow *E. coli* OxyR to be responsive to H_2O_2 (■) (Imlay 2008).

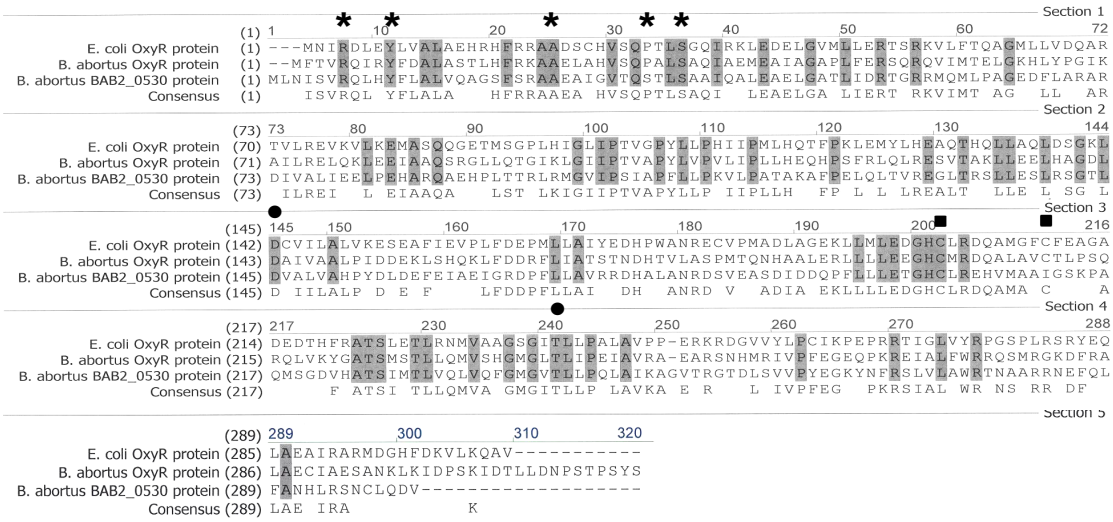

Section 1
Section 2
Section 3
Section 4
Section 5
E. coli OxyR protein
B. abortus OxyR protein
B. abortus BAB2_0530 protein
Consensus

Figure 3.1

Figure 3.2. Genetic organization of the BAB2_0530 – BAB2_0533 locus in *B. abortus* 2308. BAB2_0530 encodes a LysR-type transcriptional regulator that has significant amino acid homology with bacterial OxyR homologs; BAB2_0531 encodes a perioxiredoxin protein named AhpC that is involved in detoxifying H_2O_2 in *B. abortus*; BAB2_0532 encodes a hydroperoxidase named AhpD which functions to reduce the AhpC protein; and BAB2_0533 encodes a protein belonging to the major facilitator superfamily (MFS), which is a diverse group of transporters that includes uniporters, symporters, and antiporters (Law et al., 2008). The transcriptional start site for *ahpC* is noted by +1 and the corresponding G is shown in boldface. The -10 and -35 regions relative to the transcriptional site are also shown. The amino-termini of the BAB2_0530 and *ahpC* coding regions are labeled by their amino acids. There are two 17 bp direct repeats (shaded regions) in the intergenic region between BAB2_0530 and *ahpC*. A predicted Irr box is underlined, and the five predicted OxyR binding sites are shown in boldface.

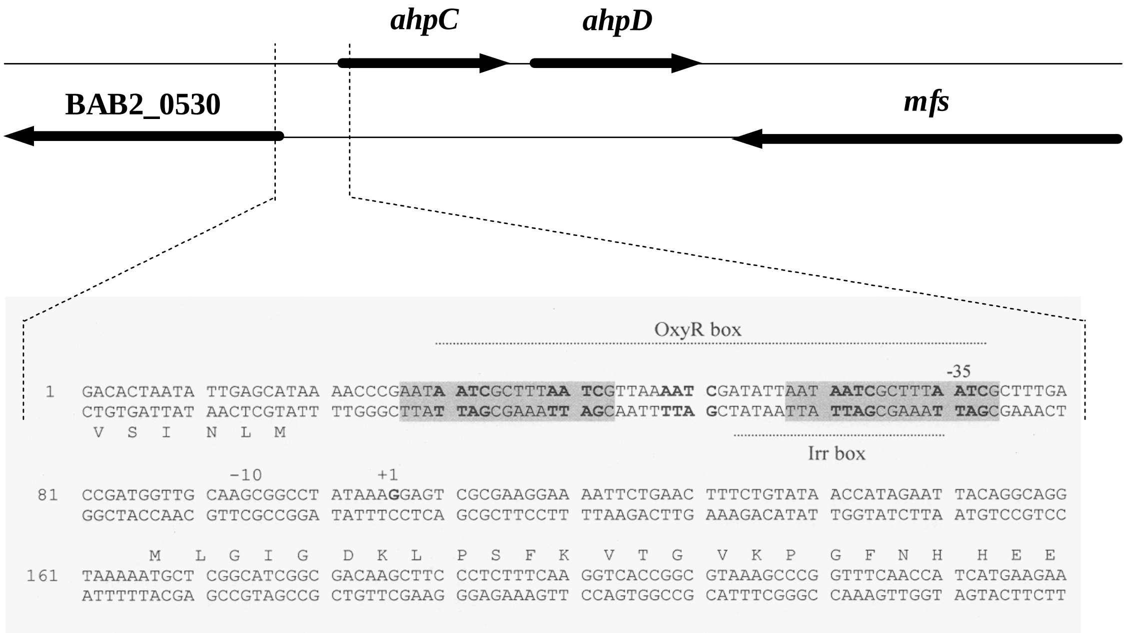

ahpC
ahpD
BAB2_0530
mfs
OxyR box
-35
1 GACACTAATA TTGAGCATAA AACCCGAATA ATCGCTTTAA TCGTTAAAAT CGATATTAAT AATCGCTTTA ATCGCTTTGA
CTGTGATTAT AACTCGTATT TTGGGCTTAT TAGCGAAATT AGCAATTTTA GCTATAATTA TTAGCGAAAT TAGCGAAACT
V S I N L M
Irr box
-10
+1
81 CCGATGGTTG CAAGCGGCCT ATAAAGGAGT CGCGAAGGAA AATTCTGAAC TTTCTGTATA ACCATAGAAT TACAGGCAGG
GGCTACCAAC GTTCGCCGGA TATTTCCTCA GCGCTTCCTT TTAAGACTTG AAAGACATAT TGGTATCTTA ATGTCCGTCC
M L G I G D K L P S F K V T G V K P G F N H H E E
161 TAAAAATGCT CGGCATCGGC GACAAGCTTC CCTCTTTCAA GGTCACCGGC GTAAAGCCCG GTTTCAACCA TCATGAAGAA
ATTTTTACGA GCCGTAGCCG CTGTTCGAAG GGAGAAAGTT CCAGTGGCCG CATTTCGGGC CAAAGTTGGT AGTACTTCTT

Figure 3.2

Figure 3.3 Reduction of endogenous H_2O_2 levels (black bars) in *B. abortus* 2308 results in reduced β-galactosidase production from an *ahpC-lacZ* fusion (white bars). The data presented are means and standard deviations for triplicate determinations from a single culture in a single experiment. The data presented here are representative of multiple (≥ 3) experiments performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test is represented by an asterisk and is for the comparison of 2308 (pMEK21; pKHS15) versus 2308 (pBBR1MCS4; pKHS15).

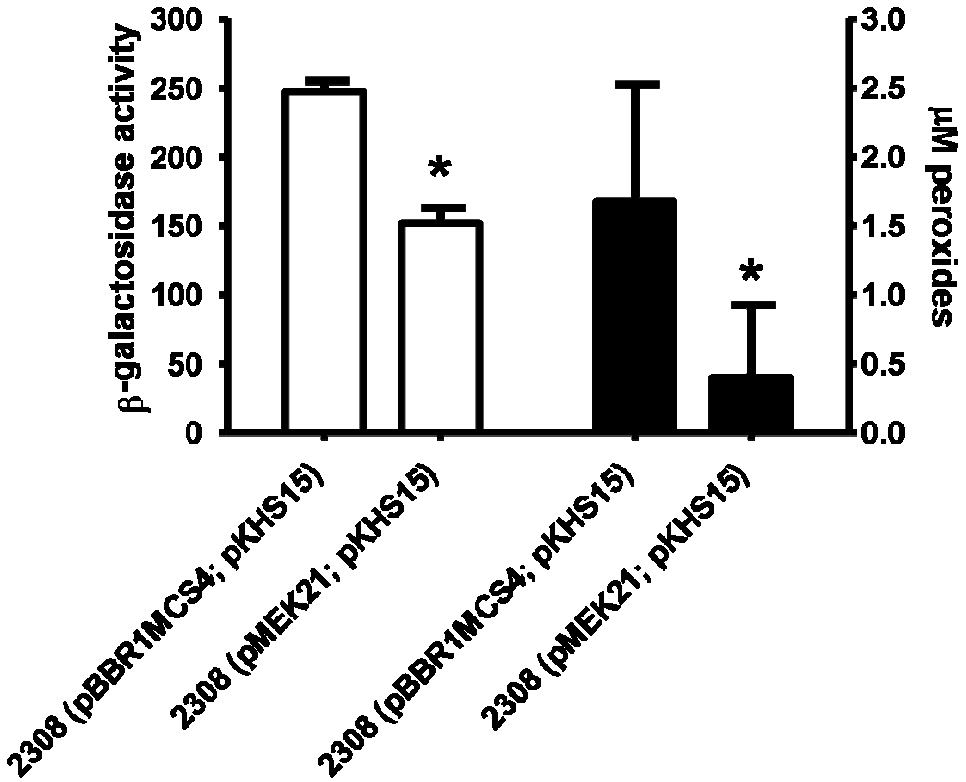

β-galactosidase activity
300
250
200
150
100
50
0
μM peroxides
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
2308 (pBBR1MCS4; pKHS15)
2308 (pMEK21; pKHS15)
2308 (pBBR1MCS4; pKHS15)
2308 (pMEK21; pKHS15)

Figure 3.3

Figure 3.4 The *B. abortus ahpCD* mutant KH16 exhibits increased levels of endogenous H_2O_2 (black bars) and *ahpC* expression (white bars) compared to the parental 2308 strain. The data presented are means and standard deviations for triplicate determinations from a single culture in a single experiment. The data presented here are representative of multiple ($\geq$ 7) experiments performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test is represented by an asterisk and is for the comparison of 2308 versus KH16.

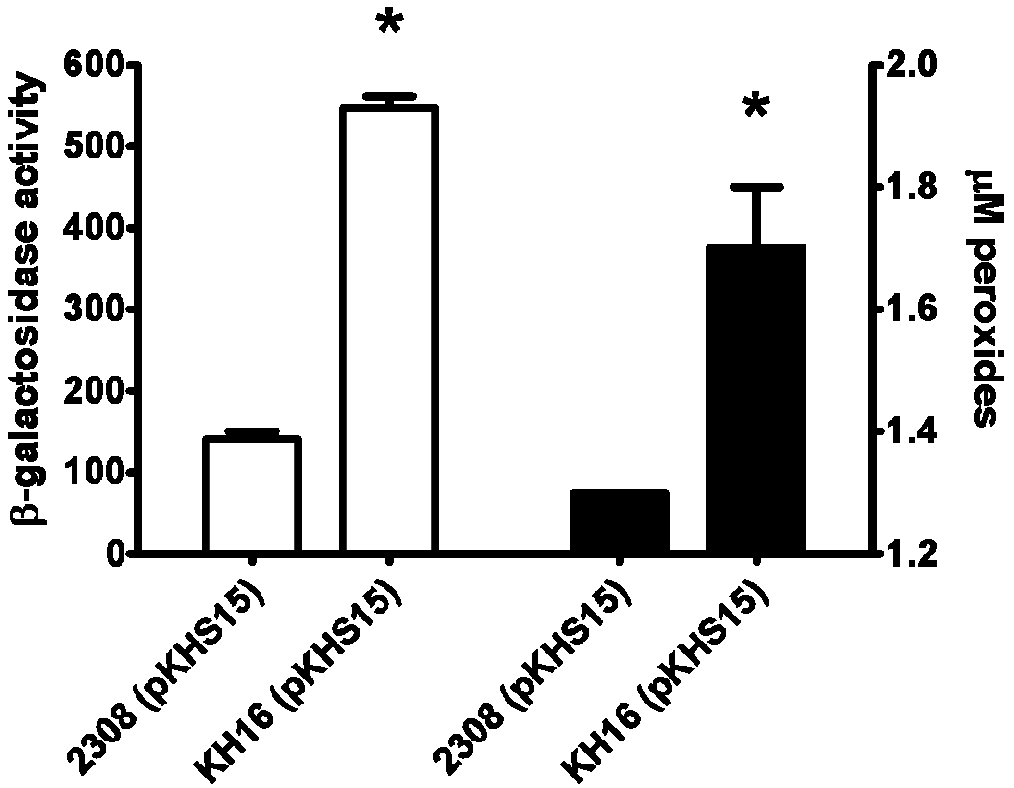
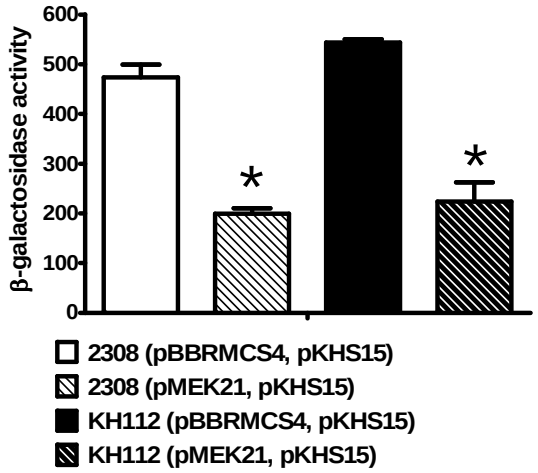

β-galactosidase activity
600
500
400
300
200
100
0
2.0
1.8
1.6
1.4
1.2
µM peroxides
*
*
2308 (pKHS15)
KH16 (pKHS15)
2308 (pKHS15)
KH16 (pKHS15)

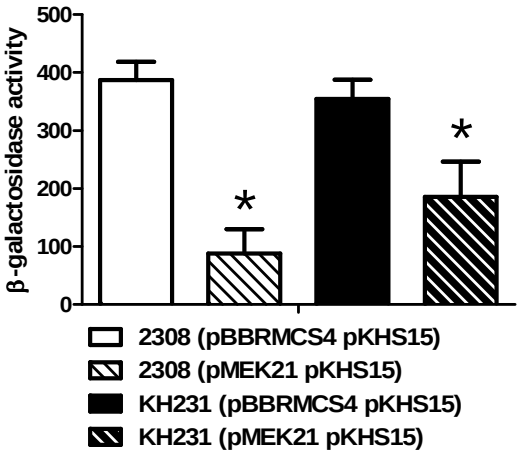
Figure 3.4

Figure 3.5 Overproduction of *katE* does not reduce *ahpC* expression in the *B. abortus irr* mutant BEA2. β-galactosidase activity from an *ahpC-lacZ* fusion in derivatives of *B. abortus* 2308 (A-C), KH112 (BAB2_0530) (A), KH231 (*oxyR*) (B), and BEA2 (*irr*) (C) carrying pBBR1MCS4 or a version of this plasmid containing the *Brucella katE* gene (pMEK21). The data presented are means and standard deviations for triplicate determinations from a single culture in a single experiment. The data presented here are representative of multiple (≥ 2 (A) and ≥ 3 (B and C)) experiments performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test is represented by an asterisk and is for the comparison of wild-type 2308 to KH112, KH231, or BEA2.

A.



B.



C.

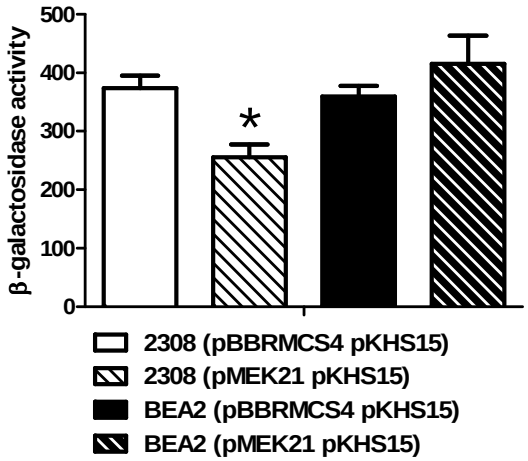


Figure 3.5

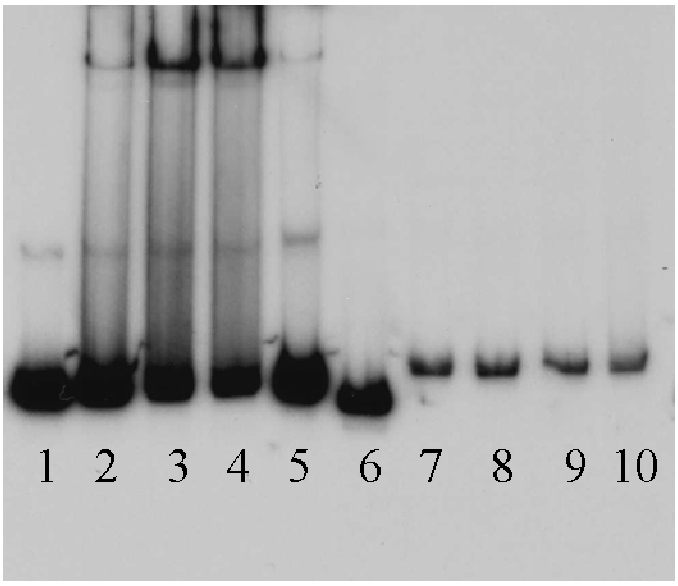
Figure 3.6 Irr binds to the *Brucella rbr* promoter region but not to the *ahpC* promoter region in an EMSA. Lanes 1, α^{32}P-labeled *rbr* promoter DNA; lane 2, α^{32}P-labeled *rbr* promoter DNA plus 50 ng Irr; lane 3, α^{32}P-labeled *rbr* promoter DNA plus 100 ng Irr; lane 4, α^{32}P-labeled *ahpC* promoter DNA; lane 5, α^{32}P-labeled *ahpC* promoter DNA plus 50 ng Irr; lane 6, α^{32}P-labeled *ahpC* promoter DNA plus 100 ng Irr The results presented here are from a single experiment that is representative of multiple ($\geq$ 2) experiments performed from which equivalent results were obtained.

1 2 3 4 5 6

Figure 3.6

Figure 3.7 The OxyR-MBP fusion protein binds to the *Brucella katE* promoter region but neither OxyR-MBP (A) nor BAB2_0530-MBP fusion proteins (B) bind to the *ahpC* promoter region in an EMSA. (A) Lane 1, α^{32}P-labeled *katE* promoter DNA; lane 2, α^{32}P-labeled *katE* promoter DNA plus 1 μg OxyR-MBP fusion protein; lane 3, α^{32}P-labeled *katE* promoter DNA plus 2 μg OxyR-MBP fusion protein; lane 4, α^{32}P-labeled *katE* promoter DNA plus 3 μg OxyR-MBP fusion protein; lane 5 specific competitor control, α^{32}P-labeled *katE* promoter DNA plus 1 μg OxyR-MBP fusion protein and 20 × unlabeled *katE* promoter-specific DNA fragment; lane 6 non-specific competitor control, α^{32}P-labeled *serA* promoter DNA plus 1 μg OxyR-MBP fusion protein; lane 7, α^{32}P-labeled *ahpC* promoter DNA; lane 8, α^{32}P-labeled *ahpC* promoter DNA plus 1 μg OxyR-MBP fusion protein; lane 9, α^{32}P-labeled *ahpC* promoter DNA plus 2 μg OxyR-MBP fusion protein; lane 10, α^{32}P-labeled *ahpC* promoter DNA plus 3 μg OxyR-MBP fusion protein. The results presented here are from a single experiment that is representative of multiple (≥ 3) experiments performed from which equivalent results were obtained. (B) Lanes 1-5, α^{32}P-labeled *ahpC* promoter DNA; lane 2, 0.5 μg BAB2_0530-MBP fusion protein; lane 3, 1 μg BAB2_0530-MBP fusion protein; lane 4, 2 μg BAB2_0530-MBP fusion protein; lane 5, 3 μg BAB2_0530-MBP fusion protein. The data presented here are representative of multiple (≥ 3) experiments performed from which equivalent results were obtained.

A.



B.

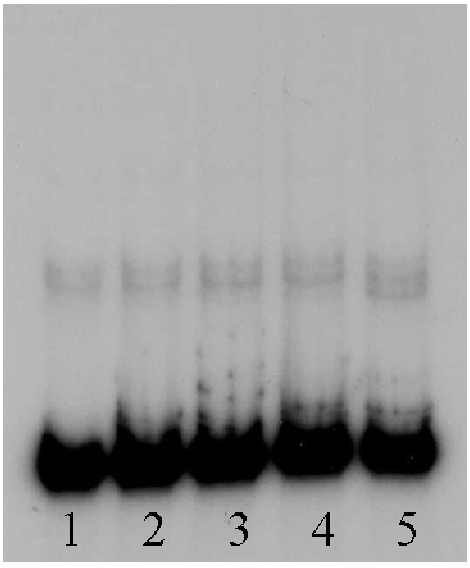


Figure 3.7

Figure 3.8 The original working model proposing that Irr directly represses *ahpCD* expression when cellular H_2O_2 levels are low, but repression is relieved when cellular H_2O_2 levels increase because H_2O_2 destabilizes Irr.

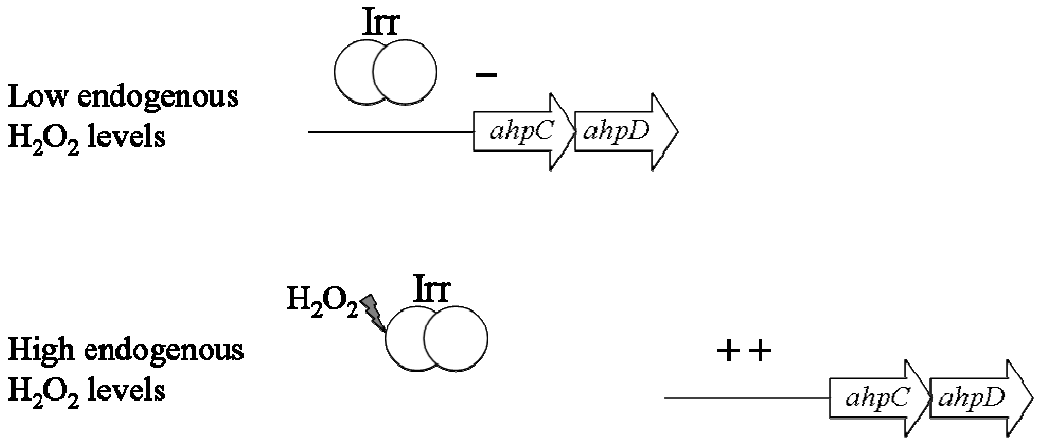

Low endogenous
H_2O_2 levels
Irr
–
ahpC
ahpD
High endogenous
H_2O_2 levels
H_2O_2
Irr
++
ahpC
ahpD

Figure 3.8

Figure 3.9 *B. abortus* Irr can affect *ahpC* expression by repressing (A) or activating (B) the gene encoding *ahpC*'s regulator or by keeping cellular iron levels high enough to keep *ahpC*'s regulator in an active state (C). In this model, the *ahpC* direct regulator is termed AhpR(A) [alkyl hydroperoxidase repressor (activator)]. Ahp is an activator in (A) and a repressor in (B) and (C). In figure C, AhpR is in an inactive state when iron is not bound to the sulfur group.

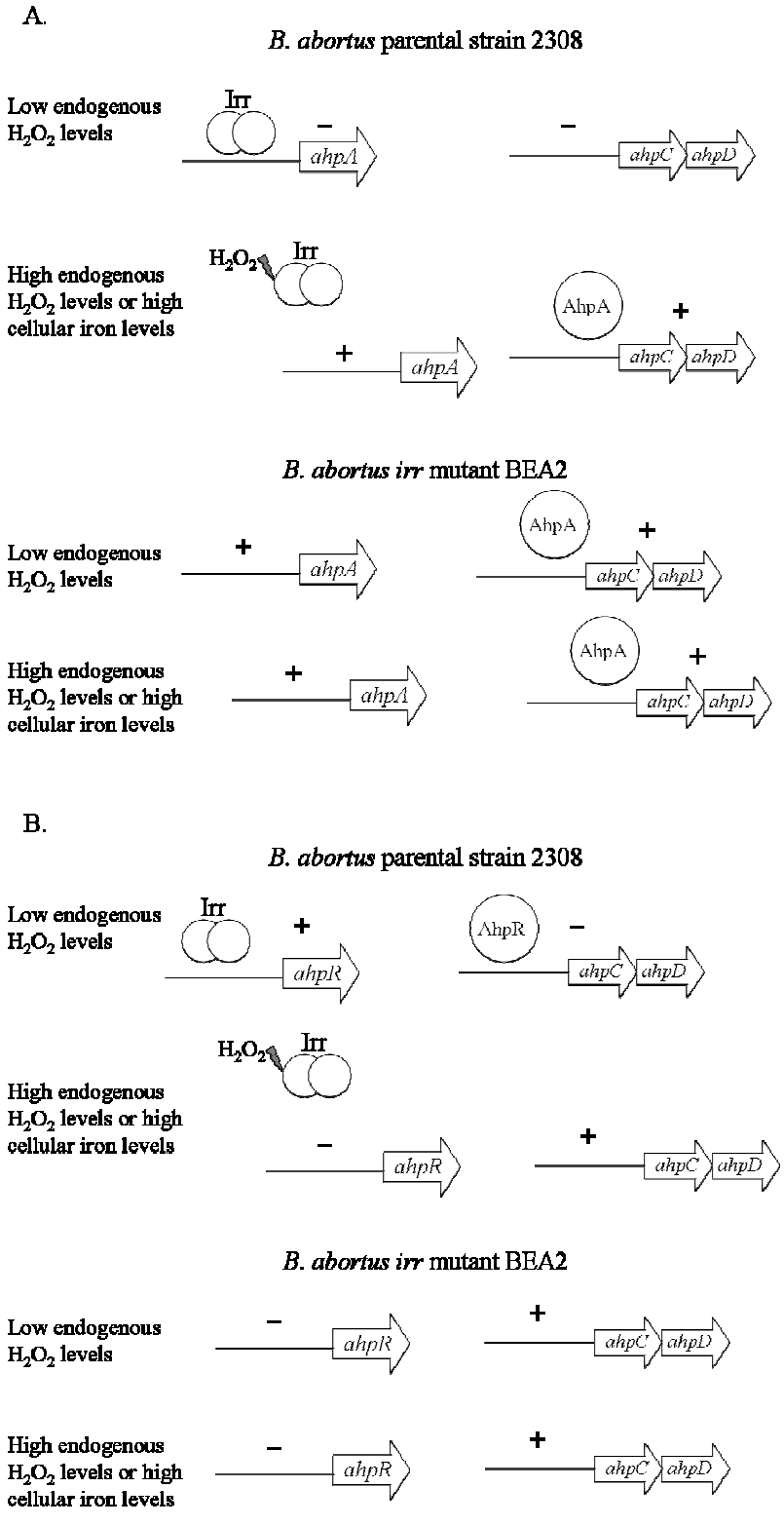

A.
B. abortus parental strain 2308
Low endogenous H2O2 levels
Irr
ahpA
ahpC
ahpD
High endogenous H2O2 levels or high cellular iron levels
H2O2
AhpA
B. abortus irr mutant BEA2
B.
AhpR
ahpR

Figure 3.9

C.

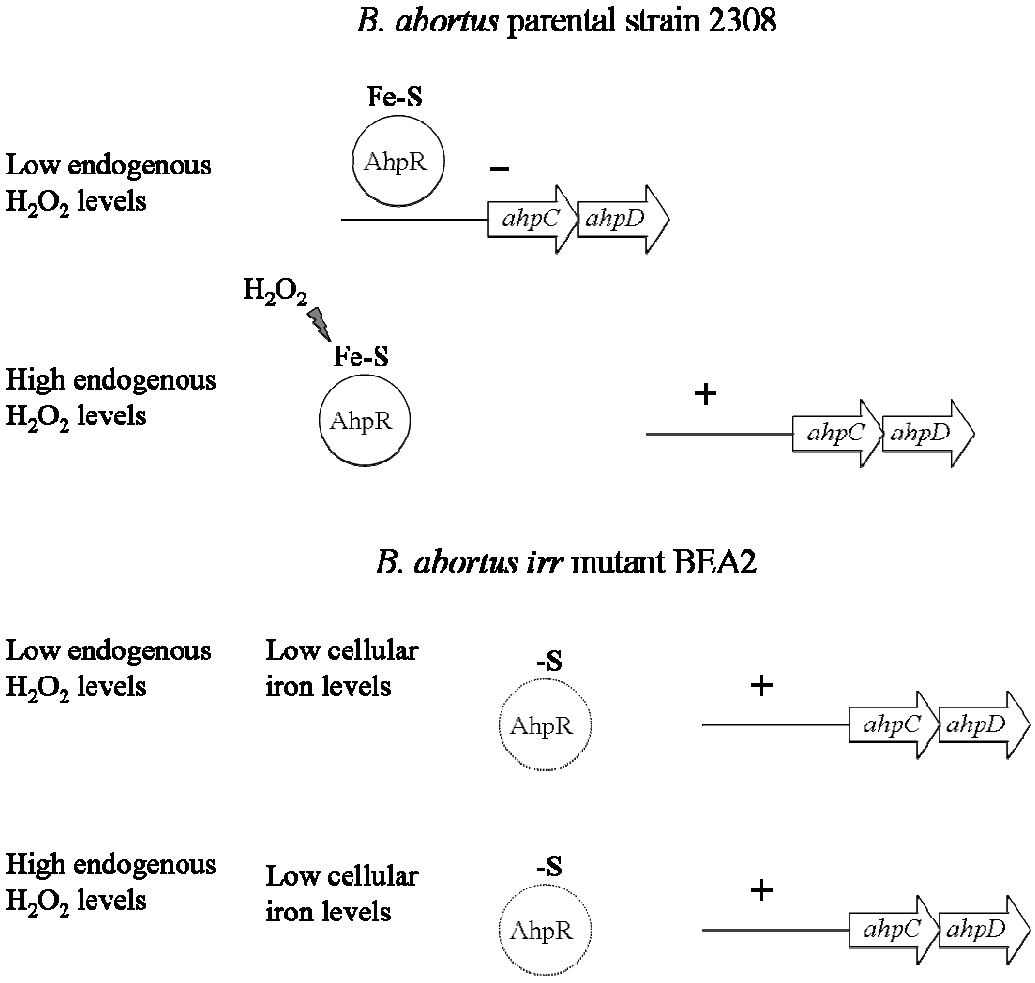


Figure 3.9 (continued)

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Chapter 4

Our current understanding of the roles of BAB2_0530, Irr, and OxyR in oxidative stress defense in *Brucella abortus* 2308

The OxyR-like protein BAB2_0530 regulates genes whose products detoxify superoxide and organic peroxides, but not hydrogen peroxide in *B. abortus* 2308.

Identification of the OxyR homolog BAB2_0530 in *B. abortus* 2308. Analysis of the *B. abortus* 2308 genome sequence reveals the presence of two genes that are predicted to encode LysR-type transcriptional regulators with high homology to OxyR. OxyR is an oxidative stress regulator in Gram-negative bacteria that allows bacteria to adapt to lethal levels of H_2O_2 by activating antioxidant genes (Christman et al., 1985; Storz et al., 1987). One of the *Brucella* OxyR homologs is designated as BAB2_0849 and shares 37% amino acid identity with the *Escherichia coli* K12 OxyR (Figure 3.1). A study describing its characterization has been published (Kim and Mayfield, 2000), and this homolog was named OxyR. The second potential *oxyR* gene in *B. abortus*, BAB2_0530, is found divergently transcribed from the *ahpCD* operon. BAB2_0530 shares 33% amino acid identity with the *E. coli* K12 OxyR. Amino acid alignment indicates that BAB2_0530 is a 1-Cys OxyR homolog that contains most of the critical residues for OxyR activity (Figure 3.1; Chen et al., 2008). The D142 and T238 residues that are important for binding to RNA polymerase in the *E. coli* OxyR are conserved as D145 and T241 in *B. abortus* BAB2_0530 (Paget and Buttner, 2003). Four of the five residues that are important for DNA binding (R4, Y8, A22, S33, P30) in the *E. coli* OxyR are also conserved in *B. abortus* BAB2_0530 (R7, Y11, A25, S36) (Paget and Buttner, 2003). There are two cysteine residues in the *E. coli* OxyR (C199 and C208) that are critical for its H_2O_2-responsiveness (Imlay 2008; Zheng et al., 1998; Mongkolsuk and Helmann, 2002), but *B. abortus* BAB2_0530 only contains the first cysteine (corresponding to C202, Figure 3.1). This means that if BAB2_0530 functions as an OxyR homolog, it only requires one cysteine for oxidation and thus is similar to the *Deinococcus radiodurans* OxyR. *D. radiodurans oxyR* mutants are sensitive to H_2O_2, and

the *D. radiodurans* OxyR protein activates *katE* expression and represses *dps* and *mntH* expression in response to H_2O_2 (Chen et al., 2008). Researchers showed that the *D. radiodurans* OxyR C210 is oxidized to a sulfenic acid by H_2O_2 and this oxidation is sufficient to fully activate OxyR (Chen et al., 2008). Therefore BAB2_0530 could be effective at responding to H_2O_2 even though BAB2_0530 contains one redox-active cysteine residue.

B. abortus BAB2_0530 does not impact the removal of H_2O_2 from the bacterial cell. Earlier work showed that BAB2_0530 has a two-fold repressive effect on the expression of *ahpCD* in *B. abortus* 2308 under all conditions tested (Chapter 3). BAB2_0530 continues to have this effect even when endogenous H_2O_2 levels change, suggesting that H_2O_2 does not affect the regulatory activity of BAB2_0530. To determine if BAB2_0530 regulates other genes involved in detoxifying H_2O_2, we compared the sensitivities of *B. abortus* 2308 and the isogenic BAB2_0530 mutant KH112 to H_2O_2 in *in vitro* assays. KH112 and 2308 displayed equivalent resistance to H_2O_2 in both disk inhibition assays on a solid medium (Figure 4.1A) and in broth culture susceptibility assays (Figure 4.1B) indicating that the loss of BAB2_0530 does not impact the survival of *B. abortus* 2308 after an exposure to H_2O_2.

These results are further supported by the observation that the *B. abortus* BAB2_0530 mutant produces the same levels of endogenous peroxides as the parent strain during aerobic cultivation (Figure 4.2A) and can detoxify 50 μM exogenous H_2O_2 with the same efficiency as 2308 (Figure 4.2B). In summary, the phenotype exhibited in these assays indicates that BAB2_0530 does not regulate genes in *B. abortus* 2308 that are essential for H_2O_2 resistance.

BAB2_0530 is necessary for the detoxification of superoxide and organic peroxides. To determine if BAB2_0530 regulates genes involved in $O_2^{\bullet-}$ or organic peroxide resistance, we asked if a mutation in the BAB2_0530 gene would affect the viability of *B. abortus* 2308 in response to superoxide or organic peroxides. As shown in Figure 4.3, the BAB2_0530 mutant KH112 displays an increased sensitivity to the $O_2^{\bullet-}$ generator paraquat (PQ) compared to the parental 2308 strain. The addition of catalase to the test medium did not relieve the increased sensitivity of KH112 to PQ indicating that this toxicity is due to the generation of endogenous $O_2^{\bullet-}$ and not the result of generation of endogenous H_2O_2 as a by-product from the PQ reaction. The *B. abortus* BAB2_0530 mutant also exhibits an increased susceptibility to the organic peroxides cumene hydroperoxide (CHP) and tert-butyl hydroperoxide (tBOOH) (Figure 4.4). Thus it appears that BAB2_0530 plays a role in the regulation of genes involved in protecting *B. abortus* 2308 from $O_2^{\bullet-}$ and organic peroxides. The identity of these genes remains to be determined. The *B. abortus* BAB2_0530 mutant demonstrates a wild-type spleen colonization profile in C57BL/6 mice through eight weeks post infection (data not shown) indicating that regulation by BAB2_0530 is not critical for virulence in the mouse model of chronic infection.

BAB2_0530 summary and future directions. BAB2_0530 is a predicted LysR-type transcriptional regulator that has a two-fold repressive effect on both the expression of its own gene (data not shown) and that of *ahpCD* under all conditions tested (Chapter 3). The data shown here illustrate that despite BAB2_0530 being an OxyR-like protein, BAB2_0530 has no role in the removal of H_2O_2 from *Brucella* cells: BAB2_0530 mutants scavenge H_2O_2 as quickly as wild-type cells, and BAB2_0530 mutants have the same sensitivity to H_2O_2 as wild-type cells. Furthermore, the two-fold repressive effect BAB2_0530 has on *ahpCD* expression is not affected when endogenous H_2O_2 levels decrease.

OxyR regulates *sod* in the anaerobe *Porphyromonas gingivalis* (Ohara et al., 2006; Wu et al., 2008) and *tpx* (which encodes a thiol peroxidase) in the anaerobe *Bacteroides fragilis* (Herren et al., 2003), but OxyR does not seem to have a direct role in the regulation of these genes in aerobic bacteria (Nakjarung et al., 2003). Aerobes have other regulators like SoxRS and OhrR to regulate genes whose products are involved in detoxifying superoxide and organic peroxides (Imlay 2006). If BAB2_0530 functions as a regulator, it may target the potential organic peroxide detoxifier genes *bcp, tpx,* and *ohr* and the superoxide detoxifier genes *sodC* and *sodA.* More studies are necessary to define the role BAB2_0530 has in the *Brucella* oxidative stress defense against superoxide and organic peroxides.

Irr regulates oxidative defense genes in *Brucella* and other α-proteobacteria.

Irr is an iron responsive regulator that regulates genes whose products are involved in iron metabolism (Hamza et al., 1998). In *B. abortus*, Irr represses the expression of its own gene and activates expression of the siderophore biosynthesis operon *dhbCEBA* (Rudolph et al., 2006b; Martinez et al., 2006). In *Bradyrhizobium japonicum*, Irr regulates over eighty genes including a heme biosynthesis gene *hemB* (Hamza et al., 1998), the heme uptake gene cluster *hmuTUV-hmuR-exbBD-tonB,* and the gene encoding bacterioferritin (Rudolph et al., 2006a). In *Rhizobium leguminosarum,* Irr regulates the heme biosynthesis gene *hemA1*, a gene encoding the iron-responsive regulator RirA, the iron-sulfur synthesis operon *sufS2BCDS1XA*, and a ferrisiderophore ABC transporter gene *rrp1* (Todd et al., 2006).

Iron and H_2O_2 work in concert to degrade Irr in *B. japonicum* and indeed experimental evidence suggests that in addition to its role in regulating iron metabolism genes, Irr also

regulates genes involved in oxidative defense (Yang et al., 2006). Martinez et al. (2006) for example showed that *B. abortus irr* mutants have increased catalase activity. Irr also affects *ahpCD* expression in *B. abortus* in response to H_2O_2 (Chapter 3). In *B. japonicum*, Irr directly binds to the rubrerythrin gene promoter to repress *rbr* expression (Rudolph et al., 2006a; Sangwan et al., 2008).

To further investigate the role *B. abortus* Irr plays in protecting bacteria against oxidative stress, we determined cell viability of *B. abortus irr* mutants and wild-type 2308 after an exposure to H_2O_2, $O_2^{\bullet -}$, and organic peroxides. We first performed a broth sensitivity assay to compare sensitivities of wild-type 2308, *B. abortus katE* mutant MEK6, and *B. abortus irr* mutant BEA2 to varying concentrations of H_2O_2. The results showed that the *B. abortus irr* mutant BEA2 is more sensitive to H_2O_2 than wild-type but not as sensitive as the *B. abortus katE* mutant MEK6 (Figure 4.5). These results agree with the data that show that the *B. abortus irr* mutant BEA2 has a defect scavenging H_2O_2 (Figure 4.2). Since there are predicted Irr binding sites upstream of the *Brucella katE, bcp, tpx,* and *rbr* genes (Figure 4.7), it is plausible that Irr has a role in regulating genes involved in removing H_2O_2 from the bacterial cells. Our data contrast with the data published by Martinez et al. (2006), though, who showed that a *B. abortus irr* mutant is more resistant to H_2O_2 and scavenges H_2O_2 more quickly than wild-type. It should be noted, though, that their experiments were performed in low iron media, at a different cell density, and the authors used different experimental assays. Further analysis must be done to work out these discrepancies and to determine if iron availability is the reason for these differences.

We next asked if the presence of Irr affects the viability of the brucellae after an exposure to organic peroxides (OP), which would indicate that Irr affects genes whose products are

involved in detoxifying OP. The results of broth sensitivity assays showed the *B. abortus irr* mutant is more sensitive to cumene hydroperoxide and tert-butyl hydroperoxide than wild-type (Figure 4.6). These results indicate that the Irr regulon may include genes whose products detoxify OP and if this is true, that Irr is an important oxidative stress regulator in *B. abortus*. Irr could be directly regulating either *tpx* or *bcp*, because there is a predicted Irr binding sequence with 67% and 76% identity in the DNA upstream of *tpx* and *bcp* genes, respectively (Figure 4.7).

Since our evidence suggests that *B. abortus* Irr regulates genes whose products are involved in detoxifying H_2O_2 and OP, it is possible that Irr may regulate genes whose products detoxify another important ROS, superoxide. So we next asked if the *B. abortus irr* mutant is more sensitive than wild-type to superoxide. In this experiment, we used the endogenous superoxide generator paraquat. Paraquat is a redox-cycling drug that increases superoxide production from the respiratory chain (Bus and Gibson, 1984). Thus, the effects of paraquat are more obvious during cellular growth. Our data showed that while wild-type 2308 is sensitive to an exposure to paraquat during growth, the *B. abortus irr* mutant BEA2 is much more sensitive to the addition of paraquat (Figure 4.8). These results indicate that Irr is involved in the removal of endogenous superoxide and/or superoxide's downstream product H_2O_2. Further analysis must be done to distinguish if *irr* mutants are sensitive to superoxide and/or to H_2O_2 in this assay, and if Irr regulates genes whose protein products are responsible for this phenotype.

A *B. abortus* strain lacking *irr* is attenuated compared to wild-type in mice. Since *B. abortus* Irr plays an important role in the brucellae's resistance to oxidative stress, we next asked

whether *B. abortus* Irr is critical for establishing and maintaining an infection in the experimental murine host. Martinez et al. (2006) showed that *B. abortus irr* mutants are not attenuated by three weeks post infection in BALB/c mice. Our results agree, but preliminary evidence showed that *B. abortus irr* mutants are significantly less virulent than wild-type in the mouse model starting at four weeks post-infection (Steele unpublished). These results indicate that regulation by Irr is critical for maintaining an infection *in vivo*, and further study is warranted to identify Irr-regulated genes and Irr's contribution to the survival of this bacterium in its host.

Irr summary and future studies. Martinez et al. (2006) showed that the *B. abortus* iron responsive regulator Irr is involved in removing H_2O_2, most likely through its regulation of antioxidant genes. For this reason and the fact that iron-responsive regulators have been linked to oxidative stress defense in other bacteria (Zheng et al., 1999), we examined the effect Irr has on *ahpCD* expression in *B. abortus* 2308. Iron metabolism and oxidative stress defense are coupled because of Fenton chemistry. Fenton chemistry is the creation of hydroxyl radical from the interaction of ferrous iron and H_2O_2 (Reaction 6 p. 12). Hydroxyl radical reacts instantaneously with cellular macromolecules causing irreversible DNA, protein, and lipid damage (Babior 1978; Hassett and Cohen, 1989; Dubbs and Mongkolsuk, 2007). Since there are no known antioxidants that remove hydroxyl radical from the bacterial cell before damage can occur, bacteria use regulators that respond to high iron and high H_2O_2 levels and repress genes whose proteins bring iron into the bacterial cell and activate genes whose products remove H_2O_2 (Zheng et al., 1999). Irr is a well-equipped regulator capable of responding to both high iron and H_2O_2 levels, because Irr is degraded in the presence of iron (through the iron-dependent heme biosynthesis process) and H_2O_2 simultaneously (Small et al., 2009). Indeed, in *B.*

japonicum, Irr degrades when cellular H_2O_2 and iron levels are high, and Irr's degradation causes an increase in expression of *rbr* (Sangwan et al., 2008), whose gene product removes H_2O_2 from the bacterial cell.

We know Irr plays a role in the oxidative stress defense of *B. abortus* 2308, because its presence increases the resistance of these bacteria to exposures to superoxide, hydrogen peroxide, and organic peroxides. Circumstantial evidence suggests that Irr is able to sense redox changes within the bacterial cell and directly regulate antioxidant genes. One example of this is Irr's ability to bind to the DNA upstream of the *rbr* open reading frame (Chapter 3). Rubrerythrin degrades H_2O_2 (Lumppio et al., 2001). As seen in Figure 4.9, Irr may repress *rbr* expression when cellular H_2O_2 levels are low, and as H_2O_2 levels increase, Irr repression is relieved. Further experiments have to be done to determine if Rbr degrades H_2O_2 in *B. abortus*, and if Irr regulates *rbr* expression.

It is possible that Irr has an indirect role in the oxidative stress defense of *B. abortus*. An example of this is Irr's affect on *ahpCD* expression. *B. abortus irr* mutants have abnormal iron concentrations: they have lower cellular iron levels but higher heme levels than wild-type (Martinez et al., 2006). Thus, because the presence of Irr impacts cellular iron levels, Irr would affect the activity of any iron containing proteins. Figure 4.10 illustrates how Irr could affect the activity of *Brucella*'s primary oxidative stress regulator if the regulator requires iron to function. Further experiments must be done to distinguish between the direct and indirect models.

***ahpCD* expression in *B. abortus* 2308 is responsive to supraphysiologic levels of H_2O_2 through OxyR.**

Earlier work has shown that *B. abortus ahpCD* expression increases in response to an increase in endogenous H_2O_2 levels (Chapter 3). Since the expression of *ahpC* increases in response to 50 μM exogenous H_2O_2 in *Bacteroides fragilis* (Rocha and Smith, 1999), to 200 μM exogenous H_2O_2 in *Streptomyces coelicolor* (Hahn et al., 2002), and to 2 mM exogenous H_2O_2 in *Mycobacterium marinum* and *M. xenopi* (Pagán-Ramos et al., 1998), we next asked if *B. abortus ahpCD* expression is responsive to the addition of exogenous H_2O_2. To answer this question we measured β-galactosidase production from an *ahpC-lacZ* transcriptional fusion in *B. abortus* 2308 to determine *ahpC* promoter activity in response to the addition of varying concentrations of H_2O_2. The results show that *ahpCD* expression does not increase in response to an exposure with low nanomolar levels of H_2O_2 (Figure 4.11A). Since KatE is probably detoxifying the exogenous H_2O_2 before it can oxidize a regulator, we measured β-galactosidase production from the *ahpC-lacZ* transcriptional fusion in a *B. abortus katE* mutant. These results show that *ahpCD* expression slightly increases in response to 100 μM exogenous H_2O_2, but returns to basal levels in response to 500 μM and 1000 μM H_2O_2 (Figure 4.11B). We next discovered that *ahpCD* expression in wild-type 2308 decreases in a dose-dependent fashion in response to millimolar, non-lethal levels of exogenous H_2O_2 (Figure 4.11C). These results demonstrate that *ahpCD* expression is responsive to the addition of supraphysiologic levels of exogenous H_2O_2, but it is somewhat surprising that *B. abortus* 2308 would decrease expression of an antioxidant gene in response to H_2O_2.

OxyR is considered to be an important oxidative stress regulator in *B. abortus*, because an *oxyR* mutation makes the brucellae more sensitive to H_2O_2 (Figure 4.1a), and because OxyR

regulates *katE*, whose gene product detoxifies H_2O_2 (Kim J-A and Mayfield, 2000). Furthermore, OxyR has an established role for defending the brucellae against high concentrations of exogenous H_2O_2 (30 mM) (Kim J-A and Mayfield, 2000). Since OxyR directly regulates *ahpC* expression in response to exogenous H_2O_2 exposure in other bacteria (*E. coli* [Christman et al., 1985], *Neisseria gonorrhoeae* [Sieb et al., 2007], *Pseudomonas aeruginosa* [Ochsner et al., 2000]), we next asked if OxyR was involved in the H_2O_2-responsive repression of *ahpC* in *B. abortus* 2308 caused by millimolar levels of exogenous H_2O_2. Using the *B. abortus* 2308 strain carrying the *ahpC-lacZ* fusion to measure *ahpC* promoter activity, we found that *ahpCD* expression decreases in response to 10 mM H_2O_2 in 2308 but not in the *B. abortus oxyR* mutant KH231 (Figure 4.12). These results indicate that OxyR is responsible for repressing *ahpCD* expression in response to supraphysiologic levels of H_2O_2 in *B. abortus*. However our electrophoretic mobility shift analysis revealed that OxyR does not bind to the *ahpC* promoter DNA, so OxyR is likely affecting *ahpC* expression in *B. abortus* 2308 indirectly (Chapter 3). Since we know that OxyR activates *katE* expression in response to 10 mM H_2O_2 (Kim and Mayfield, 2000; Kim et al., 2000), and that the overexpression of *katE* causes *ahpCD* expression to decrease (Chapter 3), we propose in Figure 4.13 that OxyR indirectly affects the *ahpC* H_2O_2-responsive expression by leading to an increase in KatE protein. Further work must be done to verify this model.

OxyR summary and future directions. Our data suggest that the basal level of catalase protein being produced in *B. abortus* 2308 is sufficient to protect the cell against H_2O_2 toxicity even without the presence of OxyR to enhance *katE* expression: *B. abortus oxyR* mutants are not as sensitive as *katE* mutants to H_2O_2 (Figure 4.1A), *B. abortus oxyR* mutants have catalase

activity (Figure 4.14 and personal observation), and *oxyR* mutants do not have the same defect as *katE* mutants in scavenging 50 μM and 100 μM exogenous H_2O_2 (Figure 4.15). So we question what role OxyR plays in *Brucella*'s oxidative defense. Does OxyR only activate *katE* expression in response to high levels of H_2O_2 (e.g. mM)? Does OxyR regulate other antioxidant genes in *B. abortus*? Is OxyR necessary for the survival of *B. abortus* within the host? Our phenotypic analysis suggests that OxyR may not have the important role that OxyR has in *E. coli* and *Salmonella typhimurium* (Storz et al., 1987), *Xanthomonas campestris* (Mongkolsuk et al., 1998), *N. gonorrhoeae* (Sieb et al., 2007), *Porphyromonas gingivalis* (Ohara et al., 2006; Meuric et al., 2008), and many other bacteria. Instead, *Brucella* OxyR resembles the *B. japonicum* OxyR. While a mutation in *B. japonicum*'s sole catalase gene, *katG*, results in the absence of catalase activity, the inability to grow in the presence of O_2, and the failure to scavenge 1.5 μM and 150 μM exogenous H_2O_2, *B. japonicum oxyR* mutants have catalase activity, have only a small aerobic growth defect, and have no defect in scavenging 1.5 μM and 150 μM H_2O_2 (Panek and O'Brian, 2004). Therefore the relationship OxyR has with *katG* expression in *B. japonicum* is similar to the relationship OxyR has with *katE* expression in *B. abortus*. Both OxyR proteins may only have a limited role in oxidative stress defense in these bacteria.

The indirect effect of OxyR on *ahpC* expression in response to high H_2O_2 levels implies that OxyR does have a role in oxidative stress defense in *B. abortus*, but we do not know how universal that role is or how OxyR affects *ahpC* expression. There are predicted antioxidants such as Bcp (BAB1_0941), Rbr (BAB1_1691), and Tpx (BAB1_0504) that have not been characterized in *Brucella*. These antioxidants may be important for the degradation of H_2O_2 and/or OP, and OxyR may have an important role in the regulation of their genes. To understand how important OxyR is to *B. abortus*, it is necessary to know what the OxyR regulon

is. Two-dimensional SDS-PAGE analysis of protein lysates can be a valuable tool for this purpose. We can compare H_2O_2-exposed 2308 protein lysates to H_2O_2-exposed *oxyR* mutant cell lysates to identify proteins that are induced by OxyR in response to H_2O_2. We can also use microarray analysis to identify OxyR regulated genes.

Conclusions.

B. abortus has the ability to detoxify H_2O_2 and $O_2^{\bullet -}$ produced from aerobic metabolism and by the macrophage before these ROS can cause irreversible damage to the bacterial cell. In order to learn how *Brucella* can sense increased levels of ROS and use antioxidants to prevent oxidative damage, we made mutations in genes encoding three putative oxidative stress regulators and studied how these mutations affected the viability of *B. abortus* in response to increased levels of H_2O_2, $O_2^{\bullet -}$, and OP. We have learned that BAB2_0530 may regulate genes encoding proteins that detoxify $O_2^{\bullet -}$ and OP. The effect BAB2_0530 has on these genes, though, does not seem to be critical for survival within the experimental host since BAB2_0530 mutants are fully virulent in C57BL/6 mice. It would be interesting to learn what the target genes of BAB2_0530 are, and if BAB2_0530 is important for survival of *B. abortus* 2308 within its natural host cattle.

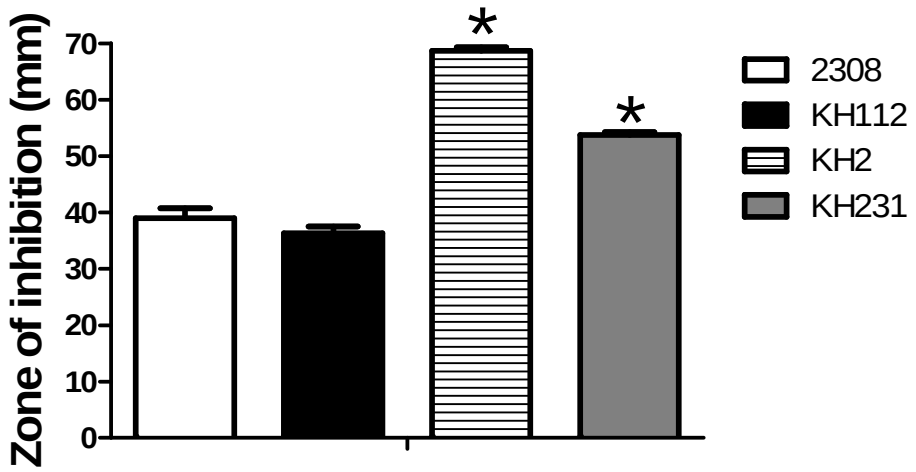
OxyR does not seem to affect *katE* expression during *in vitro* growth unless the brucellae are exposed to millimolar levels of exogenous H_2O_2. Furthermore, OxyR only represses *ahpC* expression in response to millimolar levels of H_2O_2. Therefore, it seems that OxyR's role in the *Brucella* oxidative stress defense is to affect antioxidant gene expression in response to supraphysiological levels of H_2O_2. But we question if *B. abortus* is ever exposed to these levels

of H_2O_2 in nature. The *E. coli* oxidative stress response is activated by 20 nM H_2O_2 (Imlay 2008). We estimate that the *B. abortus* oxidative stress response is activated by similar levels of H_2O_2 based on the levels of endogenous H_2O_2 that activate *ahpC* expression in *B. abortus* 2308. Macrophages are estimated to produce 100 nM H_2O_2 from their oxidative burst (Jang and Imlay, 2007). Even the combination of H_2O_2 produced from aerobic metabolism and by the macrophage do not reach millimolar levels of H_2O_2, so again *B. abortus* may never be exposed to the high levels of H_2O_2 that OxyR responds to. But since *B. abortus* produces OxyR, it is either advantageous for the brucellae to have this secondary line of oxidative defense, or OxyR is important for regulating other genes.

The data presented in chapter 3 show that Irr affects the regulator that directly controls *ahpC* expression in response to H_2O_2 in *B. abortus*. Irr may affect the expression of the regulator's gene or may affect the regulator's activity if it requires iron or iron-sulfur clusters (Figure 3.9). The data in this chapter show that Irr not only has a role in *Brucella*'s oxidative defense against H_2O_2 toxicity, but also against $O_2^{\bullet -}$ and OP damage. Therefore either Irr or the *B. abortus* unidentified oxidative stress regulator (which Irr affects) directly regulates other antioxidant genes whose products detoxify $O_2^{\bullet -}$ and OP. Further experiments must be done to identify *ahpC*'s regulator and its regulon. We must also identify the Irr regulon to really understand Irr's role in the *Brucella* oxidative stress defense.

Figure 4.1 The *B. abortus oxyR* mutant KH231 exhibits increased sensitivity to H_2O_2 in *in vitro* assays, but the BAB2_0530 mutant KH112 does not. (A) *B. abortus katE* KH2 and *B. abortus oxyR* KH231 mutants are more sensitive than wild-type 2308 and the *B. abortus* BAB2_0530 mutant KH112 to 7.5 μL 30% H_2O_2 at a cell density of 10^8 cfu/mL in a disk inhibition assay, and (B) KH2 is more sensitive than KH112 and wild-type 2308 to a one hour exposure to 50 mM H_2O_2 at a starting cell density of 10^9 cfu/mL in a liquid broth assay. The experimental procedures used are described in detail in Chapter 2. The data presented are means and standard deviations for triplicate determinations from a single culture in a single experiment. The data presented here are representative of multiple experiments (A- ≥ 2; B- ≥ 5) performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test is represented by an asterisk and is for the comparison of 2308 versus the other strains.

A.


Zone of inhibition (mm)
70
60
50
40
30
20
10
0
*
*
2308
KH112
KH2
KH231

B.

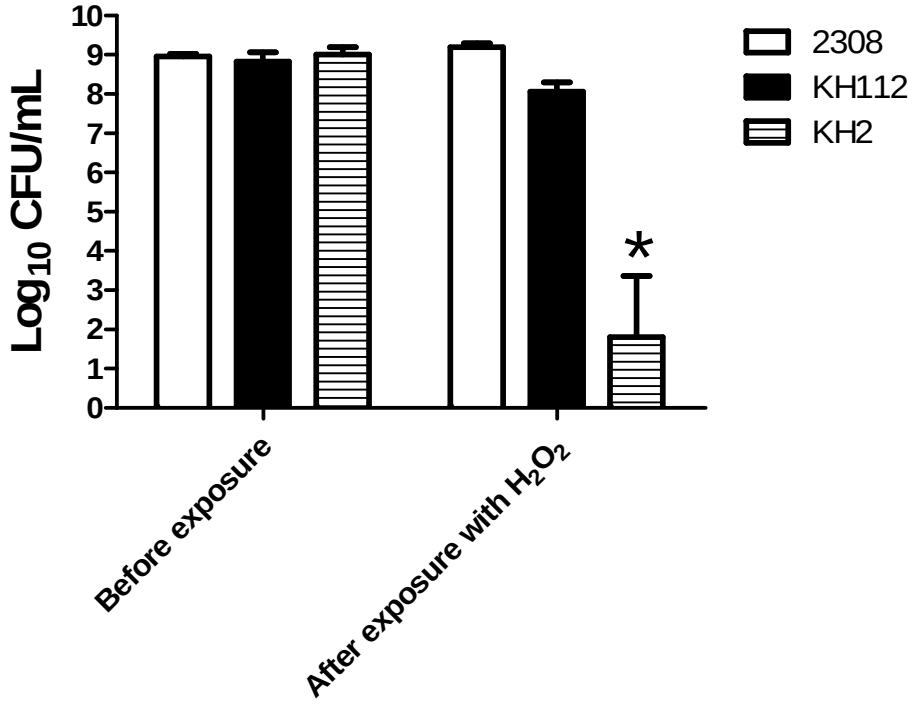
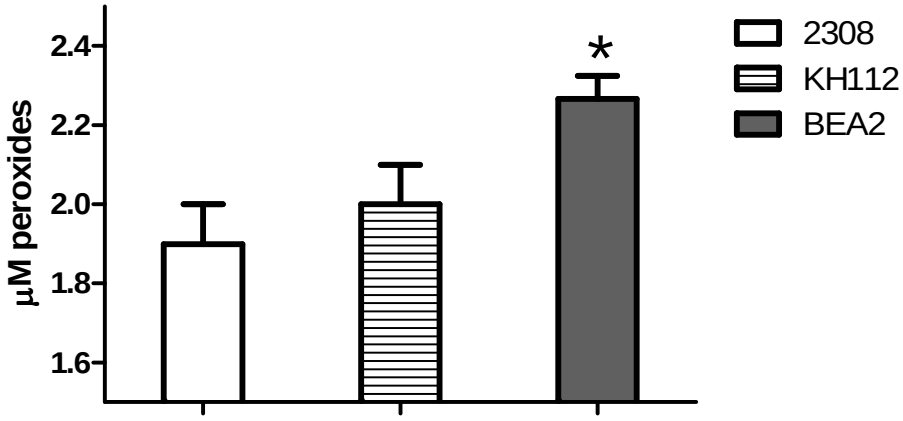

Log10 CFU/mL
10
9
8
7
6
5
4
3
2
1
0
*
2308
KH112
KH2
Before exposure
After exposure with H2O2

Figure 4.1

Figure 4.2 The *B. abortus irr* mutant BEA2 exhibits a defect scavenging both endogenous and exogenous H_2O_2, but the isogenic BAB2_0530 mutant KH112 does not. (A) The amount of endogenous peroxides in *B. abortus* 2308, the *B. abortus* BAB2_0530 mutant KH112, and the *B. abortus irr* mutant BEA2, and (B) the rate of degradation of 50 μM exogenous H_2O_2 by *B. abortus* 2308, the *B. abortus* BAB2_0530 mutant KH112, and the *B. abortus irr* mutant BEA2. The experimental method employed are described in detail in chapter 2. The data presented are means and standard deviations for triplicate determinations from a single culture in a single experiment. The data presented here are representative of multiple (≥ 2) experiments performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test is represented by an asterisk and is for the comparison of (A) 2308 versus KH112 and BEA2 and (B) the amount of cellular peroxides at 0, 5, and 10 minutes versus the amount of intracellular peroxides in each strain before the addition of H_2O_2.

A.


2.4
2.2
2.0
1.8
1.6
µM peroxides
*
2308
KH112
BEA2

B.

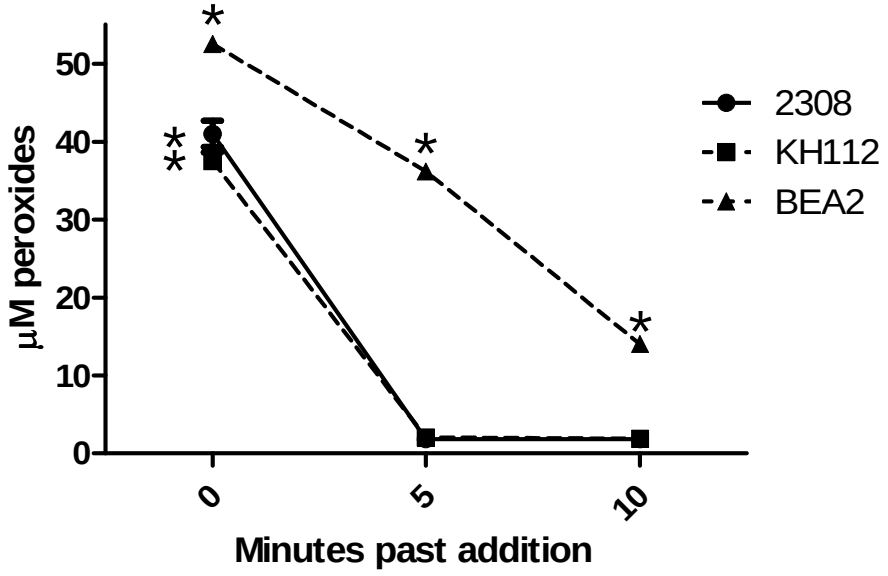

50
40
30
20
10
0
µM peroxides
*
**
*
*
0
5
10
Minutes past addition
2308
KH112
BEA2

Figure 4.2

Figure 4.3 The *B. abortus* BAB2_0530 mutant KH112 displays an increased sensitivity to endogenous $O_2^{\bullet -}$ or H_2O_2 generated by the redox cycler paraquat (PQ). In this assay, the *B. abortus sodA* mutant JB12 is used as a positive control. *B. abortus* strains were harvested into brucella broth and adjusted to a cell density of 10^9 CFU per ml ($OD_{600\ nm}$ = 0.15). 600 µl aliquots of each cell suspension was then added to 18 ml prewarmed (55°C) brucella broth supplemented with 0.7% agar, and 3 ml portions of the resulting cell suspensions plated onto three Schaedler agar (SA) plates and three SA plates containing 195,000 U bovine catalase (Sigma). A sterile 7-mm Whatman no. 3 filter paper disk was placed in the center of each plate and 10 µL of a fresh 0.5 M solution of paraquat (PQ; Acrōs Organics) was added to each disk. Plates were incubated for three days and the zones of inhibition surrounding each disk were measured in millimeters. The data presented are means and standard deviations for triplicate determinations from a single culture in a single experiment. The data presented here are representative of multiple (≥ 3) experiments performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test is represented by an asterisk and is for the comparison of 2308 versus KH112 and JB12.

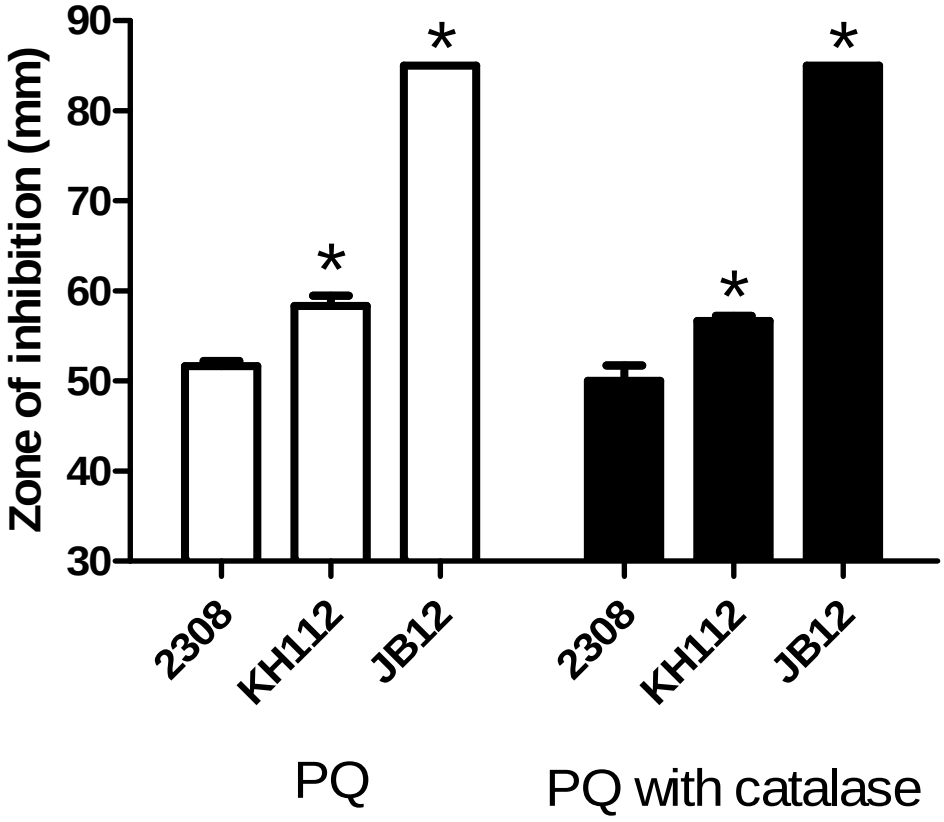

Zone of inhibition (mm)
90
80
70
60
50
40
30
*
*
*
*
2308
KH112
JB12
2308
KH112
JB12
PQ
PQ with catalase

Figure 4.3

Figure 4.4 The *B. abortus* BAB2_0530 mutant KH112 displays an increased susceptibility to organic peroxides. Viability of *B. abortus* 2308, the *B. abortus* BAB2_0530 mutant KH112, and the *B. abortus ohr* mutant JB11 to organic peroxides. Bacterial cells were harvested and resuspended to a cell density of 10^8 in 1 ml Gerhardt's minimal medium (GMM) in 17 × 100 mm culture tubes. Cumene hydroperoxide (CHP) and tert-butyl hydroperoxide (tBOOH) were added at a final concentration of 3 mM and 50 mM, respectively, and the mixtures incubated for 1 h at 165 rpm at 37°C. The number of viable brucellae for *B. abortus* 2308, was measured before and after exposure by serial dilution and plating on SBA. The data presented are means and standard deviations for triplicate determinations from a single culture in a single experiment. The data presented here are representative of multiple (≥ 2) experiments performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test is represented by an asterisk and is for the comparison of 2308 versus the other strains.

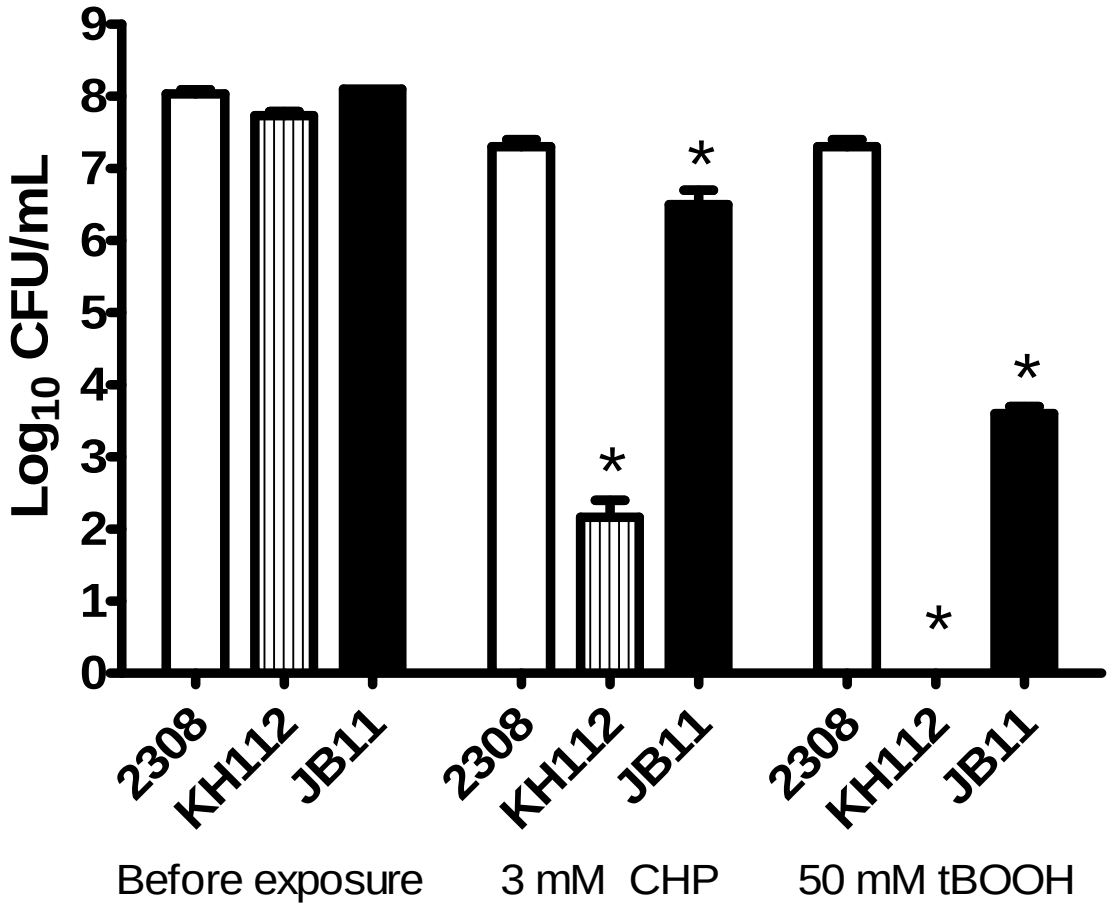

Log10 CFU/mL
9
8
7
6
5
4
3
2
1
0
*
*
*
2308
KH112
JB11
2308
KH112
JB11
2308
KH112
JB11
Before exposure
3 mM CHP
50 mM tBOOH

Figure 4.4

Figure 4.5 The *B. abortus irr* mutant BEA2 exhibits an increased sensitivity to supraphysiologic levels of H_2O_2 in a broth assay. Viability of *B. abortus* 2308, the *B. abortus irr* mutant BEA2, and the *B. abortus katE* mutant MEK6 to increasing concentrations of H_2O_2. Bacterial cells were harvested and resuspended to a cell density of 10^8 in 1 ml GMM in 17 × 100 mm culture tubes. Varying concentrations of H_2O_2 were added to the cell suspensions and the mixtures incubated for 1 h at 165 rpm at 37°C. The number of viable brucellae in these cultures before and after exposure was then determined by serial dilution and plating on SBA. The data presented are means and standard deviations for triplicate determinations from a single culture in a single experiment. The data presented here are representative of multiple (≥ 9) experiments performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test is represented by an asterisk and is for the comparison of 2308 versus the other strains.

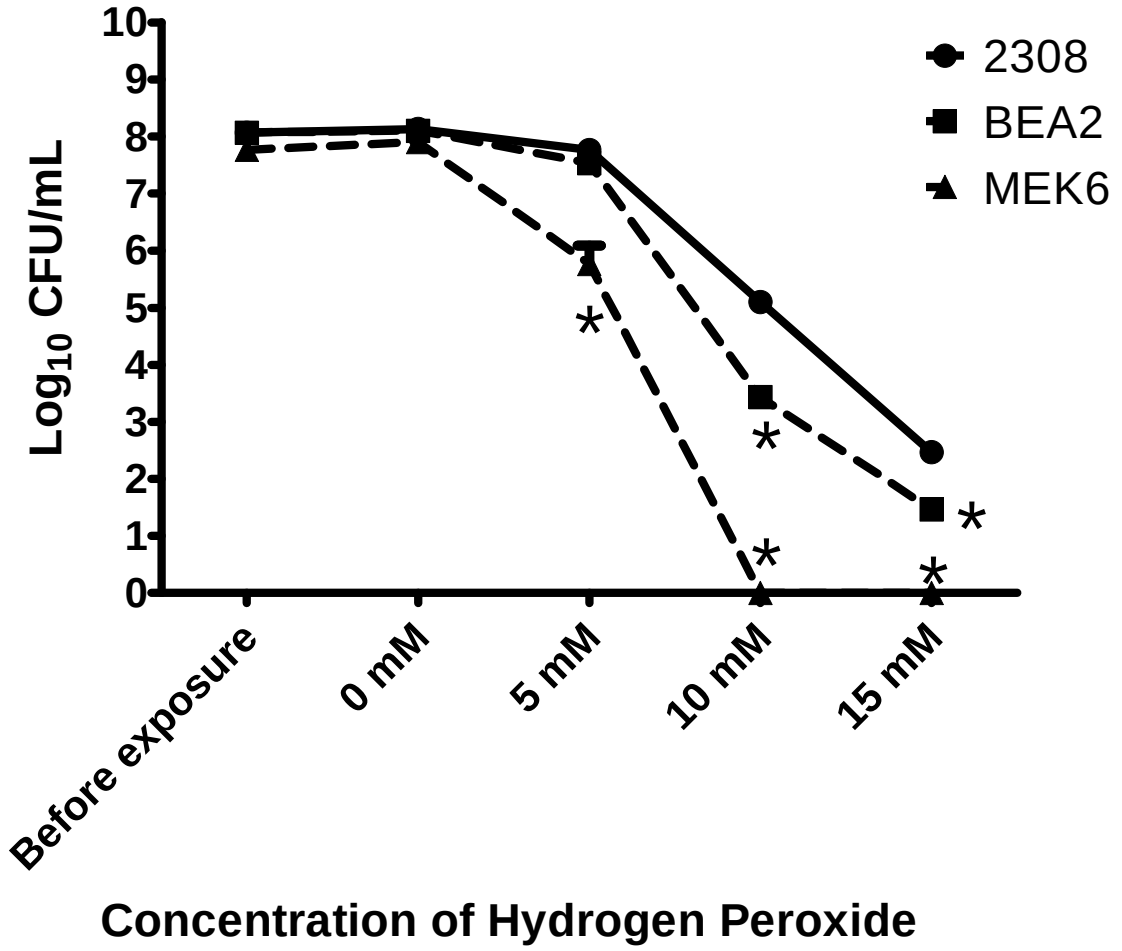

10
9
8
7
6
5
4
3
2
1
0
Log10 CFU/mL
2308
BEA2
MEK6
*
*
*
*
Before exposure
0 mM
5 mM
10 mM
15 mM
Concentration of Hydrogen Peroxide

Figure 4.5

Figure 4.6 The *B. abortus irr* mutant BEA2 is more sensitive to organic peroxides than the parental 2308 strain. Viability of *B. abortus* 2308 (white bars) and *B. abortus irr* mutant BEA2 (black bars) before exposure and after a one hour exposure to either (A) cumene hydroperoxide (CHP) or (B) tert-butyl hydroperoxide (tBOOH). Bacterial cells were harvested and resuspended to a cell density of 10^8 in 1 ml GMM in 17 × 100 mm culture tubes. CHP and tBOOH were added at a final concentration of 3 mM and 50 mM, respectively, and the mixtures incubated for 1 h at 165 rpm at 37°C. The number of viable brucellae for *B. abortus* 2308, was measured before and after exposure by serial dilution and plating on SBA. The data presented are means and standard deviations for triplicate determinations from a single culture in a single experiment. The data presented here are representative of multiple experiments (A- $\geq$ 3, B- $\geq$ 6) performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test is represented by an asterisk and is for the comparison of 2308 versus *B. abortus irr* mutant BEA2.

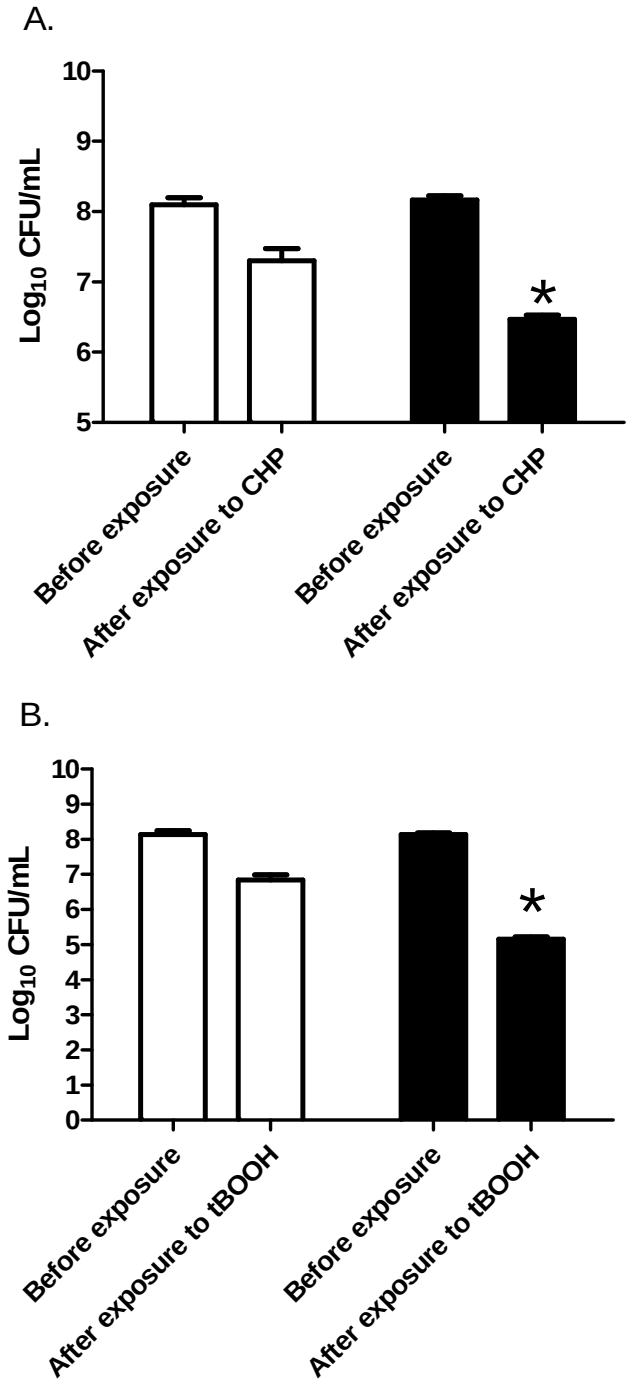

A.
Log10 CFU/mL
10
9
8
7
6
5
*
Before exposure
After exposure to CHP
Before exposure
After exposure to CHP
B.
Log10 CFU/mL
10
9
8
7
6
5
4
3
2
1
0
*
Before exposure
After exposure to tBOOH
Before exposure
After exposure to tBOOH

Figure 4.6

Figure 4.7 Predicted Irr binding sites (termed ICE boxes) found in promoter regions of antioxidant genes in *B. abortus* 2308. (A) The ICE boxes are shown in bold and in brackets. (B) Percent identity of predicted ICE boxes to the *B. abortus* consensus ICE box is noted in parentheses (Rodionov et al., 2006).

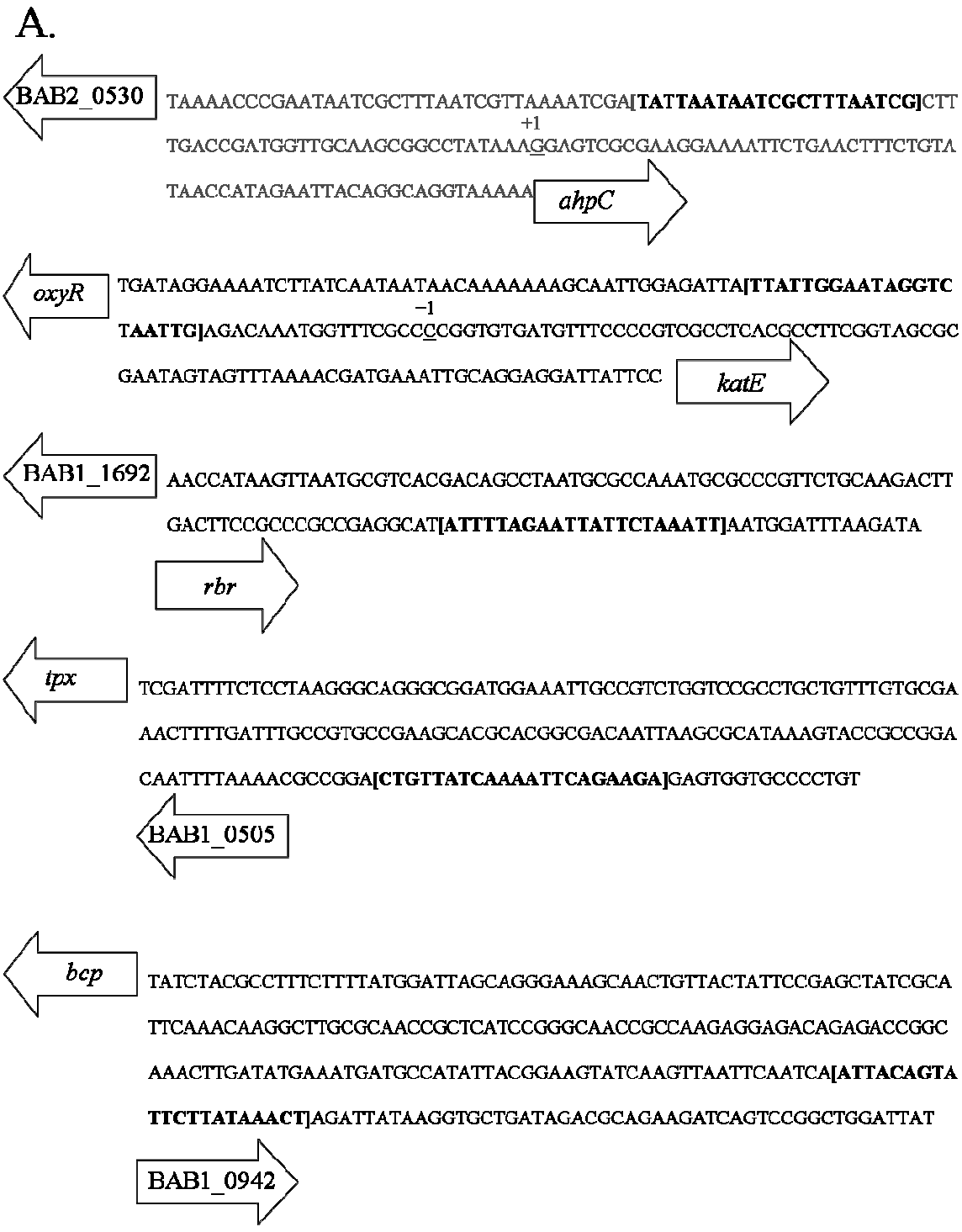

A.
BAB2_0530
TAAAACCCGAATAATCGCTTTAATCGTTAAAATCGA[TATTAATAATCGCTTTAATCG]CTT
+1
TGACCGATGGTTGCAAGCGGCCTATAAAGGAGTCGCGAAGGAAAATTCTGAACTTTCTGTA
TAACCATAGAATTACAGGCAGGTAAAAA
ahpC
oxyR
TGATAGGAAAATCTTATCAATAATAACAAAAAAAGCAATTGGAGATTA[TTATTGGAATAGGTC
−1
TAATTG]AGACAAATGGTTTCGCCCCGGTGTGATGTTTCCCCGTCGCCTCACGCCTTCGGTAGCGC
GAATAGTAGTTTAAAACGATGAAATTGCAGGAGGATTATTCC
katE
BAB1_1692
AACCATAAGTTAATGCGTCACGACAGCCTAATGCGCCAAATGCGCCCGTTCTGCAAGACTT
GACTTCCGCCCGCCGAGGCAT[ATTTTAGAATTATTCTAAATT]AATGGATTTAAGATA
rbr
tpx
TCGATTTTCTCCTAAGGGCAGGGCGGATGGAAATTGCCGTCTGGTCCGCCTGCTGTTTGTGCGA
AACTTTTGATTTGCCGTGCCGAAGCACGCACGGCGACAATTAAGCGCATAAAGTACCGCCGGA
CAATTTTAAAACGCCGGA[CTGTTATCAAAATTCAGAAGA]GAGTGGTGCCCCTGT
BAB1_0505
bcp
TATCTACGCCTTTCTTTTATGGATTAGCAGGGAAAGCAACTGTTACTATTCCGAGCTATCGCA
TTCAAACAAGGCTTGCGCAACCGCTCATCCGGGCAACCGCCAAGAGGAGACAGAGACCGGC
AAACTTGATATGAAATGATGCCATATTACGGAAGTATCAAGTTAATTCAATCA[ATTACAGTA
TTCTTATAAACT]AGATTATAAGGTGCTGATAGACGCAGAAGATCAGTCCGGCTGGATTAT
BAB1_0942

Figure 4.7

B.

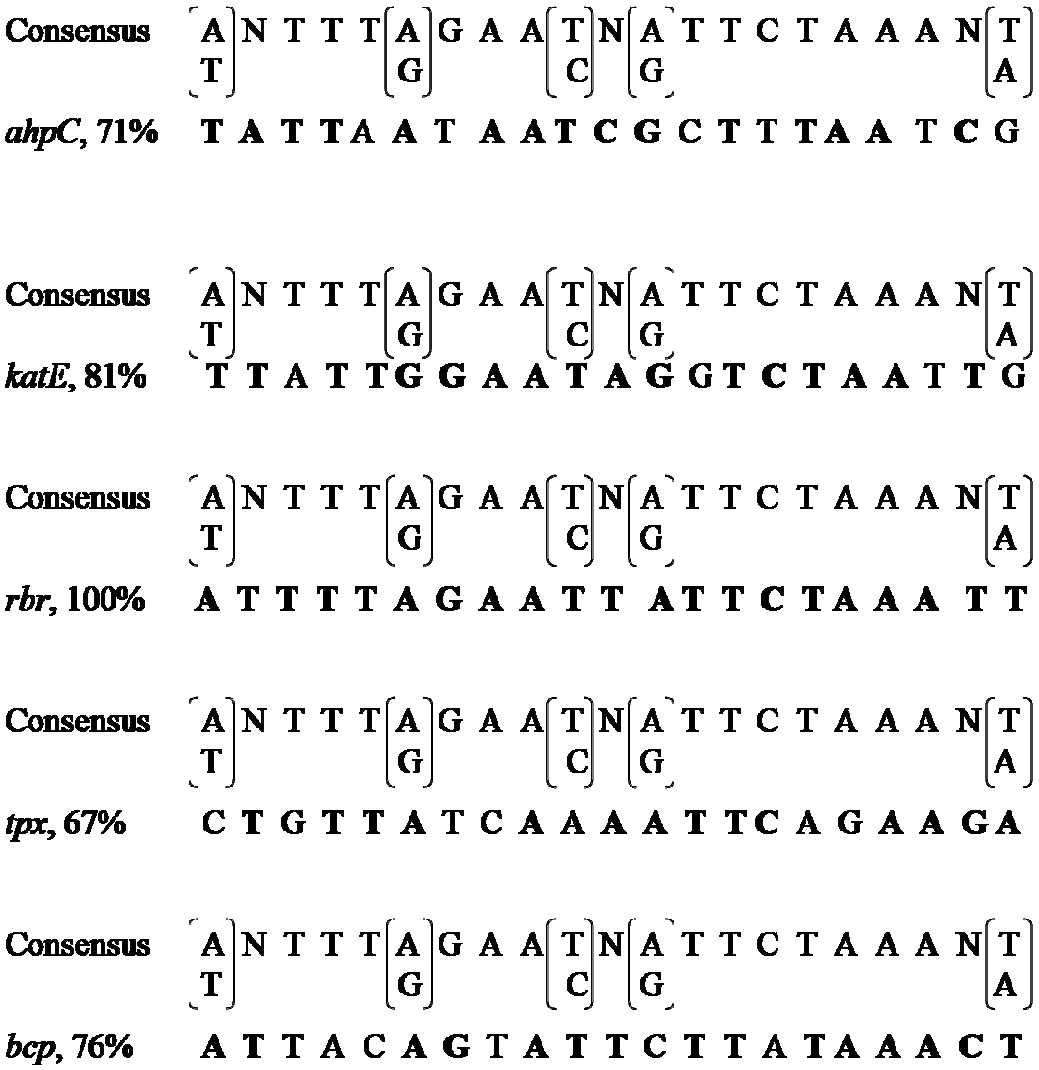


Figure 4.7 (continued)

Figure 4.8 The *B. abortus irr* mutant BEA2 is more sensitive to paraquat (PQ) than the parental 2308 strain. *B. abortus* strains were grown overnight in 3 ml brucella broth in 17 × 100 mm culture tubes incubated at 37°C with shaking at 165 rpm. The resulting cultures were inoculated into 500 ml flasks containing 100 ml of brucella broth at a cell density of approximately 10^3 CFU per ml, and the flasks were incubated at 37°C while shaking at 165 rpm. Some cultures were exposed to 100 μM PQ after 24 h of growth and are represented by dashed lines. Cultures that were not exposed to PQ are represented by solid lines. The number of viable brucellae in these cultures was determined at selected times after inoculation by serial dilution and plating on SBA or SBA containing the appropriate antibiotic. The data presented are means and standard deviations for triplicate determinations from a single culture in a single experiment. The data presented here are representative of multiple (≥ 4) experiments performed from which equivalent results and statistical trends were obtained. Statistical significance as determined by the Student two-tailed *t* test is represented by one asterisk ($P \leq 0.05$) or two asterisks ($P \leq 0.001$) and is for the comparison of cultures not exposed to PQ versus cultures exposed to PQ.

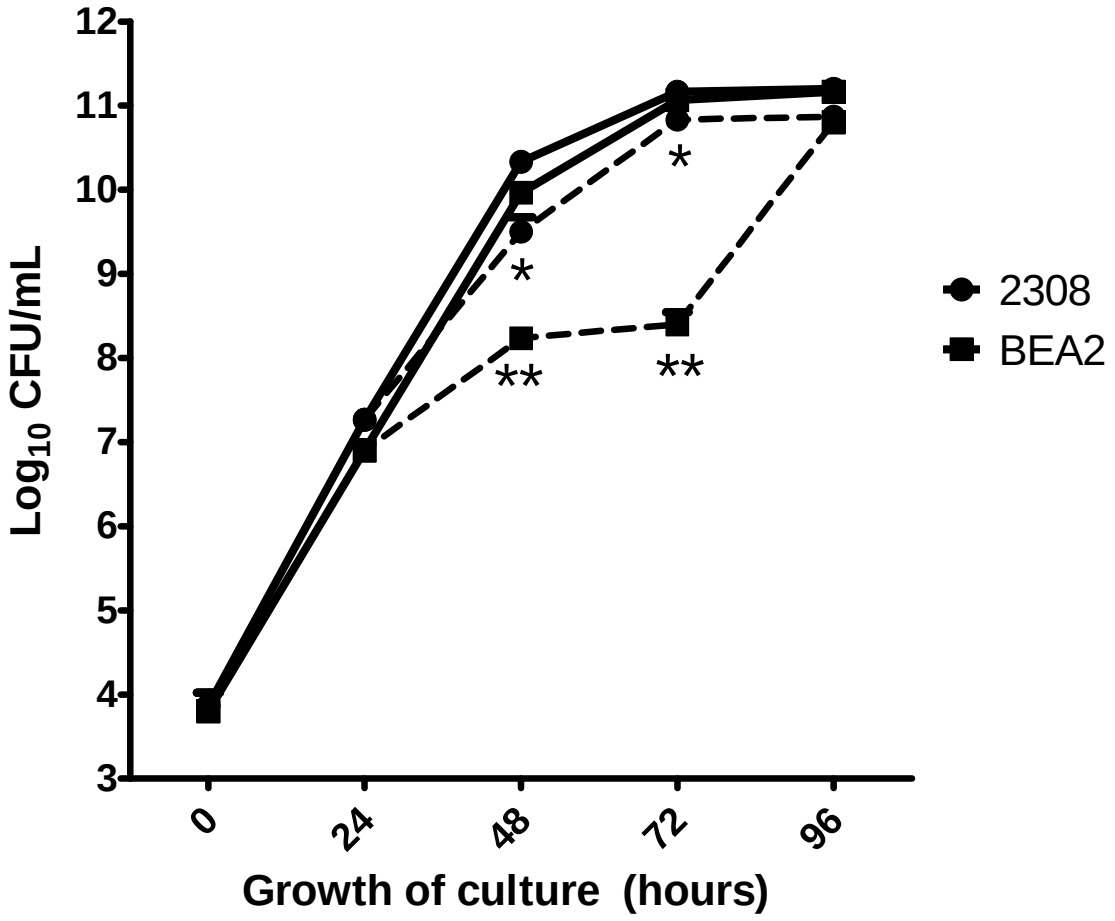

12
11
10
9
8
7
6
5
4
3
Log10 CFU/mL
0
24
48
72
96
Growth of culture (hours)
*
*
**
**
2308
BEA2

Figure 4.8

Figure 4.9 A model depicting how Irr could be directly affecting *rbr* expression in response to H_2O_2 in *B. abortus* 2308. In this figure, Irr is directly repressing *rbr* (rubrerythrin) expression when cellular H_2O_2 levels are low. When H_2O_2 levels increase, Irr is degraded in response to the H_2O_2 and Irr repression on *rbr* is relieved. The corresponding increase in Rbr levels would be beneficial for the bacterial cell because Rbr detoxifies H_2O_2.

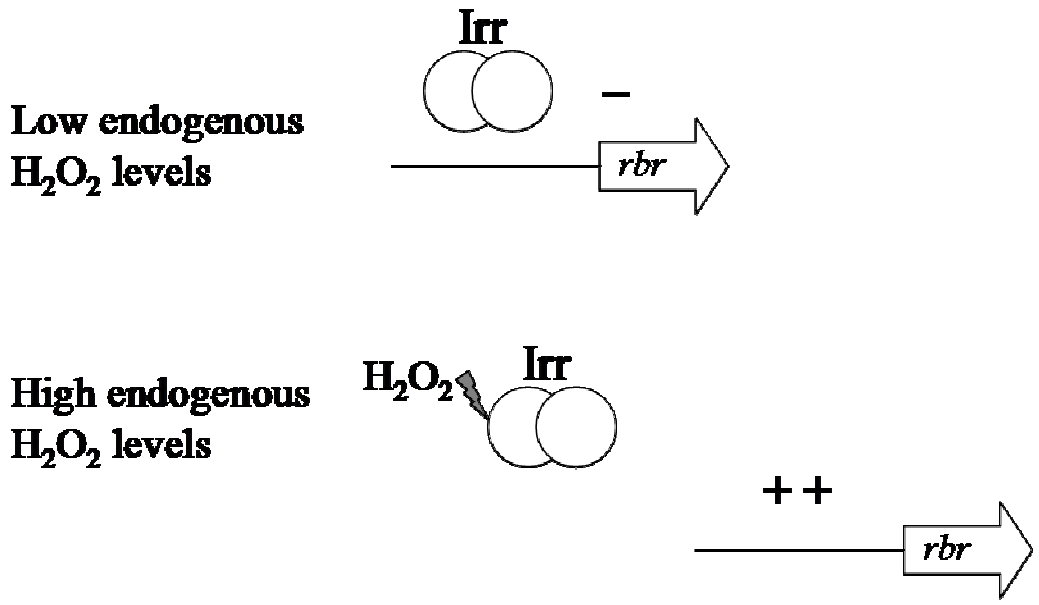

Irr
–
Low endogenous
H_2O_2 levels
rbr
H_2O_2
Irr
High endogenous
H_2O_2 levels
++
rbr

Figure 4.9

Figure 4.10 A model depicting how Irr could indirectly affect the *Brucella* oxidative stress response. Irr directly activates the *dhbCEBA* operon in *B. abortus* (Martinez et al., 2006), which encodes the siderophore 2.3-DHBA. The bacterial cell can take in more iron with increased levels of 2,3-DHBA. Iron is a cofactor for many proteins, and the *Brucella* unidentified oxidative stress regulator (Osr) may require iron for its activity. In this model, Osr represses *ahpCD* expression when iron levels are high. In the absence of Irr, cellular iron levels decrease, Osr is inactive, and *ahpCD* expression increases in the absence of Osr.

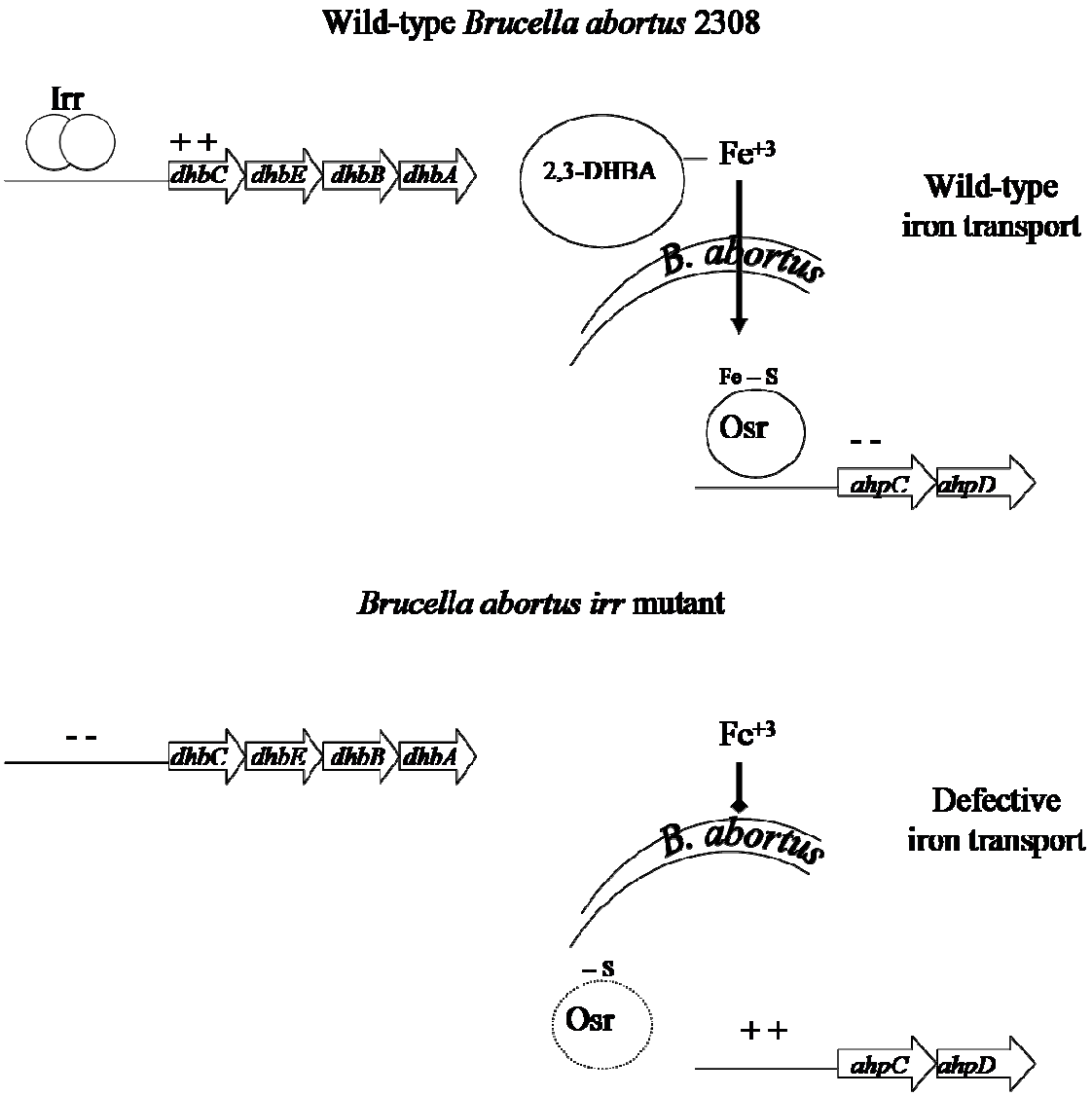

Wild-type *Brucella abortus* 2308
Irr
++
dhbC
dhbE
dhbB
dhbA
2,3-DHBA
Fe+3
Wild-type
iron transport
B. abortus
Fe – S
Osr
--
ahpC
ahpD
Brucella abortus irr mutant
--
dhbC
dhbE
dhbB
dhbA
Fe+3
Defective
iron transport
B. abortus
– s
Osr
++
ahpC
ahpD

Figure 4.10

Figure 4.11 *ahpC* expression in *B. abortus* 2308 inversely correlates with increasing supraphysiologic levels of H_2O_2 in the mM range. β-galactosidase production from the *ahpC-lacZ* fusion in *B. abortus* 2308 (Chapter 3) in response to exposure to nanomolar concentrations of H_2O_2 (A) and millimolar concentrations of H_2O_2 (C). (B) β-galactosidase production from the *ahpC-lacZ* fusion in a *B. abortus katE* mutant in response to μM H_2O_2. *B. abortus* strains were grown on SBA for 48 h and inoculated into 3 ml brucella broth in 17 × 100 mm tubes and incubated at 37°C with shaking at 165 rpm. Following overnight incubation, the bacterial cells were inoculated into 500 ml flasks containing 100 ml of brucella broth at a cell density of 10^4 CFU per ml, and the flasks were incubated at 37°C while shaking at 165 rpm. After 30 h of growth, bacterial cells were adjusted to a cell density of 10^9 CFU/mL in GMM in 17 × 100 mm tubes and exposed to varying concentrations of H_2O_2 for one hour while incubating at 37°C with shaking at 165 rpm. β-galactosidase production by these cultures was then measured using the methods of Miller as described in Chapter 2. There was no loss in cell viability after any of these exposures. The data presented are means and standard deviations for triplicate determinations from a single culture in a single experiment. The data presented here are representative of multiple (≥ 3) experiments performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test is represented by an asterisk and is for the comparison of *ahpCD* promoter activity levels exposed to no H_2O_2 versus levels exposed to H_2O_2.

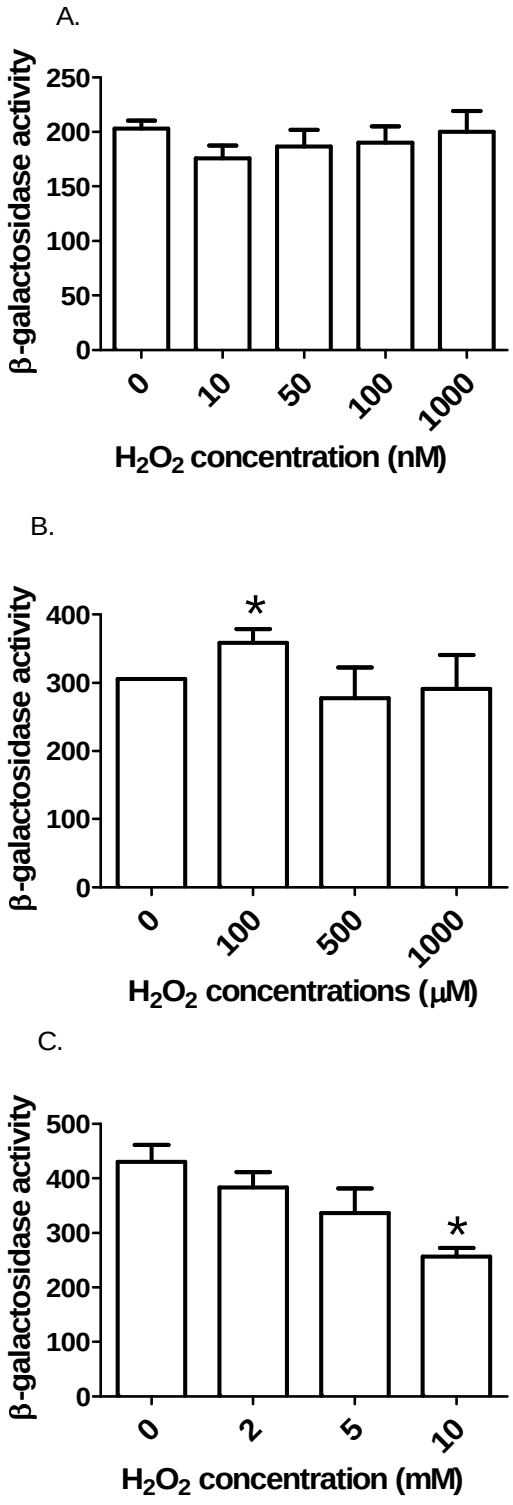

A.
β-galactosidase activity
250
200
150
100
50
0
0
10
50
100
1000
H2O2 concentration (nM)
B.
β-galactosidase activity
400
300
200
100
0
*
0
100
500
1000
H2O2 concentrations (μM)
C.
β-galactosidase activity
500
400
300
200
100
0
*
0
2
5
10
H2O2 concentration (mM)

Figure 4.11

Figure 4.12 *ahpC* expression does not decrease in the *B. abortus oxyR* mutant KH231 in response to exposure to 10 mM H_2O_2. β-galactosidase production from the *ahpC-lacZ* fusion in *B. abortus* 2308 and the *B. abortus oxyR* mutant KH231 in response to exposure to millimolar concentrations of H_2O_2 (C). *B. abortus* strains were grown on SBA for 48 h and inoculated into 3 ml brucella broth in 17 × 100 mm tubes and incubated at 37°C with shaking at 165 rpm. Following overnight incubation, the bacterial cells were inoculated into 500 ml flasks containing 100 ml of brucella broth at a cell density of 10^4 CFU per ml, and the flasks were incubated at 37°C while shaking at 165 rpm. After 30 h of growth, bacterial cells were adjusted to a cell density of 10^9 CFU/mL in GMM in 17 × 100 mm tubes and exposed to 0 mM or 10 mM H_2O_2 for one hour while incubating at 37°C with shaking at 165 rpm. β-galactosidase production by these cultures was then measured using the methods described by Miller in chapter 2. There was no loss in cell viability after any of these exposures. The data presented are means and standard deviations for triplicate determinations from a single culture in a single experiment. The data presented here are representative of multiple (≥ 3) experiments performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test is represented by an asterisk and is for the comparison of *ahpCD* promoter activity levels when not exposed to H_2O_2 versus levels when exposed to H_2O_2.

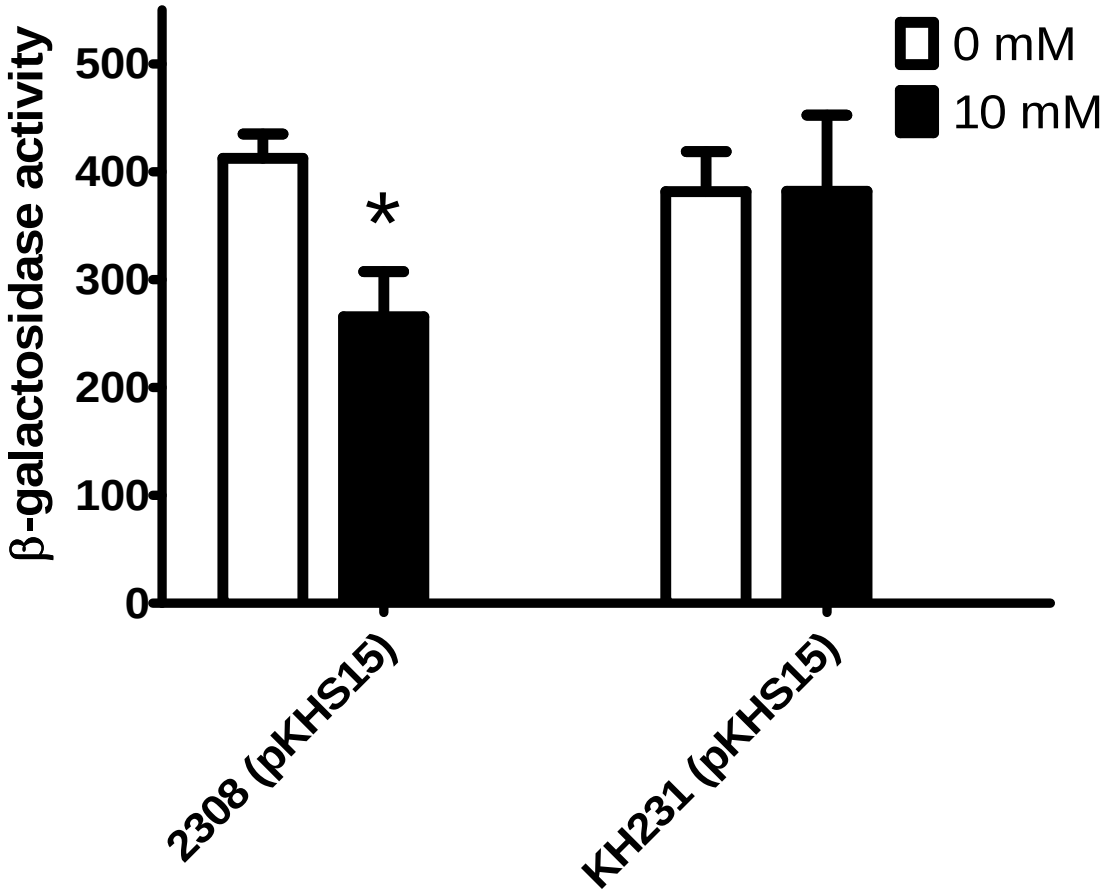

β-galactosidase activity
500
400
300
200
100
0
0 mM
10 mM
*
2308 (pKHS15)
KH231 (pKHS15)

Figure 4.12

Figure 4.13 A model illustrating that OxyR indirectly affects *ahpC* expression in *B. abortus* 2308 by upregulating *katE* expression whose protein will change cellular H_2O_2 levels. (A) During routine growth, *B. abortus*' primary oxidative stress regulator, Osr, activates *ahpCD* expression in response to endogenous H_2O_2, and basal levels of KatE are being produced. (B) When *B. abortus* is exposed to high levels of exogenous H_2O_2 (e.g. mM), OxyR is oxidized by the H_2O_2 and activates *katE* expression (Kim and Mayfield, 2000). KatE detoxifies H_2O_2 into H_2O and O_2, and thus removes the exogenous H_2O_2 that activates OxyR and the endogenous H_2O_2 that activates Osr. When Osr is not active it cannot activate *ahpC* expression in response to endogenous H_2O_2.

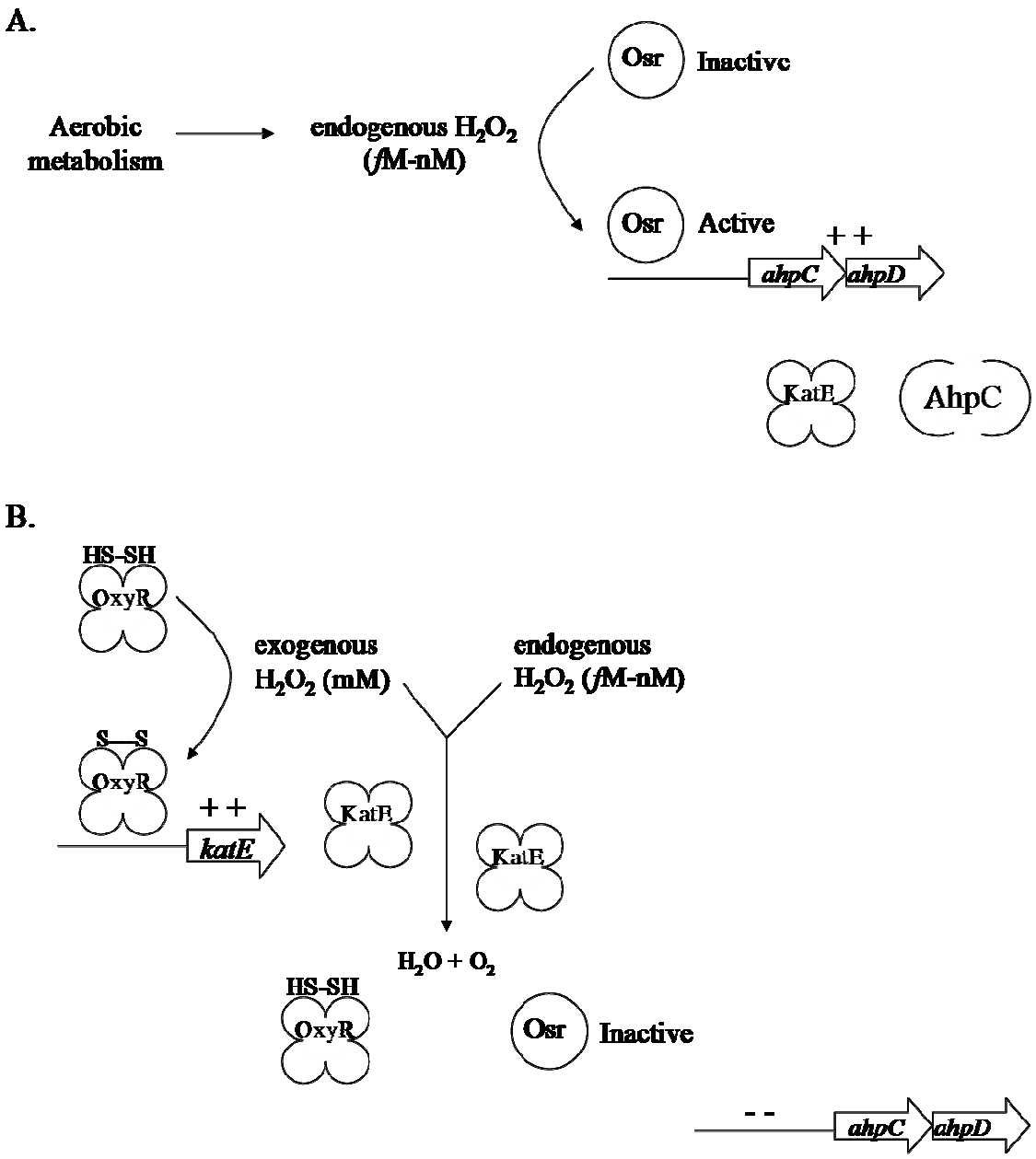

A.
Aerobic metabolism
endogenous H_2O_2 (*f*M-nM)
Osr
Inactive
Osr
Active
+ +
ahpC
ahpD
KatE
AhpC
B.
HS-SH
OxyR
exogenous H_2O_2 (mM)
endogenous H_2O_2 (*f*M-nM)
S—S
OxyR
+ +
katE
KatE
KatE
$H_2O + O_2$
HS-SH
OxyR
Osr
Inactive
- -
ahpC
ahpD

Figure 4.13

Figure 4.14 *B. abortus* 2308 (white) and the isogenic *oxyR* mutant KH231 (black) display similar levels of catalase activity during routine *in vitro* cultivation. *B. abortus* strains grown on SBA supplemented with the appropriate antibiotics for 48 h were inoculated into 3 ml brucella broth in 17 × 100 mm tubes and incubated at 37°C with shaking at 165 rpm. After overnight incubation, the bacterial cells were inoculated into 500 ml flasks containing 100 ml of brucella broth at a cell density of 10^3 CFU per ml and the flasks were incubated at 37°C while shaking at 165 rpm. At specific time intervals, bacterial cells were harvested by centrifugation (12,100 × *g*, 10 mins, room temperature) and resuspended in 1× reaction buffer to an optical density at 600 nm (OD_{600}) of 0.5. Reaction buffer and other solutions were provided by a commercial version of the catalase detection assay (Cell Technology, #FLOCAT 100-3), which was used to measure the level of catalase activity following the manufacturer's directions. Briefly, 50 µl of bacterial cell suspension was mixed with 50 µl of a 40 µM H_2O_2 solution and incubated at room temperature for 30 minutes. Next 100 µL of reaction cocktail (detection reagent, horseradish peroxidase, 1× reaction buffer) was added to the reaction mixture and incubated at room temperature for an additional 10 minutes. The reaction mixtures were then excited at an absorbance of 570 nM, and the absorbance of the reaction mixtures were measured in a spectrophotometer at 590 nm. Cell-free catalase standards (0 U/mL, 1 U/mL, 2 U/mL, 3 U/mL, 4 U/mL) were used to construct a standard curve, and the levels of catalase activity in the cell suspensions were determined by comparison to the standard curve. The data presented are means and standard deviations for triplicate determinations from a single culture in a single experiment. The data presented here are a representative of multiple (≥ 2) experiments performed from which equivalent results and statistical trends were obtained. Statistical

significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test is represented by an asterisk and is for the comparison of the levels of catalase activity in 2308 versus KH231.

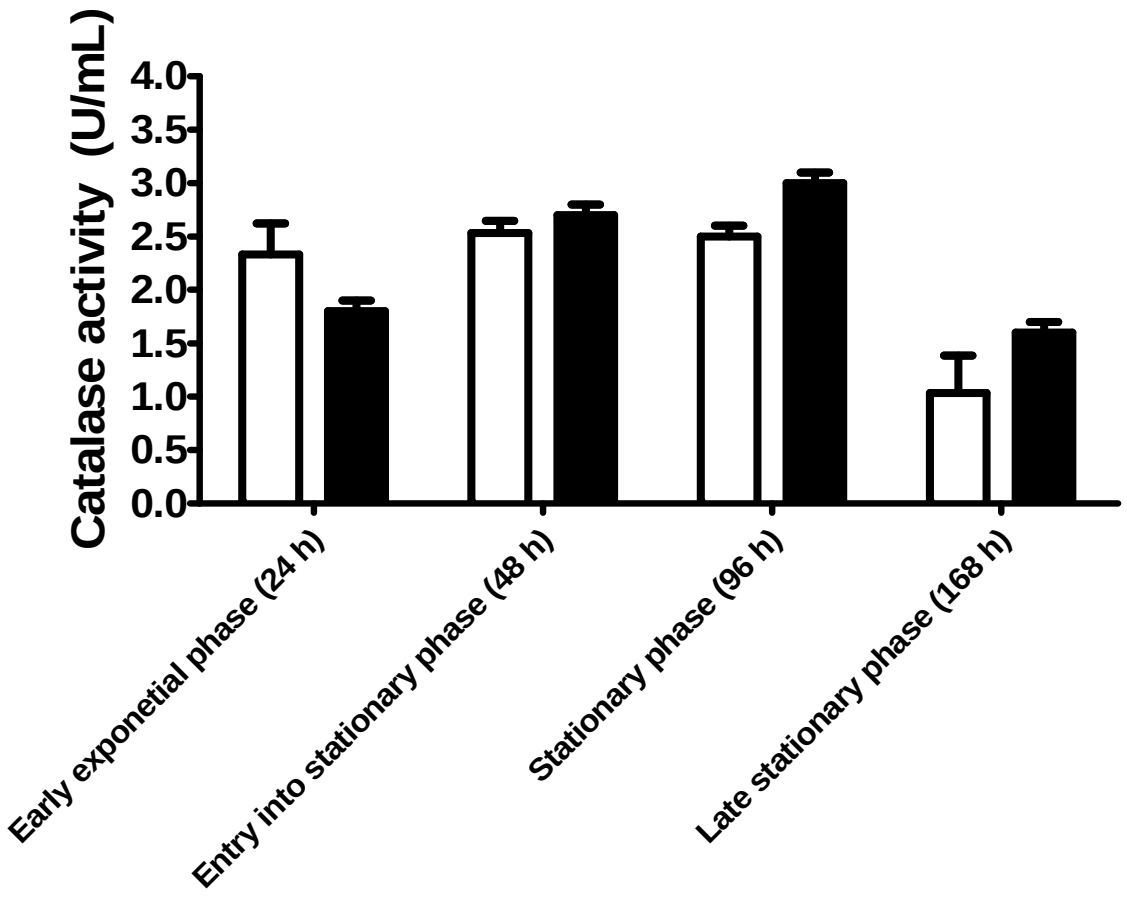
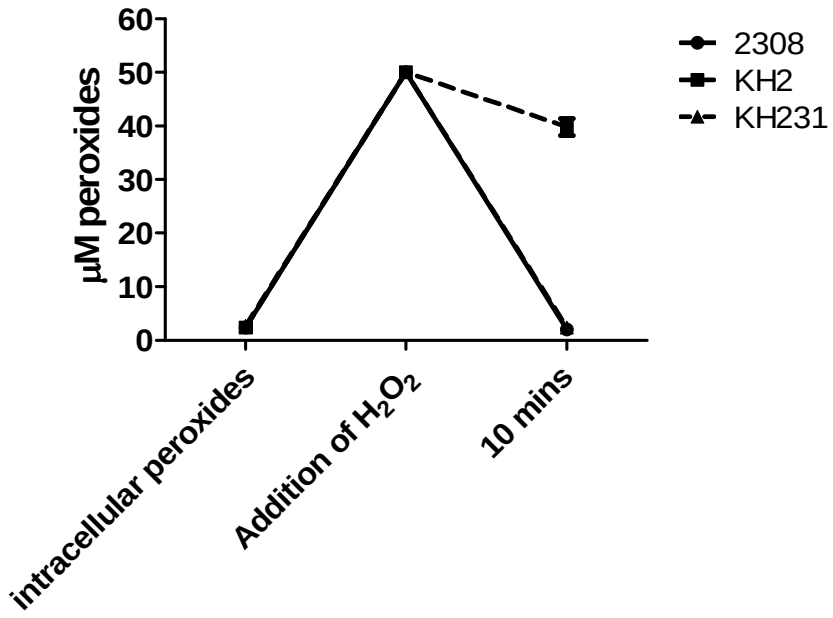

Catalase activity (U/mL)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Early exponetial phase (24 h)
Entry into stationary phase (48 h)
Stationary phase (96 h)
Late stationary phase (168 h)

Figure 4.14

Figure 4.15 The *B. abortus oxyR* mutant KH231 degrades 50 and 100 μM exogenous H_2O_2 with the same efficiency as the parental 2308 strain. The levels of peroxides were measured using the xylenol orange/ferrous iron assay (Chapter 2) in *B. abortus* 2308 (●), KH2 (2308 *katE*) (■), and KH231 (2308 *oxyR*) (▲) cell suspensions at selected times after the addition of 50 μM H_2O_2 (A) and 100 μM H_2O_2 (B). The data presented are means and standard deviations for triplicate determinations from a single culture in a single experiment. The data presented here are representative of multiple (≥ 2) experiments performed from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$) as determined by the Student two-tailed *t* test is represented by an asterisk and is for the comparison of 2308 versus KH2 and KH231.

A.



B.

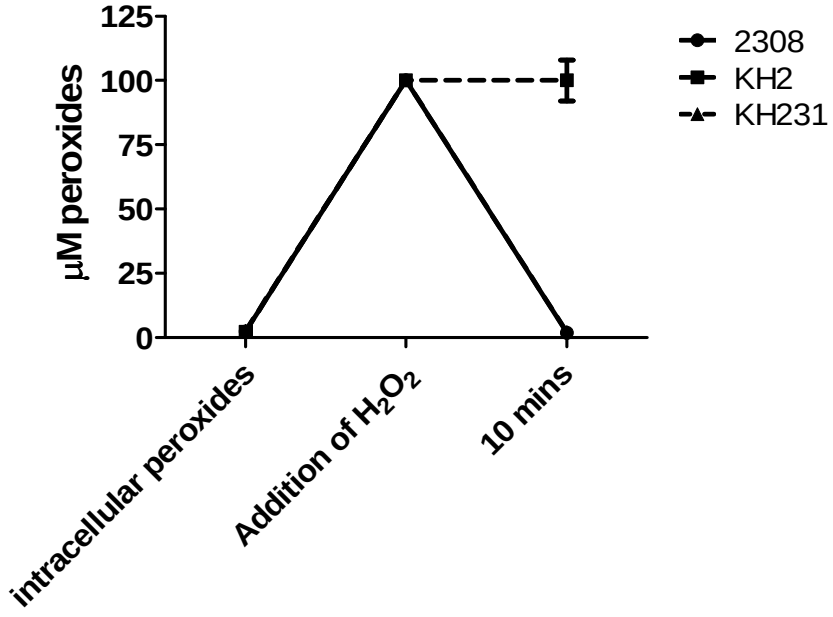


Figure 4.15

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Chapter 5

Summary and Conclusions

AhpC is a major detoxifier of metabolic H_2O_2 during routine aerobic growth of *Brucella abortus* 2308. KatE plays a minimal, if any role in removing this endogenous H_2O_2, since only an *ahpCD* mutation results in an aerobic growth defect, and the additional loss of *katE* does not amplify this defect. The need for AhpC is enhanced in nutrient limited conditions compared to growth in rich media, which suggests that certain metabolic pathways used in *B. abortus* 2308 are prone to H_2O_2-mediated damage. Our evidence shows that there are other H_2O_2 detoxifiers capable of working in conjunction with AhpC to remove endogenous H_2O_2. These potential H_2O_2 scavengers are bacterioferritin comigratory protein (Bcp), thiol peroxidase (Tpx), and rubrerythrin (Rbr). Mutational studies are necessary to learn if these antioxidants are effective scavengers of H_2O_2 in *B. abortus* 2308 and if so, how they work in combination with AhpC to protect the brucellae from H_2O_2-mediated damage.

KatE is an effective detoxifier of all levels of exogenous H_2O_2 examined in *B. abortus* 2308 in this study. This is expected based on catalase's high Km for H_2O_2. One source of exogenous H_2O_2 that the brucellae must cope with is H_2O_2 produced from the macrophage's oxidative burst. However, we have no evidence that KatE or AhpC is required for protection against this source of H_2O_2. Instead, the roles of KatE and AhpC in the host appear to be detoxifying H_2O_2 produced from respiratory metabolism in *B. abortus*. It is interesting that the *B. abortus ahpCD katE* double mutant cannot survive in mice but retains viability when growing aerobically in the laboratory. One explanation for this is that higher levels of H_2O_2 are produced in the host than during routine growth in the laboratory. It is possible that there are other sources of exogenous H_2O_2 other than the respiratory burst of macrophages that are creating a high concentration of H_2O_2. AhpC alone or KatE alone can detoxify these levels of H_2O_2, but the combination of Bcp, Tpx, and/or Rbr cannot.

We have provided evidence that *B. abortus* 2308 uses a H_2O_2-responsive regulator to modulate *ahpC* expression according to the bacterial cell's needs. This is beneficial to *Brucella*, because this method conserves NADH and NADPH reducing equivalents that are required to reduce AhpC. If Bcp, Tpx, and/or Rbr work in conjunction with AhpC to remove endogenous H_2O_2, we propose that this H_2O_2-responsive regulator may also regulate *bcp*, *tpx*, and *rbr* expression. It is important to identify this regulator, since it is a major oxidative stress regulator in *B. abortus* 2308. NolR, PerR, and RirA are possible candidates to be this oxidative stress regulator.

Compared to its role in other bacteria, OxyR is atypical in *B. abortus* 2308 because it does not regulate *ahpC*. Moreover, it appears that OxyR-regulated genes do not participate in scavenging endogenous H_2O_2 in this strain. Virulence studies in mice can be performed to learn if OxyR is critical for the survival of this bacterium in the host, and microarray analysis can be used to define the OxyR regulon. This will help us learn if OxyR contributes to the *Brucella* cell physiology by regulating other genes whose proteins are important for *Brucella* survival in the host. Irr regulates *ahpC* expression in response to H_2O_2 levels and/or cellular iron in *B. abortus* 2308. This indicates that *Brucella* strains link iron metabolism to oxidative defense, which is beneficial to the bacterial cell because iron exacerbates H_2O_2 toxicity. BAB2_0530's failure to regulate *ahpC* in a H_2O_2-responsive manner is puzzling, because BAB2_0530 is an OxyR homolog and its gene is divergently transcribed from the *ahpCD* operon. We presently do not know what genes BAB2_0530 regulates in *B. abortus* 2308, but our data suggest that these genes may be linked to organic peroxide and superoxide resistance.

APPENDIX

EAST CAROLINA UNIVERSITY

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

April 21, 2009

R. Martin Roop, Ph.D.
Department of Micro/Immuno
Biotechnology Building
ECU Brody School of Medicine

Dear Dr. Roop:

Your Animal Use Protocol entitled, "*Brucella* Stationary Phase Gene Expression and Virulence," (AUP #K145a) was reviewed by this institution's Animal Care and Use Committee on 4/21/09. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to biohazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure