

Chapter 4

Ochrobactrum anthropi: gain of function model

Chapter 4-1

***Ochrobactrum anthropi*: a soil bacterium for studying *Brucella* virulence genes** Under review Journal of Molecular Microbiology

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Abstract:

To date, little is known about the bacterial factors contributing to the persistence and multiplication of *Brucella*, a facultative intracellular pathogen, within phagocytic cells. The presumable importance of alkyl hydroperoxide reductases encoded by *ahpC* and *ahpD* genes and the contribution to intracellular survival of *Brucella* were studied by their over-expression in the phylogenically related soil bacterium *Ochrobactrum anthropi*. Recombinant *O. anthropi* over-expressing *B. suis ahpC* and *ahpD* genes resisted *in vitro* killing by H₂O₂ and or cumene hydroperoxide and survived longer in the murine macrophage cell line J774 A.1. The control *O. anthropi* was cleared from BALB/c mice in five days while the strains over-expressing *ahpC/D* were recovered from spleens, livers and lungs of infected mice up to eight days post-infection. Gain of function studies in this closely related soil bacterium appears to represent a novel way to assess the effects of putative virulence genes from *Brucella spp.* and may have utility to other species in the alphaproteobacteria order.

Introduction

Brucella spp. are facultative intracellular gram-negative bacteria that are pathogenic for a variety of mammalian species including humans. They belong to the alpha-2 subdivision of the Proteobacteria, along with *Ochrobactrum*, *Rhizobium*, *Rhodobacter*, *Agrobacterium*, *Bartonella*, and *Rickettsia*. *Brucella* cause a chronic infectious disease known as brucellosis, a major zoonosis in many countries (Corbel, 1997).

One feature that distinguishes *Brucella* is that they do not express classic virulence factors. Thus identification of virulence factors and corresponding genes has been elusive and at best putative. Disruption of putative virulence genes and studying the corresponding effect on attenuation in cell lines or mouse models is a widely used method. However, in most cases it is not apparent whether the mutated genes encode virulence factors or merely affect normal metabolic or biological functions. Furthermore, as there is redundancy or backup mechanisms within some species, such as *Brucella*, it can be challenging to accurately define whether the function of a particular gene contributes to virulence (Gee et al., 2004).

One method for identifying putative virulence genes is to express them in a nonpathogenic host and to determine whether the recombinants have increased virulence or survivability either in cell culture or animal models (Miller and Shinnick, 2001). This technique was first established by using a non pathogenic *Escherichia coli* K-12 strain to identify *inv* gene that enables *Yersinia pseudotuberculosis* to invade HEP-2 cells (Isberg and Falkow, 1985). The ability of *M. tuberculosis* to gain entry into mammalian cells and survive inside the macrophage was revealed by cloning two distinct loci of *M. tuberculosis* into nonpathogenic *E. coli* (Arruda et al., 1993). And again the *M. leprae* and *M. tuberculosis* virulence genes were identified using *E. coli* and *M. smegmatis* respectively as expression hosts by screening for enhanced survival (Mundayoor and Shinnick, 1994; Wei et al., 2000).

We hypothesized that over-expression of putative *Brucella* virulence genes in the non-pathogenic and close phylogenetic relative soil bacterium *O. anthropi* should enhance its survival ability in *in-vivo* models.

The genus *Ochrobactrum* belongs to the family *Brucellaceae* within the alphaproteobacterial order Rhizobiales, and currently comprises five described species, *O. anthropi*, *O. intermedium*, *O. tritici*, *O. grignonense* and *O. gallinifaecis* (Lebuhn et al., 2006) The species are non-fermentative, strictly aerobic, motile, oxidase-positive and indole-negative, Gram-negative rods (Teyssier et al., 2005) *O. anthropi* is one of the closest *Brucella* relatives based on DNA, rRNA, and protein analyses (Cloeckart et al., 1999; Velasco et al., 1997; Velasco et al., 1998; Yanagi and Yamasato, 1993). However, they, unlike *Brucella*, are unable to establish a chronic infection. They are considered opportunistic pathogens, which under certain circumstances, can produce disease in immunocompromised humans (Gill et al., 1997; Saavedra et al., 1999).

Alkyl hydroperoxide reductases encoded by (*ahpC* & *ahpD*) belong to a family of peroxidases that are beginning to receive intense research attention due to their roles in degrading damaging hydrogen peroxides and related hydroperoxides (Sherman et al., 1996). Inactivation of *ahpC* gene in various bacteria results in increased sensitivity to organic peroxide killing and to spontaneous mutagenesis (Mongkolsuk et al., 2000). sequencing *Brucella* genome (DelVecchio et al., 2002; Halling et al., 2005; Paulsen et al., 2002) revealed the presence of *ahpC* and *ahpD* genes. However, very little is known about these *Brucella* peroxidases in terms of their protein structure, catalytic mechanism and contribution to virulence if any, as they have not yet been purified and investigated. There are conflicting preliminary results about the contribution of *ahpC* & *ahpD* to virulence of *Brucella*. *B. suis ahpC* and *ahpD* mutants were not attenuated in BALB/c mice while a *B. abortus ahpC* mutant was attenuated in C57BL/6J mice (Roop and Bandara, unpublished data).

We tested the contribution of *B. suis ahpC* and *ahpD* genes to virulence of *Brucella* by over-expressing them in a *O. anthropi*. We demonstrate an enhanced ability of the recombinant *O. anthropi* to resist killing *in vitro* and *in vivo*.

Materials and Methods

Bacterial strains, plasmids and oligonucleotides.

The bacterial strains and plasmids used in this study are listed in Table 4-1-1 and the oligonucleotides are listed in Table 4-1-2. *Brucella* strains were routinely grown at 37°C in tryptic soy broth (TSB) or on tryptic soy agar (TSA) (Difco). *O. anthropi* was routinely grown at 30°C in TSB or TSA. Chloramphenicol (Cm) was used at a final concentration of 30µg/ml.

Recombinant DNA methods

DNA ligations, restriction endonuclease digestions, and agarose gel electrophoresis were performed according to standard techniques (Sambrook J, 1989). The polymerase chain reactions (PCR) were performed using Platinum PCR SuperMix High Fidelity (Invitrogen) and a Gradient Mastercycler® (Eppendorf). Oligonucleotides were purchased from Sigma-Genosys (Sigma). Restriction and modification enzymes were purchased from Promega. QIAprep Spin Miniprep Kit from QIAGEN was used for plasmid extractions and QIAGEN PCR cleanup kit was used for restriction enzymes removal and DNA gel extraction.

DNA extraction:

An overnight culture (200ml) of *Brucella* strains (Table 4-1-1) was used for DNA extraction using Qiagen Maxi DNA kit (Qiagen) and according to the manufacturer's procedure. The cells were pretreated with TE/Citrate/Zwittergent 3-14 (10mM Tris, 1mM EDTA, pH adjusted to 4 with Citric acid, 1% Zwittergent 3-14) for 2 h at 55°C prior to purification.

Amplification:

The *ahpC*, *ahpD* and the *ahp* operon were amplified from the extracted *B. suis* DNA using primers ahpC-F and ahpC-R for *ahpC*, ahpD-F and ahpD-R for *ahpD*, and ahpC-F and ahp-RHis for *ahp* operon. The *Bam*HI and *Xba*I restriction sites were designed in the forward and reverse primers for directional cloning.

Differential PCR:

The primers ahpC-F and ahpC-R for *ahpC*, ahpD-F and ahpD-R for *ahpD* were used to detect the presence of *ahpC* and *ahpD* genes in various *Brucella* species (*B. suis* 1330, *B. melitensis* 16M, *B. canis*, *B. abortus* 2303, *B. abortus* strain 19, *B. abortus* strain RB51, *B. melitensis* Egyptian field strain) and *O. anthropi* strain 49237

Expression vector construction:

After restriction digestion and purification the amplified *ahpC* and *ahpD* genes were cloned into pNSGroE a broad host range expression vector (Seleem et al., 2004). The *ahp* operon was cloned downstream of the *Brucella groE* promoter with the N-terminus RNA stem loop in the pNSGroE2His expression vector (Genbank accession number DQ412049). The three expression plasmids (pNSGroE/*ahpC*, pNSGroE/*ahpD* and pNSGroE2His/*ahp*) were transformed into *O. anthropi* by electroporation with a Gene Pulser (BTX) set at 2.4 KV, 25 μ F and 200 Ω . The transformation mixture was diluted and plated on several TSA/Cm plates. Colonies growing on the selective plates after 36h were checked for the presence of the plasmid by PCR.

Western blot:

To test the fusion of the His-tag and efficiency of detection, Western blot analysis was performed by loading the total cell lysates prepared from recombinant *O. anthropi* expressing *ahpC*, *ahpD* and *ahp* operon onto a 12% SDS-PAGE and after separation, the proteins were transferred to nitrocellulose membrane. The membrane was incubated overnight with Anti-His6 antibody (Roche Diagnostics) (1:1000) and Anti-mouse-HRP secondary antibodies (1:1000) (KPL) for 1 hour.

H₂O₂ and cumene hydroperoxide sensitivity:

O. anthropi strains expressing either *ahpC*, *ahpD* or the *ahp* operon were tested for sensitivity to H₂O₂ and cumene hydroperoxide by antimicrobial disk diffusion assay and liquid culture method.

Antimicrobial disk diffusion assay: 10 μ l of either 3% H₂O₂ or 4% cumene hydroperoxide were applied to a 3-mm-diameter filter paper disk (Difco) placed on a TSA/Cm streaked with an overnight culture of a specific *O. anthropi* strain. The clear

zone of killing due to diffusion of H₂O₂ or cumene hydroperoxide was measured after 24 h of incubation.

Liquid culture: 100 µl of early log phase (OD₆₀₀ of 0.2) of an *O. anthropi* strain was added to 10 ml TSB containing either H₂O₂ or cumene hydroperoxide (0.5mM, 0.25, 0.125mM or 0.075mM). Cultures were incubated for 12 hours and the optical densities (600 nm) of the cells were measured every 4 hours.

Effect of cell densities on resistance to H₂O₂:

O. anthropi strains were grown on TSB and the cell density were adjusted to approximately ~10¹⁰ CFU/ml (high density) or ~10⁵ CFU/ml (low density) (Hornback and Roop, 2006). 100 µl portions of the bacterial cell suspensions were incubated with H₂O₂ at final concentrations of 1, 2.5, and 4 mM for 1 h in a 37°C shaking incubator. Bovine catalase (Sigma Chemical Co) at a final concentration of 2,000 U/ml was added to the cell suspensions to detoxify any remaining H₂O₂. The cell suspensions were serially diluted in phosphate buffer saline and plated on TSA and the CFU were determined.

Cell culture and infection of macrophages:

The murine macrophage-like cell line J774 A.1 was seeded ~1 x 10⁵ cells per well in 24-well plates (Corning Incorporated) 24h prior to infection. The cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Sigma Chemical Co) in a humidified 5% CO₂ atmosphere at 37°C. The J774 A.1 cells were infected with *O. anthropi* strains for 30 minutes at a 1:200 multiplicity of infection. The exact CFU of bacteria used for macrophage infection were determined retrospectively by plating serial dilutions of the bacterial suspensions used for infection on 30 µg/ml Cm TSA plates. The infected macrophages were washed three times with DMEM medium containing 50 µg/ml gentamicin (Sigma Chemical Co) to wash off non-phagocytized bacteria prior to incubation with DMEM medium supplemented with 10% FCS and 50 µg/ml gentamicin. At 3, 8, 18, 24, 30 and 42 hours post infection, the infected cells were washed with PBS and lysed with 1% TritonX 100 (Sigma Chemical Co) and plated after serial dilution on TSA/Cm plates.

Animal Experiment:

Survival of recombinants *O. anthropi* expressing either *ahpC*, *ahpD* or the *ahp* operon were done in six-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA) in two different experiments.

In the first experiment four groups of 8 mice each were inoculated i.p. with either approximately 5×10^8 CFU *O. anthropi*, 4.8×10^8 CFU *O. anthropi/ahpC*, 6.8×10^8 CFU of *O. anthropi/ahpD* or 3.8×10^8 CFU of *O. anthropi/ahp*. At 5 and 8 days post-inoculation, four mice from each group were euthanatized by CO₂ asphyxiation. Their spleens were collected, and bacterial CFU per individual spleen were determined by plating serial dilutions of the spleen homogenates on TSA plates as well as on TSA/Cm plates.

In the second experiment four groups of 10 mice each were inoculated i.p. with the same constructs and dose used for the first experiment. At 5 and 8 days post-inoculation, five mice from each group were euthanatized by CO₂ asphyxiation. Their spleens, livers and lungs were collected, and CFU per individual organ were determined by plating the serial dilutions of the organ homogenates on TSA plates as well as on TSA/Cm plates.

Statistical analysis:

All statistical analyses were performed using the Student two-tailed t test using Microsoft Excel. P values ≤ 0.05 were considered significant.

Results:

The *ahpC*, *ahpD* genes and the *ahp* operon were cloned individually into the broad host range expression vectors pNSGroE and pNSGroE2His. The three expression plasmids (pNSGroE/*ahpC*, pNSGroE/*ahpD* and pNSGroE/2His/*ahp*) were transformed into *O. anthropi* and the heterologous gene expression detected by reactivity with Anti-His6 antibody (Figure 4-1-2).

O. anthropi expressing either the *ahpC* or the *ahpD* or the *ahp* operon were tested for sensitivity to H₂O₂ and cumene hydroperoxide by antimicrobial disk diffusion assay and liquid culture method. Table 4-1-3 shows the sensitivity to H₂O₂ and cumene hydroperoxide by antimicrobial disk diffusion assay. Figure 4-1-3 shows the sensitivity to H₂O₂ and cumene hydroperoxide in liquid culture method. Non of the strains were able

to grow at 0.25mM or 0.5mM H₂O₂ or cumene hydroperoxide. In contrast, all strains including the control were able to grow at 0.075mM H₂O₂ or cumene hydroperoxide (data not shown). *O. anthropi* over-expressing the *ahp* operon was not able to grow at 0.125mM H₂O₂ or cumene hydroperoxide. As shown in (Figure 4-1-3 B) only *O. anthropi* expressing the *ahpD* was able to grow on a concentration of 0.125mM H₂O₂ and *O. anthropi* expressing *ahpC* and *ahpD* genes were able to grow on a concentration of 0.125mM cumene hydroperoxide (Figure 4-1-3 A).

Effect of cell density

To determine if bacterial cell density influences the resistance of the recombinant *O. anthropi* to H₂O₂, high cell density $\sim 10^{10}$ /ml and low cell density $\sim 10^5$ /ml cultures of *O. anthropi* strains were exposed to 1mM, 2.5mM and 4mM concentration of H₂O₂ for 1 hour. At the higher cell density *O. anthropi* over-expressing *ahpC* and *ahpD* exhibited higher levels of resistance to H₂O₂ than the control *Ochrobactrum*. The difference was statistically significant at 1mM and 2.5mM but not at 4 mM hydrogen peroxide (Figure 4-1-4). There was no statistically significant difference in survival between *Ochrobactrum* expressing *ahpC* or *ahpD* and the control at low cell density.

To determine if the enhanced resistance to H₂O₂ and cumene hydrogen peroxide could be correlated with enhanced survival in macrophages and/or splenic clearance we infected either the macrophage J774 A.1 cell line or BALB/c mice.

Cell line:

Intracellular survival and replication of the *O. anthropi* strains were tested in the murine macrophage-like cell line J774 A.1. Figure 4-1-5 shows the control *Ochrobactrum* strain was cleared by 30 hours post-infection while the strains over-expressing *ahp* operon survived up to 42 hours post-infection. In contrast to the *in vitro* resistance to H₂O₂, the *O. anthropi* over-expressing *ahpC* was able to resist killing by the macrophages more than *O. anthropi* over-expressing *ahpD*. Although *O. anthropi* over-expressing the *ahp* operon survived longer than the control, there was no statistically significant difference except at 2 hours post-infection.

Animal model:

The control *Ochrobactrum* was cleared from the spleens of infected mice by day 5 while the *O. anthropi* expressing *ahpC* or *ahpD* or the *ahp* operon were recovered from the spleens of the infected mice in the first experiment (Figure 4-1-6); and from the spleen, liver and lungs of infected mice in the second experiment up to day 8 post-infection (Figure 4-1-7- 4-1-9). Similar to the results from the macrophage infection, *O. anthropi* expressing *ahpC* showed much better survival than *O. anthropi* expressing *ahpD* in BALB/c mice.

Discussion:

Brucella spp. are not normally found as a free living or commensal organisms (Gorvel and Moreno, 2002). The preferred ecological niche for the brucellae is within the phagosomal compartment of host macrophages. Moreover, the capacity of this pathogen to establish and maintain chronic infections is dependent upon its ability to survive and replicate within phagocytic cells (Roop et al., 2004). Experimental evidence indicates that the production of reactive oxygen intermediates (ROIs) represents one of the primary mechanisms utilized by host macrophages for limiting the intracellular replication of the brucellae (Gee et al., 2005; Jiang et al., 1993). Reactive oxygen intermediates (ROIs) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) are harmful to *Brucella* because they damage macromolecular structures. Any mechanism that allows the brucellae to avoid oxidative killing would help them establish and maintain residence in their intracellular niche (Farr and Kogoma, 1991; Hornback and Roop, 2006). The sequences of *B. melitensis*, *B. suis*, and *B. abortus* genomes (DelVecchio et al., 2002; Halling et al., 2005; Paulsen et al., 2002) revealed the presence of *ahpC* and *ahpD* genes that share no sequence homology to each other, are organized in an operon and appear to be controlled by the same promoter. The differential PCR depicted in Figure 4-1-1 also revealed the presence of *ahpC* and *ahpD* in all tested *Brucella* species and presumably reflecting the importance of those two genes. Although inactivation of *ahpC* gene in various bacteria results in increased sensitivity to organic peroxide killing and to spontaneous mutagenesis (Mongkolsuk et al., 2000), there are conflicting results about the attenuation of *ahpC* & *ahpD* mutants in different mice

strains. A *Brucella ahpC* mutant was not attenuated in BALB/c while it was attenuated in C57BL6J mice (personal communication Roop and Bandara).

We tested the putative virulence role of the *B. suis ahpC* and *ahpD* genes by assessing gain of function in the soil bacterium *O. anthropi*, a nonpathogenic but close phylogenetic relative of *Brucella*. Although the presence of homologous *ahpC* and *ahpD* genes was excluded in *O. anthropi* by differential PCR (Figure 4-1-1) using specific primers (Table 4-1-2), the possibility of the presence of *ahpC* and *ahpD* genes or similar genes in the chromosome of *O. anthropi* can't be excluded.

The *ahpC*, *ahpD* and the *ahp* operon were over-expressed in *O. anthropi* using pNSGroE and pNSGroE2His under strong constitutive transcriptional promoter (*Brucella groE*). The *in vitro* study indicated that over-expression of *B. suis ahpC*, *ahpD* and *ahp* operon in *O. anthropi* protected the recombinant bacteria to killing by H₂O₂ and cumene hydroperoxide. These results were more noticeable using the antimicrobial disk diffusion assay in case of *ahp* operon. There was no protection provided by expression of the *ahp* operon in liquid culture against H₂O₂ or cumene hydroperoxide.

O. anthropi over-expressing *ahpD* gene was able to resist *in vitro* killing more efficiently than *O. anthropi* over-expressing *ahpC* in liquid culture. This difference could be attributed to the bacterial cell density, which is known to have a dramatic effect on the capacity of defense mechanisms to protect from damage mediated by H₂O₂ (Hornback and Roop, 2006). This conclusion was supported by the results of high cell density survival subjected to different concentration of H₂O₂. Although both *O. anthropi* over-expressing *ahpC* and *ahpD* resist killing by H₂O₂ at high cell density, the *ahpC* was able to provide more protection than *ahpD*.

The higher level of protection provided by *ahpD* in *O. anthropi* in liquid culture could be due to its higher level of heterologous expression in *Ochrobactrum*; this was excluded by demonstrating the level of expression in Western blot (Figure 4-1-10). In contrast, *ahpC* seemed to have higher level of heterologous gene expression than *ahpD*, although both genes are being driven by the same promoter. Another possibility is that *ahpD* has higher activity of degrading H₂O₂ than *ahpC*, in contrast to the *ahpD* of *Mycobacterium tuberculosis* (Nunn et al., 2002). Although *ahpD* is expressed at a lower level in *O. anthropi*, it is still more efficient in protecting the cells against H₂O₂ *in vitro*

than *ahpC*. The recombinant *ahpC* and *ahpD* were purified from *O. anthropi* and the activity of both proteins is currently under investigation to address this question.

The macrophage survival experiment revealed the ability of all recombinants *O. anthropi* over-expressing *ahpC*, *ahpD* or the *ahp* operon to survive longer in macrophage cell line J774 A.1 In contrast to the *in vitro* experiment *O. anthropi* over-expressing *ahpC* was more protected against killing by macrophage. Also, the *ahp* operon was less efficient than either *ahpC* or *ahpD* genes alone in protecting *Ochrobactrum in vivo* inside macrophages.

The mouse clearance study revealed the ability of the recombinants to survive longer in mice. Control *O. anthropi* was cleared by the 5th day post-infection while the recombinants survived up to 8 days post-infection. Recombinant *Ochrobactrum* were recovered from the spleen, liver and lungs of infected animal at days 5 and 8 postinfection. *O. anthropi* over-expressing the *ahp* operon was cleared from the lungs by day 8 post-infection and had less colonization in the spleen and liver than other recombinants.

O. anthropi over-expressing *ahpC* were able to resist killing in BALB/c mice and survived longer in organs compared to *O. anthropi* over-expressing *ahpD* (except in lungs) as indicated by the clearance study (Figure 4-1-6 - 4-1-9). Splenomegaly at day 5 post-infection was characteristic in the mice infected with *O. anthropi* over-expressing *ahpC*. This difference could be attributed to the corresponding level of the heterologous gene expression in *O. anthropi* (Figure 4-1-10).

We expected to have stronger protection (both *in vivo* and *in vitro*) of recombinant *O. anthropi* over-expressing the *ahp* operon in comparison to *O. anthropi* over-expressing either *ahpC* or *ahpD* alone. The unexpected lower protection level can be explained by the lower level of gene expression of the *ahp* operon under the control of the *groE* promoter including the downstream RNA stem loop (pNSGroE2His/*ahp*). Although the RNA stem loop downstream of *Brucella groE* promoter (GroE2His) enhanced heterologous gene expression up to 2 fold in *Brucella*, *Salmonella* and *E. coli*, it unexpectedly decreased expression more than 3 folds in *O. anthropi*. Replacing *ahpC* and *ahp* operon by *lacZ* and measuring the *lacZ* activity in *O. anthropi* under the two promoters (*groE* and GroE2His) confirmed this observation (Figure 4-1-11).

The splenic clearance data suggests that over-expression of the *Brucella ahpC* and *ahpD* genes facilitated the survival of *O. anthropi* and can be considered as virulence determinants. The mechanism of action of the enzymes encoded by these two genes is presumably through neutralization of reactive oxygen intermediates encountered inside phagocytes. Although disruption of the *ahpC* or *ahpD* in *B. suis* did not lead to attenuation in BALB/c mice (unpublished data), in this study over-expression of *Brucella ahpC* or *ahpD* extended the *in vivo* survival and enhanced the *in vitro* resistance to killing by H₂O₂ and cumene hydrogen peroxide of *Ochrobactrum*.

The conflicting observations that a *Brucella ahpC* mutant is not attenuated in BALB/c but is attenuated in C57BL/6J mice may be explained by the observation that BALB/c mice experience a cessation of IFN- γ production during infection with *Brucella* strains that is not observed in C57BL/6J mice (Murphy et al., 2001). Considering the link between IFN- γ activation, reactive oxygen intermediate production, and the brucellacidal activity of murine macrophages (Jiang and Baldwin, 1993; Jiang et al., 1993), it is possible that the *ahpC* *Brucella* mutant experiences less exposure to oxygen radical damage in the phagocytes of BALB/c mice than of C57BL/6J mice (Gee et al., 2005). Thus other cellular defenses are likely sufficient to protect the *ahpC* *Brucella* mutant from the oxidative stress encountered during its interaction with host phagocytes in BALB/c mice. A similar observation was seen with *Brucella* Cu-Zn SOD mutants that were attenuated in C57BL/6J mice and corresponding peritoneal macrophages but not in BALB/c mice and J774 A.1 macrophages. This difference was explained by the observation that the macrophages isolated from the peritoneal cavities of the C57BL/6J mice produce a more robust oxidative burst in primary culture than the J774 A.1 and HeLa cell lines (Gee et al., 2005).

The results obtained with *O. anthropi* as a gain of function model suggest that the products of the *Brucella ahpC* and *ahpD* genes participate in the resistance of the bacteria to organic peroxide killing and enhanced survival ability *in vivo* (cell line and mouse model). The capacity of the brucellae to resist the oxidative killing pathways of host macrophages has been proposed to be of critical importance in their ability to establish and maintain chronic infections in their mammalian hosts (Hornback and Roop, 2006; Jiang et al., 1993). *Brucella* as an intracellular pathogen also has additional mechanisms

to cope with ROI including Cu-Zn SOD and catalase (Sha et al., 1994) or redundancy as represented by (ureases encoded by ure-1 and ure-2, Exonuclease III encoded by *xthA1* and *xthA2*) (Hornback and Roop, 2006). Although the importance of *ahpC* and *ahpD* in *Brucella* as virulence determinants is still putative, the data presented here provide clear evidence that they play a role in the resistance to oxidative killing and could participate in the persistence and establishment of chronic infection. *O. anthropi* would appear to have some utility as a gain of function model for studying putative virulence genes of intracellular pathogens in general and *Brucella* in particular. Because *O. anthropi* is normally nonpathogenic and is cleared from cell lines and animal model very rapidly, the effects of any over-expressed putative virulence gene or genes will be very clearly represented by increased survival ability.

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Table 4-1-1. Bacterial strains and plasmids used in this study

Plasmid	Description^a	Source
pNSGroE	Cm ^r , Broad host range expression vector, bearing <i>Brucella groE</i> promoter	(Seleem et al., 2004)
pNSGroE2His	Cm ^r , Broad host range expression vector, bearing <i>Brucella groE</i> promoter and N-terminus RNA stem loop	unpublished data
pBBgroE	Cm ^r , Broad host range expression vector, bearing <i>Brucella groE</i> promoter	(Vemulapalli et al., 2000)
Bacterial Strain	Description	Source
<i>O. anthropi</i> strain 49237	Originally isolated from soil	Lab stock
<i>B. abortus</i> 2308	Smooth, virulent	Lab stock
<i>B. abortus</i> 19	Smooth, attenuated	Lab stock
<i>B. abortus</i> RB51	Rough, derived from 2308 (Schurig et al., 1991)	Lab stock
<i>B. canis</i> RM6/66	Rough	Lab stock
<i>B. melitensis</i> 16M	Smooth, virulent	Lab stock
<i>B. suis</i> strain 1330	Smooth, virulent	Lab stock
<i>B. melitensis</i> field strain	Smooth, virulent	Lab stock
<i>E. coli</i> Mach1	Chemical competent cells, <i>endA1</i> , <i>recA1</i> , <i>tonA</i> , <i>panD</i>	Invitrogen

^a Cm^r, chloramphenicol resistance

Table 4-1-2 Primers used to amplify the promoters

PCR Product	Size in bp	Source of DNA	Primer Name	Primer sequence
ahpC	555	<i>B. suis</i>	ahpC-F ahpC-R	5'-CCC GGATCC ATGCTCGGCATCGGCGACAAGCTT-3' 5'-CCCT TCTAGAT CAGGCTGCCTTGAGCGTCTCACC-3'
ahpD	528	<i>B. suis</i>	ahpD-F ahpD-R	5'-CCC GGATCC ATGTCGATTGACGACCTGAAATC-3' 5'-CCCT TCTAGAT TATTTCGGTGTCGGCAGCTTCAAG-3'
ahp operon	1161	<i>B. suis</i>	ahp-RHis	5'-CCCT TCTAGA AATGATGATGATGATGATGATGTCAGGCTGCCTTGAGCGTCTCACC-3'

Table 4-1-3. Antimicrobial diffusion assay

Construct	3% H ₂ O ₂ Zone of inhibition	4% cumene hydroperoxide Zone of inhibition
<i>ahpD</i>	24 mm	23mm
<i>ahpC</i>	22mm	22mm
<i>ahp</i> operon	24mm	24mm
Control	30mm	28mm

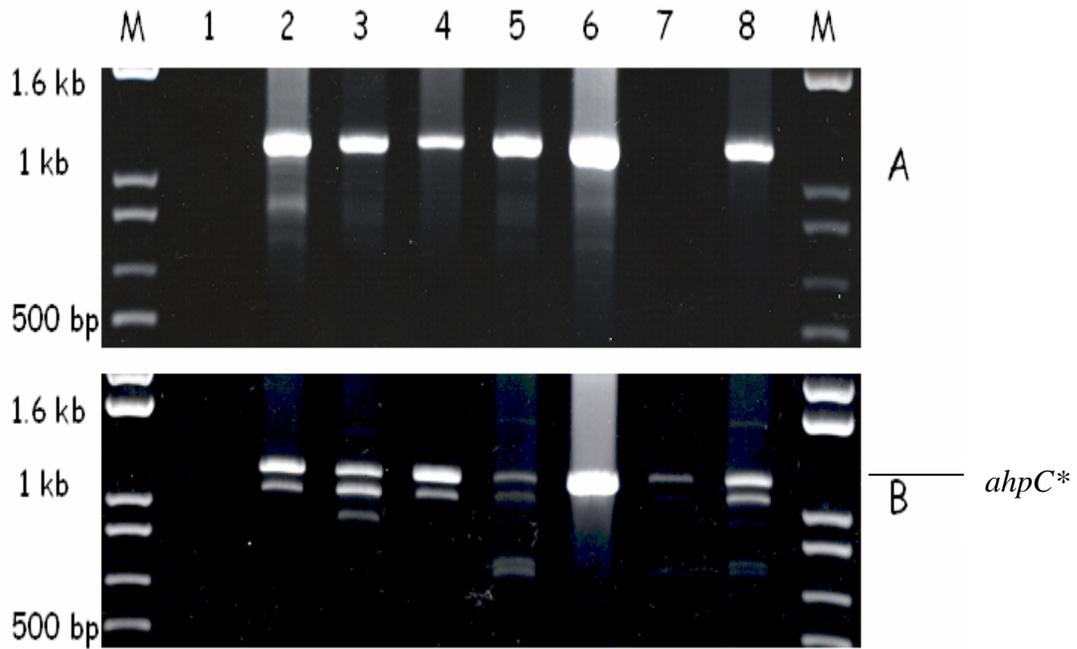


Figure 4-1-1: Differential PCR

Differential PCR of *ahpD* (A) and *ahpC* (B) using DNA extracted from 1; *O. anthropi*, 2; *B. melitensis* Egyptian field strain, 3; *B. abortus* strain RB51, 4; *B. abortus* strain 19, 5; *B. abortus* 2308, 6; *B. canis*, 7; *B. melitensis* 16M (very faint band A), 8; *B. suis* strain 1330. The multiple bands that appear on *ahpC* amplification are due to the wide range in annealing temperature between the forward and reverse primers.

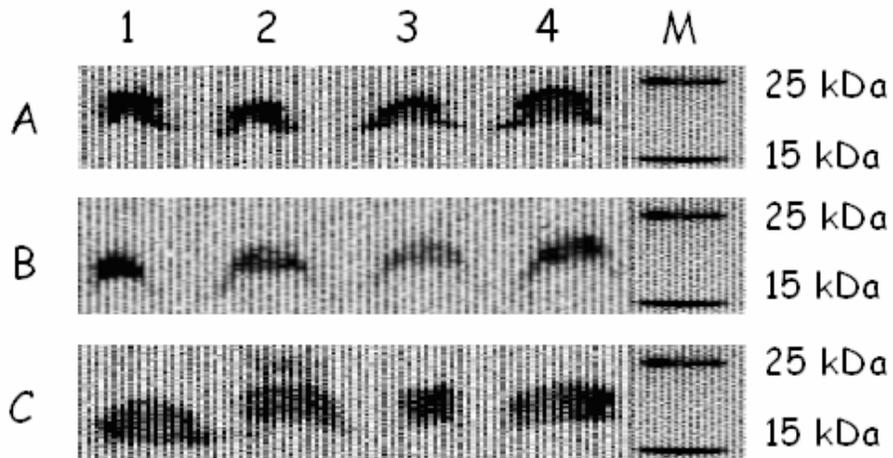


Figure 4-1-2; Western Blot

Western blot of *O. anthropi* expressing *ahpC* (A), *ahpD* (B) and *ahp* operon (C). Membranes were incubated with Anti-His6 antibody (1:1000) overnight and Anti-mouse-HRP secondary antibodies (1:1000) for 1 hour. lanes 1-4 represent different colonies.

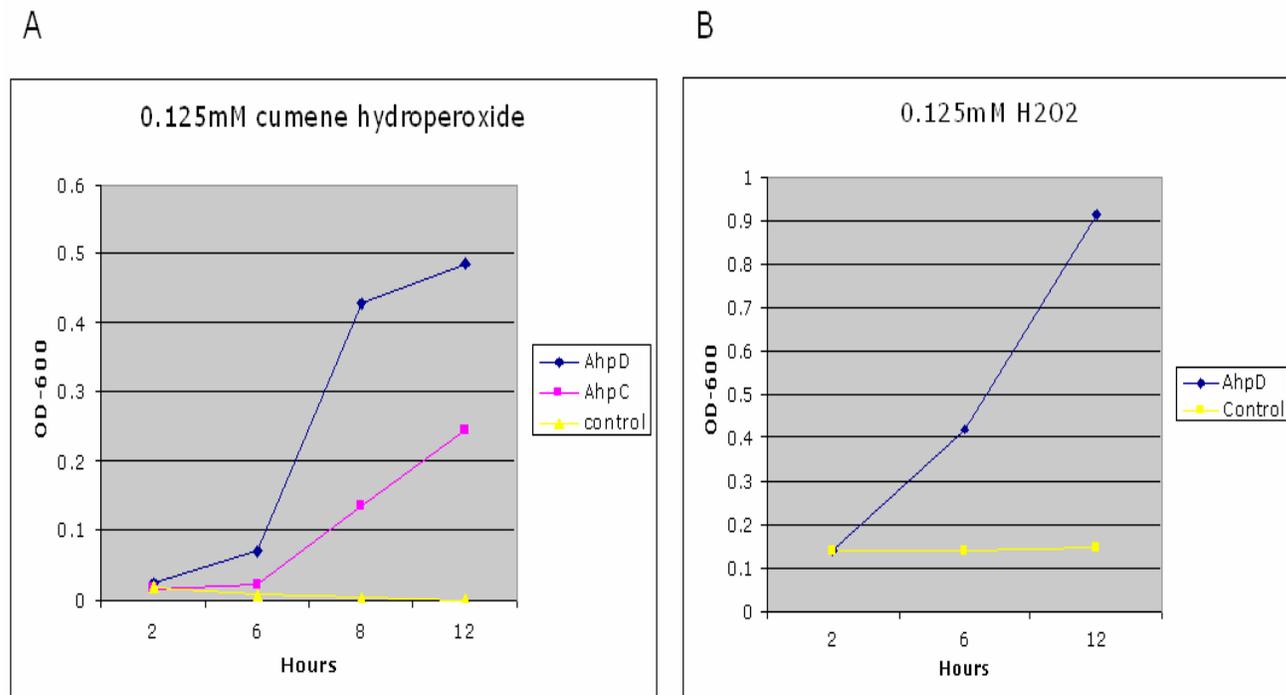


Figure 4-1-3

Ability of the recombinant *O. anthropi* over-expressing *ahpC* and *ahpD* to grow in 0.125 mM cumene hydroperoxide (A) and 0.125 mM H₂O₂ (B).

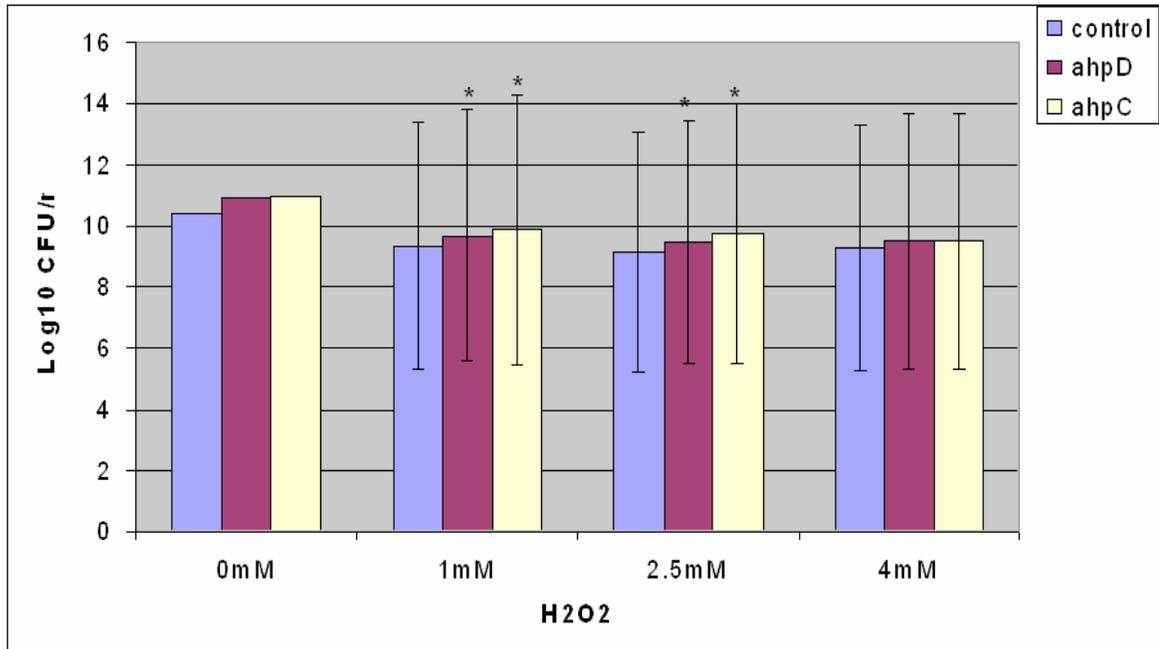


Figure 4-1-4

Ability of the high cell density of recombinant *O. anthropi* over-expressing *ahpC* and *ahpD* to resist killing at 1 mM, 2.5mM and 4 mM H₂O₂. *, $P < 0.05$. Error bars represent the standard error of the mean.

*Although the value of the *t* test was significant, using of the log₁₀ values makes the shape of the error bars contradicting the results. This is an artifact from Excel.

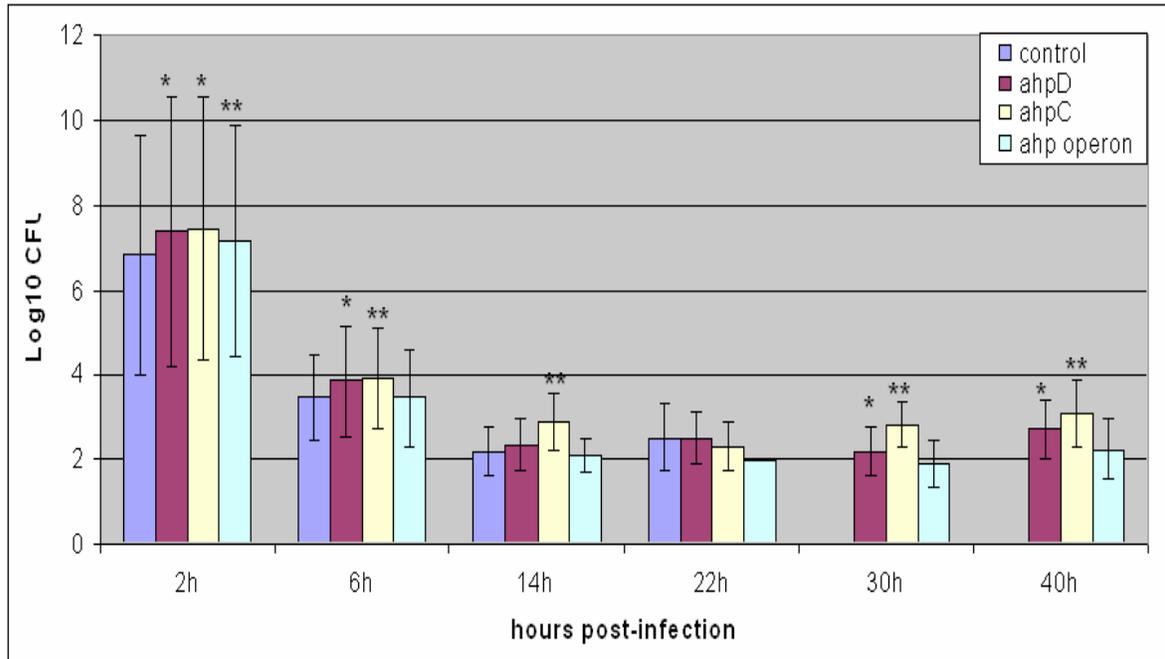


Figure 4-1-5:

Intracellular survival of *O. anthropi* over-expressing *ahpC*, *ahpD* and *ahp* operon. *, $P < 0.05$ **, $P < 0.01$. Error bars represent the standard error of the mean.

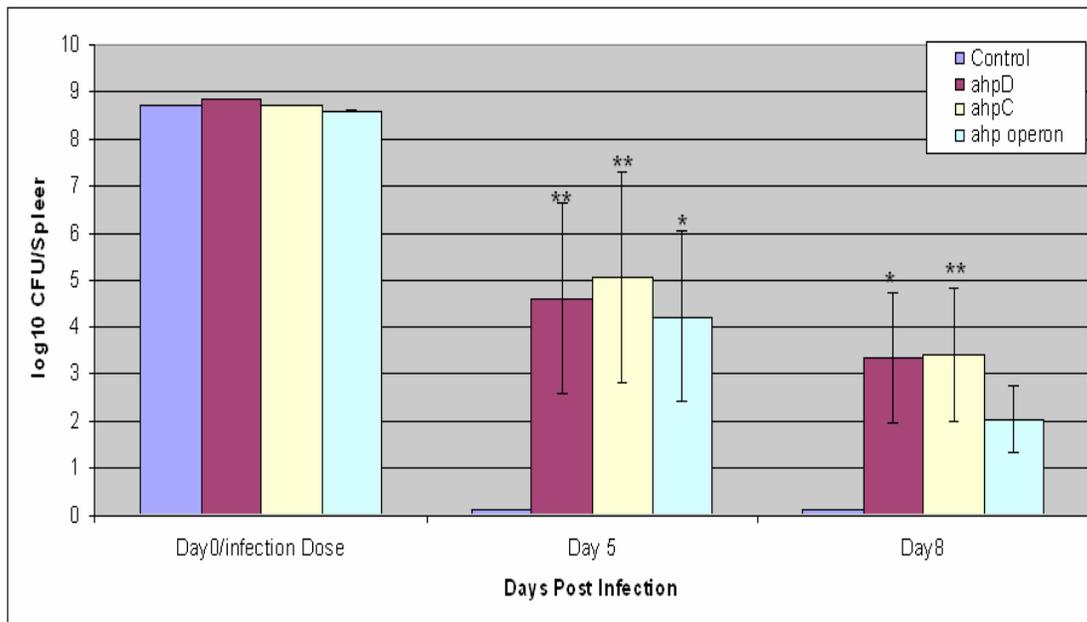


Figure 4-1-6: Spleen colonization.

Spleen colonization levels of recombinant *O. anthropi* over-expressing *ahpC*, *ahpD* and *ahp* operon at 5 and 8 days postinoculation. Columns indicate the average \log_{10} CFU colonization levels of spleen of 4 mice at each time point, and error bars indicate standard errors of the mean. Analysis for mean variation employed Student's *t* test in an unpaired, two-tailed test. *, $P < 0.05$ **, $P < 0.01$.

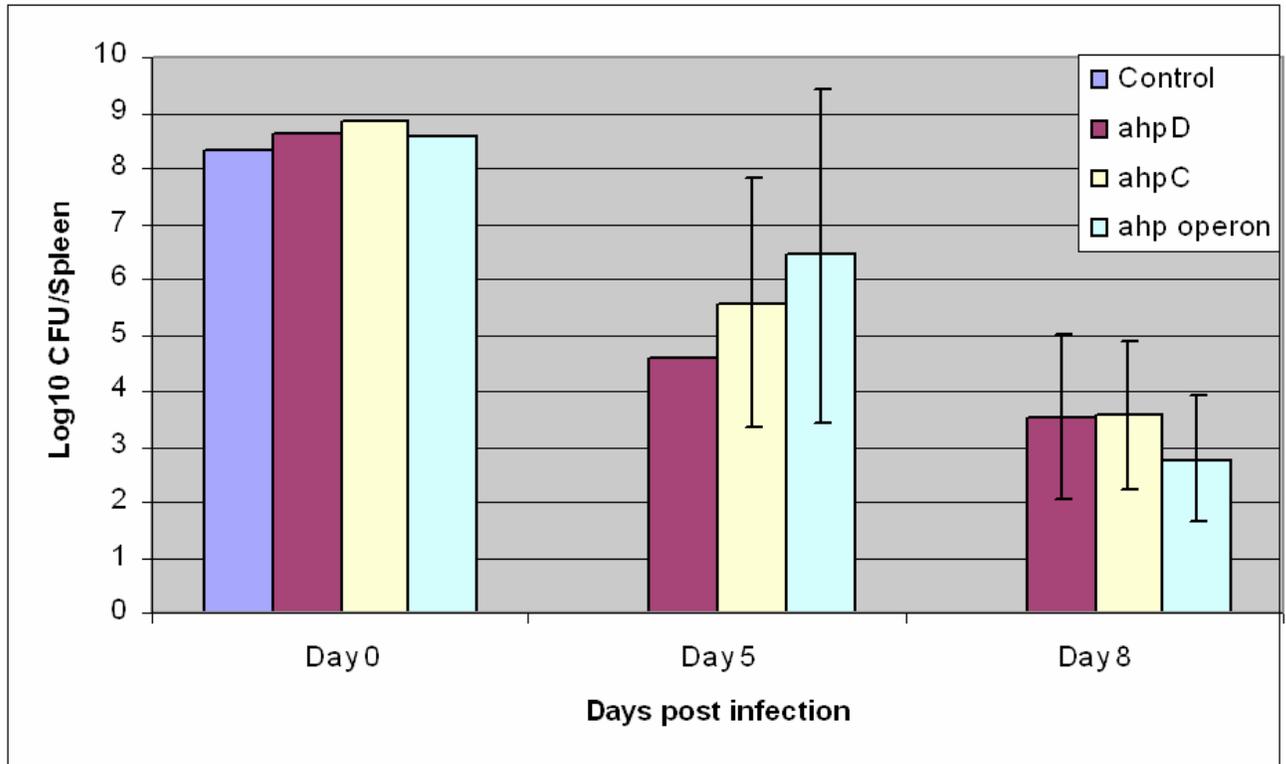


Figure 4-1-7; Spleen colonization:

Spleen colonization levels of recombinant *O. anthropi* over-expressing *ahpC*, *ahpD* and *ahp operon* at 5 and 8 days postinoculation. Columns indicate the average log₁₀ CFU colonization levels of spleen of 5 mice at each time point, and error bars indicate standard errors of the mean. Analysis for mean variation employed Student's *t* test in an unpaired, two-tailed test. *, $P < 0.05$ **, $P < 0.01$.

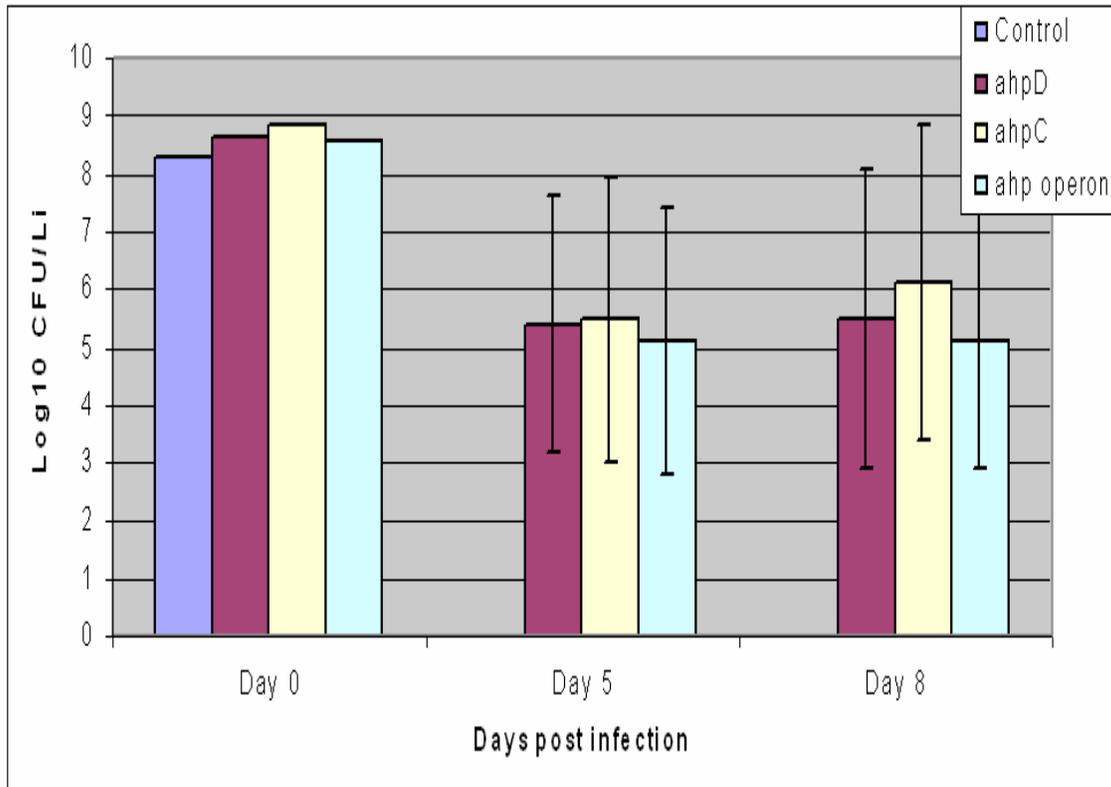


Figure 4-1-8. Liver colonization:

Liver colonization levels of recombinant *O. anthropi* over-expressing *ahpC*, *ahpD* and *ahp operon* at 5 and 8 days postinoculation. Columns indicate the average \log_{10} CFU colonization levels of liver of 5 mice at each time point, and error bars indicate standard errors of the mean. Analysis for mean variation employed Student's *t* test in an unpaired, two-tailed test.

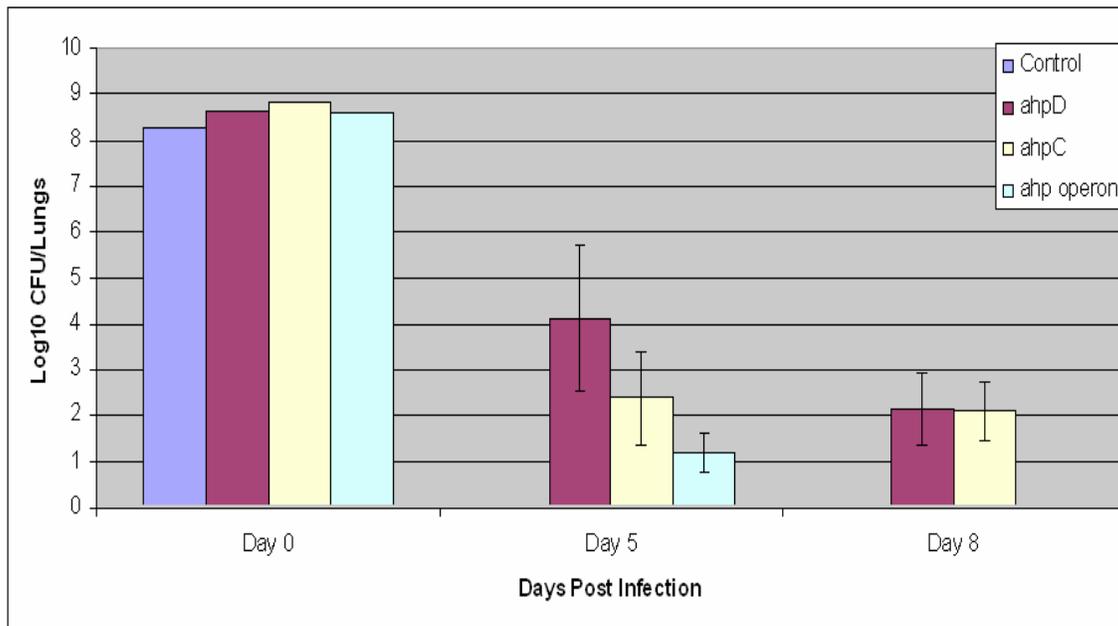


Figure 4-1-9 Lungs colonization:

Lung colonization levels of recombinant *O. anthropi* over-expressing *ahpC*, *ahpD* and *ahp operon* 5 and 8 days postinoculation. Columns indicate the average \log_{10} CFU colonization levels of lungs of 5 mice at each time point, and error bars indicate standard errors of the mean. Analysis for mean variation employed Student's *t* test in an unpaired, two-tailed test.

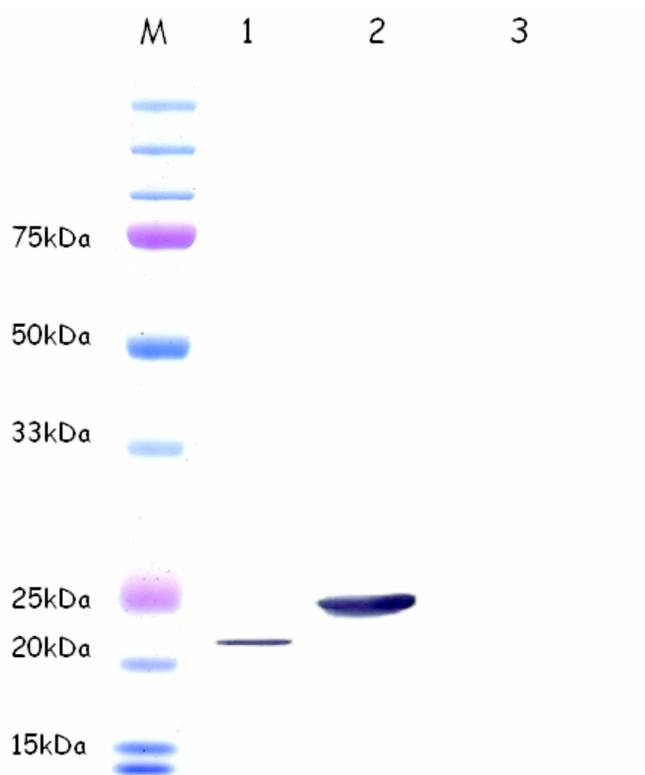


Figure 4-1-10

Western Blot of *O. anthropi* expressing *ahpD* (1); *ahpC* (2) and control (3), same amount of total cell lysates were loaded on the gel and Western blot developed with 1:3000 anti-His₆ antibodies (Invitrogen).

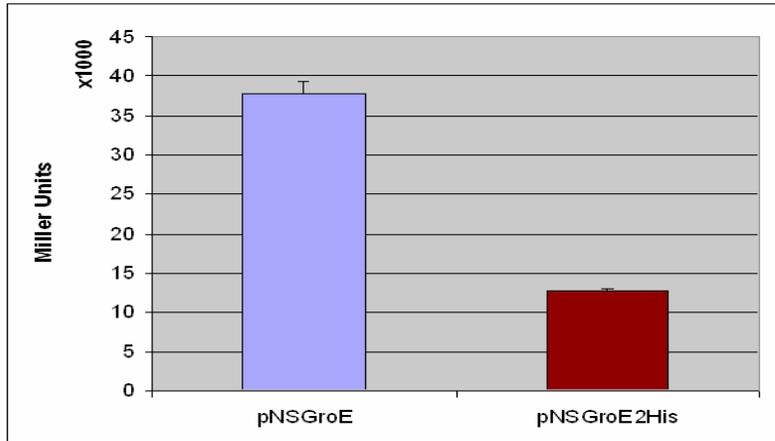


Figure 4-1-11: Promoter strength

LacZ gene was cloned downstream of *groE* promoter in pNSGroE expression vector and GroE2His with the RNA stem loop in pNSGroE2His expression vector. *lacZ* activity of each construct in *O. anthropi* is represented as Miller units. The activity represents the average of triplicate cultures.

Chapter 4

Ochrobactrum anthropi: gain of function model

Chapter 4-2

How many genes does it take to change soil bacterium into a deadly pathogen?

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Introduction

Brucella are Gram negative, facultative intracellular pathogens, and one of the most common causes of abortion in animals; in addition they cause undulant fever in humans (Corbel, 1997; Kottom and Limper, 2000). Five species, *B. abortus*, *B. melitensis*, *B. suis*, *B. canis* and *B. cetaceae* have been recognized as human pathogens each preferentially associated with a different natural host (Verger et al., 1987). These small cocco-bacilli are mainly localized intracellularly within phagocytic cells making treatment difficult, since most antibiotics and antibodies, although highly active *in vitro*, do not pass through cellular membranes (Hall, 1990; Kohler et al., 2003; Roop et al., 2004). Following uptake into macrophages, the brucellae that survive are able to prevent phagosome-lysosome fusion and multiply inside acidified phagosomes. The mechanisms by which *Brucella* survive intracellularly and establish a chronic infection are not completely understood. Recently it was demonstrated that cyclic glucan synthetase (*cgs*) plays a major role in virulence by acting on lipid rafts found on host cell membranes and interferes with cellular trafficking and prevents phagosome-lysosome fusion cycle (Arellano-Reynoso et al., 2005). The vacuoles containing *Brucella* follow a novel

intracellular trafficking pathway, which interacts with the endoplasmic reticulum (ER), leading to the creation of a specialized replicative vacuole in which the pathogen multiplies.

Osmoregulated periplasmic glucans (OPGs) are constituents of the envelopes of Gram negative bacteria and can be divided into 4 families (I-IV) on the basis of their backbone organization. Mutants deficient in OPG synthesis show altered chemotaxis and motility and reduced outer membrane stability and synthesis of exopolysaccharides (Bohin, 2000). Moreover, the mutants are unable to establish successful pathogenic or symbiotic associations with their animal or plant hosts (Arellano-Reynoso et al., 2005). *Brucella* cyclic β -1,2-glucans (C β G) are categorized as family II OPGs and are neither O-substituted nor osmoregulated (Bohin, 2000; Briones et al., 1997). *B. abortus* mutants deficient in C β G or cyclic glucan transporter gene (*cgt*), responsible for the periplasmic location of cyclic glucans, are attenuated in mice and unable to multiply in HeLa cells (Briones et al., 2001). *Brucella* synthesizes the cyclic β -1,2-glucan by a novel mechanism in which the enzyme itself acts as a protein intermediate, using UDP-glucose as a sugar donor. *Cgs* has the three enzymatic activities necessary for synthesis of the cyclic polysaccharide (initiation, elongation, and cyclization)(Ciocchini et al., 2004).

The periplasmic cyclic glucans of *Brucella* spp. consist of a cyclic backbone with a degree of polymerization ranging from 17 to 25, in which all the glucose units are linked by β -1,2 linkages (Bundle et al., 1988). The presence of cyclic glucan in the periplasm is required for full *B. abortus* virulence, as strains with mutated *cgs* or *cgt* exhibited reduced survival in a macrophage cell line and a mouse splenic clearance assay (Briones et al., 2001; Roset et al., 2004).

The genus *Ochrobactrum* belongs to the family *Brucellaceae* within the alphaproteobacterial order Rhizobiales, and currently comprises five described species, *Ochrobactrum anthropi*, *O. intermedium*, *O. tritici*, *O. grignonense* and *O. gallinifaecis* (Lebuhn et al., 2006) They are non-fermentative, strictly aerobic, motile, oxidase-positive and indole-negative, Gram-negative rods (Teyssier et al., 2005) *O. anthropi* is one of the closest *Brucella* relatives based on DNA, rRNA, and protein analyses (Cloekaert et al., 1999; Velasco et al., 1997; Velasco et al., 1998; Yanagi and Yamasato, 1993). In contrast to the *Brucella*, they are unable to establish chronic infection and considered as

opportunistic pathogens in immunocompromised humans (Gill et al., 1997; Saavedra et al., 1999). We hypothesize that over-expression of *Brucella cgs* in the non-pathogenic and close phylogenically relative *O. anthropi* should enhance its survival in infection models *in vivo*.

Materials and Methods

Bacterial strains, plasmids and oligonucleotides.

The bacterial strains and plasmids used in this study are listed in Table 4-2-1 and the oligonucleotides are listed in Table 4-2-2. *O. anthropi* was routinely grown at 30°C in tryptic soy broth (TSB) or on tryptic soy agar (TSA) (Difco). Chloramphenicol (Cm) was used at a final concentration of 30µg/ml.

Recombinant DNA methods

DNA ligations, restriction endonuclease digestions, and agarose gel electrophoresis were performed according to standard techniques (Sambrook J, 1989). The polymerase chain reactions (PCR) were performed using Platinum PCR SuperMix High Fidelity (Invitrogen) and a Gradient Mastercycler® (Eppendorf). Oligonucleotides were purchased from Sigma-Genosys (Sigma). Restriction and modification enzymes were purchased from Promega. QIAprep Spin Miniprep Kit from QIAGEN was used for plasmid extractions and QIAGEN PCR cleanup kit was used for restriction enzymes removal and DNA gel extraction.

Protein sequence analyses:

Homology of the *B. suis cgs* to proteins of the EMBL/GenBank/DDBJ databases was analyzed using the BLAST software (Altschul et al., 1990) at the National Center for Biotechnology Information (Bethesda, MD).

DNA extraction:

An overnight culture (200ml) of *B. suis* 1330 was used to extract DNA using Qiagen Maxi DNA kit (Qiagen) and according to the manufacturer's procedure. The cells were pretreated with TE/Citrate/Zwittergent 3-14 (10mM Tris, 1mM EDTA, pH adjusted to 4 with Citric acid, 1% Zwittergent 3-14) for 2 h at 55 °C prior to purification.

Amplification:

B. suis cyclic glucan synthetase (8199 bp) (NC_004310) was amplified from the extracted *B. suis* DNA using primers *cgsF* and *cgsR* (Table 4-2-2) containing *Bam*HI and *Xba*I restriction sites designed in the forward and reverse primers for directional cloning.

Expression vector construction;

To facilitate the ligation of the 8199 bp *cgs* amplicon into pNSGroE2His (2882 bp) (Genbank accession number DQ412049), we used temperature cycle ligations method (TCL) and tri-ligation reaction (Lund et al., 1996). After restriction digestion and gel purification of *cgs*, a tri-ligated reaction using *groE2His* promoter, *cgs* and pNS vector was performed with temperature TCL method (Lund et al., 1996) using Mastercycler® Gradient programmed indefinitely to cycle between 30s at 10° C and 30s at 30° C for 12 hours.

The ligation reaction was transformed into GeneHog® electrocompetent cells (Invitrogen) and the transformants plated on 30 µg/ml Cm LB plates for selection. Colonies were picked after 16 hours for screening of recombinant plasmid.

O. anthropi and *O. intermedium* plasmid curing:

O. anthropi and *O. intermedium* were tested for antibiotic susceptibility and for the presence of a native plasmid. In attempt of curing the native plasmid in *O. intermedium* we used three different methods:

A-Growing the bacteria on a different concentrations of ethidium bromide (Promega) (2µg, 5µg, 10µg, 20µg, 30µg, 40µg, 50µg, 125µg, 150µg, 175µg, 200µg, 225µg) / ml liquid culture.

B- Growing the bacteria on a different concentrations of acridine orange (Sigma) (25µg, 50µg, 75µg, 100µg and 125µg) /ml liquid culture.

C- Growing the bacteria at a high temperature (38°C- 44°C)

The bacteria were checked after each treatment for the presence of the plasmid and change of antibiotic susceptibility

Transformation of O. anthropi:

The *cgs* expression plasmid was extracted from *E. coli* and transformed into *O. anthropi* by electrotransformation with a Gene Pulser (BTX) set at 2.4 KV, 25 μ F and 200 Ω . The transformation mixture was diluted and plated on several TSA/Cm plates. Cm resistant colonies were checked for the transformation by plasmid extraction and PCR.

Scanning Electron Microscopy:

Recombinant *O. anthropi* over-expressing *cgs* was grown in TSB and minimal media. At early, mid and late log phases one ml of the bacterial culture was centrifuged, and cells washed twice in phosphate buffer saline (PBS) and re-suspended in fixative (5% Glutaraldehyde, 4.4% Formaldehyde, 2.75% Picric acid in 0.05M Sodium Cocodylate Buffer) for transmission electron microscopy. After 24 to 48hr the cells were washed three times 15 minutes each with 0.1 M sodium Cocodylate buffer (Cocodylate buffer). Then the samples were treated for 1 hour with 1% Osmium Oxide in 0.1 M Cocodylate buffer. Then rinsed twice for 10 minutes each with distilled water, and the enbloc (bacterial pellet) stained with filtered 1% Ruthenium red for 1 hr. The enbloc was rinsed twice with distilled water, and suspended in 2% melted agar and spun down for 3 minutes. The sample was treated with 70% ethanol over night in the refrigerator. The samples were minced and dehydrated with 15-minute steps twice each of 70%, 95% and 100% ethanol to remove all of the water. The samples were then treated with Propylene oxide for 15 minutes. The samples were mixed with (50:50) of propylene oxide and PolyBed 812, incubated overnight. Then samples were treated with 100% PolyBed 812 overnight. The samples were embedded in flat molds and cured in 60C dry block before processing for the thin sections and transmission electron microscopy. *E. coli* over-expressing *cgs* as well as *E. coli* and *O. anthropi* containing no plasmid were prepared the same way and used as controls.

Cell culture and infection of macrophages:

The murine macrophage-like cell line J774 A.1 (American Type Culture Collection, Manassas, VA) was seeded at a density of $\sim 1 \times 10^5$ cells per well in 24-well plates (Corning Incorporated) 24h prior to infection. The cells were grown in Dulbecco's

Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Sigma Chemical Co) in a humidified 5% CO₂ atmosphere at 37°C. The J774 A.1 cells were infected with control or transformed *E. coli* and *O. anthropi* for 30 minutes at a 1:200 multiplicity of infection. The infected macrophages were washed three times with DMEM medium containing 50 µg /ml gentamicin (Sigma Chemical Co) to remove non-phagocytized bacteria prior to incubation with DMEM medium supplemented with 10 % FCS and 50 µg /ml gentamicin. At 3, 8, 18, 24, 30 and 42 hours post infection, the infected cells were washed with phosphate buffered saline (PBS) and lysed with 1% TritonX 100 and plated after serial dilution on TSA/Cm plates.

BALB/c Mice Infection:

Survival of *O. anthropi* strain 49237 transformed with pNSGroE2His (control) and *O. anthropi* strain 49237 expressing *cgs* was performed in six-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA). In a pilot experiment two groups of 5 mice each were inoculated i.p. with approximately 6.6 x 10⁸ CFU of *O. anthropi* and 5.6 x 10⁸ CFU of *O. anthropi/cgs*. The exact CFU of bacteria injected were determined retrospectively by plating serial dilutions of the bacterial suspensions used for infection on TSA/Cm plates. At 5 and 9 days postinoculation, two and three mice respectively from each group were euthanatized by CO₂ asphyxiation. Their spleens were collected, and bacterial CFU per individual spleen were determined by plating serial dilutions of the spleen homogenates on TSA plates as well as on TSA/Cm plates.

In the second experiment, three groups of 11 mice each were inoculated i.p. with either 2 x 10⁸ CFU of *O. anthropi*, 8 x 10⁸ CFU of *O. anthropi/cgs* or 6.8 x 10⁸ CFU *O. anthropi/ahpD* (*O. anthropi* expressing alkyl hydroperoxide reductase D (*ahpD*) of *B. suis* as a positive control). At 5 and 8 days post inoculation, five and six mice respectively from each group were euthanatized by CO₂ asphyxiation. Their spleens, livers and lungs were collected, and CFU per individual organ were determined by plating the serial dilutions of the organ homogenates on TSA plates as well as on TSA/Cm plates.

Data analyses:

The mean and the standard deviation values from the clearance and protection studies were calculated using the Microsoft Excel 2001 program (Microsoft Corporation). The Student *t* test was performed in the analysis of CFU data in the macrophage study and the animal clearance.

Results

CGS Protein Homology:

At the amino acid level, the *cgs* gene shared 99% identity with the *cgs* gene in other *Brucella* strains. Additionally, it showed up to 58% identity with *cgs* of *Bartonella* species, and up to 55% identity with the *cgs* of *Mesorhizobium loti*, *Sinorhizobium meliloti*, *Agrobacterium tumefaciens* and *Rhizobium etli* (Table 4-2-3).

Amplification and cgs vector construction:

The 8199 bp *cgs* of *B. suis* (Figure 4-2-1A) was amplified by PCR and cloned into the pNSGroE2His broad host range expression vector using the TLC and tri-ligation method (Figure 4-2-1B).

O. anthropi and O. intermedium:

We were able to detect an ~18kb native plasmid in *O. intermedium*. No plasmids were detected in *O. anthropi*. Table 4-2-4 represents the antibiotic susceptibility results for *O. anthropi* and *O. intermedium*. We failed to cure the native plasmid from *O. intermedium*, the closest *Brucella* relative. The second closest *Brucella* relative *O. anthropi* was chosen for the study as an alternative for *O. intermedium*.

CGS transformation and expression in O. anthropi:

The plasmid pNSGroE2His/*cgs* was transformed into *O. anthropi* and *E. coli* and recombinants were examined macroscopically and microscopically for any change in the shape of the bacteria.

Cell morphology:

There was no change in macroscopic structure of *E. coli* over-expressing *cgs*. Figure 4-2-2 shows the change in macroscopic structure of *O. anthropi* over-expressing *cgs*. Both *E. coli/cgs* and *O. anthropi/cgs* had changes in the microscopic structure as seen by EM. Figures 4-2-3 to 4-2-6 reveal that the strains possess increased thickness of the periplasmic space in all stages of growth with the largest increase at late log phase (Figure 4-2-5B); in addition holes appeared in the membrane during the late log phase of growth (Figure 4-2-5, C to F). The outer membrane of these strains partially dissociated from the rest of the cell envelope (Figure 4-2-4 B and D). Growth on the minimum media was correlated with a change in the overall shape of the cells as well as accumulation of periplasmic materials (Figure 4-2-6).

Persistence in murine macrophages:

Intracellular survival and replication of the recombinant *O. anthropi* and *E. coli* over-expressing *cgs* were tested in the murine macrophage-like cell line J774 A.1. Figure 4-2-7 and 8 show the ability of the strains to survive intracellularly. The control *Ochrobactrum* was cleared by 30 hours postinfection while *O. anthropi* over-expressing *cgs* survived up to 42 hours post-infection. Although the control *E. coli* was not cleared by 42 hours, the *E. coli/cgs* had a significantly higher survival rate than the control.

Survival in mice:

In a pilot experiment, survival of the *O. anthropi* over-expressing *cgs* in BALB/c mice was compared with *O. anthropi*. The *O. anthropi/cgs* persisted in mice for more than 9 days, whereas at day 5 the control *O. anthropi* were 3.96 log₁₀ CFU lower than *O. anthropi/cgs* and was cleared by day 9 (Figure 4-2-9). One mouse died on day 4 postinfection from the *O. anthropi/cgs* group.

In a second experiment, *Ochrobactrum* was cleared from the infected mice by day 5 while the *O. anthropi* over-expressing *ahpD* or *O. anthropi* over-expressing *cgs* were recovered from the spleen, liver and lungs of infected mice (Figure 4-2-10). The recovered CFU in the *O. anthropi/cgs* group were log₁₀ 4.73 (spleen), log₁₀ 4.26 (liver) and log₁₀ 4.14 (lungs) higher than the positive control (*O. anthropi/ahpD*). Eight mice

from the *O. anthropi*/cgs group died over time (two by day 2, two by day 3 and four by day 4 post inoculation). Surviving mice from that group were very sick.

Discussion

One method for studying virulence genes involved in pathogenesis is to express these genes in a nonpathogenic host and isolate recombinants with increased virulence either in cell culture or animal model (Miller and Shinnick, 2001). This technique was first established by using non pathogenic *Escherichia coli* K-12 strain to identify the *inv* gene that enables *Yersinia pseudotuberculosis* to invade HEp-2 cells (Isberg and Falkow, 1985). Using the same technique the ability of *M. tuberculosis* to gain entry into mammalian cells and survive inside the macrophage was revealed by cloning two distinct loci of *M. tuberculosis* into nonpathogenic *E. coli* (Arruda et al., 1993). In addition, *M. leprae* and *M. tuberculosis* virulence genes were identified using *E. coli* and *M. smegmatis* respectively as expression hosts and assessing enhanced survival (Mundayoor and Shinnick, 1994; Wei et al., 2000).

In This research, new vectors and methodologies for the transformation of *Ochrobactrum* were developed that allow for the study of *Brucella* putative virulent genes in their close homologous host. *O. anthropi* was selected for use in these studies because it grows rapidly in the laboratory, readily expresses genes from *Brucella* (He et al., 2002) and can be genetically manipulated by various techniques. (Chapter 2 and 3)

Although *O. intermedium* is more closely related to *Brucella* (Velasco et al., 1998) than *O. anthropi*, the presence of a native plasmid and the resistant to many antibiotics makes it difficult to express heterologous genes. We failed to cure the ~18kb native plasmid using different methods (ethidium bromide, acridine orange and high temperature) which may reflect the importance of the genes on the native plasmid to *O. intermedium*. In contrast, *O. anthropi* strain 49237, the second closest relative to *Brucella* does not contain a native plasmid and thus is more suitable host for heterologous gene expression (He et al., 2002; Velasco et al., 1998)

Comparing the sequence of *Brucella cgs* at the amino acid level with other bacteria revealed that the *Brucella cgs* gene is very unique. Although highly conserved in all sequenced *Brucella* species (99% homology), it doesn't share significant homology with the *cgs* gene of other phylogenic related bacteria (Table 4-2-3). Although the *cgs*

homology was only 55% with related strains, the *B. abortus cgs* complemented a *Rhizobium meliloti* nodule development (*ndvB*) mutant and an *Agrobacterium tumefaciens* chromosomal virulence (*chvB*) mutant (Inon de Iannino et al., 1998).

Over-expression of *B. suis cgs* in *O. anthropi* changed the macroscopic morphology of the colonies. Compared to the control, *cgs* over-expression produced larger colonies on the plate, slightly brownish pigmented and surrounded by a clear zone that looks like a capsule (Figure 4-2-2).

The microscopic appearance of *O. anthropi* and *E. coli* over-expressing *cgs* changed as revealed by the EM (Figures 4-2-3 to 4-2-6). The thickness of the periplasmic space was increased and presumably due to the deposition of the glucans. The β -glucan synthetase gene of the pathogenic fungus *Pneumocystis carinii* (a frequent cause of life-threatening pneumonia in immune-compromised patients) is responsible for the thick-walled cystic form of the organism. The pneumocandin inhibitors of β -glucan synthesis have been shown to rapidly inhibit *P. carinii* growth in rodent models (Kottom and Limper, 2000). This could explain the virulence associated with the increased glucans in the periplasmic space of *E. coli* and *O. anthropi*. The late log phase of growth in recombinant *O. anthropi* was accompanied by occasional holes in the cells (Figure 4-2-6). This is probably due to the high amount of cyclic glucans that are synthesized and accumulated in the periplasmic space. Although there was a change in the microscopic appearance of *E. coli* expressing *cgs*, unlike *O. anthropi* there was no change in the morphology of the colonies. This can be attributed to the strength of *Brucella groE* promoter during *cgs* expression *E. coli*. The *groE* promoter is almost 10 fold stronger in *O. anthropi* than in *E. coli* as measured by expression of *lacZ* (unpublished data).

The macrophage survival experiment revealed the ability of the *O. anthropi* and *E. coli* over-expressing *cgs* to survive longer in macrophage cell line J774 A.1. The control *Ochrobactrum* was cleared by 30 hours postinfection while the *O. anthropi/cgs* survived with \log_{10} 5.71 CFU up to 42 hours postinfection. The enhanced survival intracellularly can be attributed to the synthesis of the cyclic β -glucans. The *B. abortus* cyclic β -glucans was able to prevent phagosomal-lysosomal fusion. The *B. abortus cgs* mutants were defective in intracellular multiplication in HeLa cells (Arellano-Reynoso et al., 2005)

The mouse clearance study supported the same results obtained from the macrophage experiment. Infection of BALB/c mice with 5.6×10^8 CFU of *O. anthropi* over-expressing *cgs* in the first pilot experiment increased the survival of the recombinant up to 9 days post infection while the control was cleared earlier. At day 5 the *O. anthropi/cgs* had (3.96 log₁₀) higher CFU than control *Ochrobactrum*.

When the infection dose was increased in the second experiment to 8×10^8 CFU of *O. anthropi/cgs* and with enough cyclic glucans the *O. anthropi/cgs* were able to escape the killing through phagosomal-lysosomal fusion. The average of the CFUs that was recovered from the three examined organs (Spleen, liver, and lungs) was 8 times higher than the infection dose and indicates that the bacteria were multiplying inside the animal. The injection of 5×10^9 CFU control *Ochrobactrum* killed the BALB/c mice. The mortality was probably due to the high dose of bacterial LPS, as the mortality was in the first 12 hours (unpublished data). The mortality caused by *O. anthropi* over-expressing *cgs* was not during the first 24 hours, which exclude the effect of the LPS of the infection dose. Injecting the same dose (7.8×10^8 CFU) of a different construct (*O. anthropi/ahpC*) although survived longer than the control *Ochrobactrum* in mice, it didn't kill the mice (unpublished data).

In summary, the *B. suis cgs* gene over-expressed in *O. anthropi* dramatically altered the virulence of normally non-pathogenic soil bacterium. Without expressing *cgs*, *O. anthropi* cleared from mice in less than five days, but when *cgs* was expressed in *O. anthropi* the strains survived up to 9 days and at a higher dose it killed the mice. The key trait of virulence in *Brucella* is to avoid phagosomal-lysosomal fusion and reach to the nutrient rich endoplasmic reticulum (ER) (Arellano-Reynoso et al., 2005). The product of the *Brucella cgs* is responsible for that important pathway by interfering with the cellular trafficking. It is likely that over-expression of *cgs* in *O. anthropi* is allowing it to act more like *Brucella* and avoid phagosomal-lysosomal fusion, replicate and eventually kill the host cells.

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Table 4-2-1. Bacterial strains and plasmids used in this study

Plasmid	Description ^a	Source
pNSGroE2His	Cm ^r , Broad host range expression vector, bearing <i>Brucella groE</i> promoter and N-terminus RNA stem loop	unpublished data (Genbank accession number DQ412049)
Bacterial Strain	Description	Source
<i>O. anthropi</i> strain 49237	Originally isolated from soil	Lab stock
<i>O. intermedium</i>	(Velasco et al., 1998)	Lab stock
<i>B. suis</i> strain 1330	Smooth, virulent	Lab stock
<i>E. coli</i> GeneHog®	Electrocompetent cells, <i>endA1</i> , <i>recA1</i> , <i>tonA</i> , <i>panD</i>	Invitrogen

^a Cm^r, chloramphenicol resistance.

Table 4-2-2 Primers used for amplification

PCR Product	Size in bp	Source of DNA	Primers Name	Primer sequence
<i>cgs</i>	8199	<i>B. suis</i>	<i>cgs</i> -F <i>cgs</i> -R	5'-CCC GGATCC ATGCCGCGCATCTTCGCGCTGGCC-3' 5'-CCCT TAGACT ACTGTTTGGGAAGCTCTACCAC-3'

TABLE 4-2-3. Percentages of identity of *B. suis cgs* to protein sequences in GenBank

Bacterial spp	Protein	Identity to <i>B. suis cgs</i> (%)	GenBank accession no.
<i>Brucella melitensis</i> biovar Abortus 2308	Cyclic β -1-2 glucan synthetase	99	NP_697152
<i>Brucella abortus</i> biovar 1 str. 9-941	Cyclic β -1-2 glucan synthetase	99	CAJ10064
<i>Brucella melitensis</i> 16M	Cillobiose-Phosphorylase	99	NP_540754
<i>Bartonella Bacilliformis</i>	Cillobiose-Phosphorylase	58	ZP_00947133
<i>Bartonella quintana</i>	Cyclic β -1-2 glucan synthetase	57	YP_031810
<i>Bartonella henselae</i>	Cyclic β -1-2 glucan synthetase	57	YP_032955
<i>Mesorhizobium loti</i>	Cyclic β -1-2 glucan synthetase	55	NP_108444
<i>Agrobacterium tumefaciens</i>	β -1-2 glucan biosynthesis protein	54	NP_533395
<i>Sinorhizobium meliloti</i>	<i>ndvB</i>	54	P20471
<i>Rhizobium etli</i>	β -1-2 glucan production associated transmembrane protein	54	YP_471468
<i>Agrobacterium vitis</i>	β -1-2 glucan synthetase	53	AAQ08605
<i>Burkholderia xenovorans</i>	Cyclic β -1-2 glucan synthetase	45	YP_556546
<i>Yersinia mollaretii</i>	Cillobiose-Phosphorylase	45	ZP_00826730
<i>Yersinia frederiksenii</i>	Cillobiose-Phosphorylase	44	ZP_00828170

Table 4-2-4 Antibiotic Susceptibility of *O. anthropi* and *O. intermedium*.

Antibiotic	<i>O. intermedium</i>	<i>O. anthropi</i> 49237
Amikacin	S	S
Ampicillin	R	I
Carbenicillin	R	S
Ceftiofur	R	S
Cephalothin	R	S
Chloramphenicol	R	S
Clavamox	R	S
Enrofloxacin	S	S
Gentamicin	S	S
Kanamycin	R	R
Neomycin	I	S
Tetracyclin	S	S
Ticarcillin	R	I

The results were obtained through the standard diffusion test. R; resistant, I; intermediate, S: susceptible.

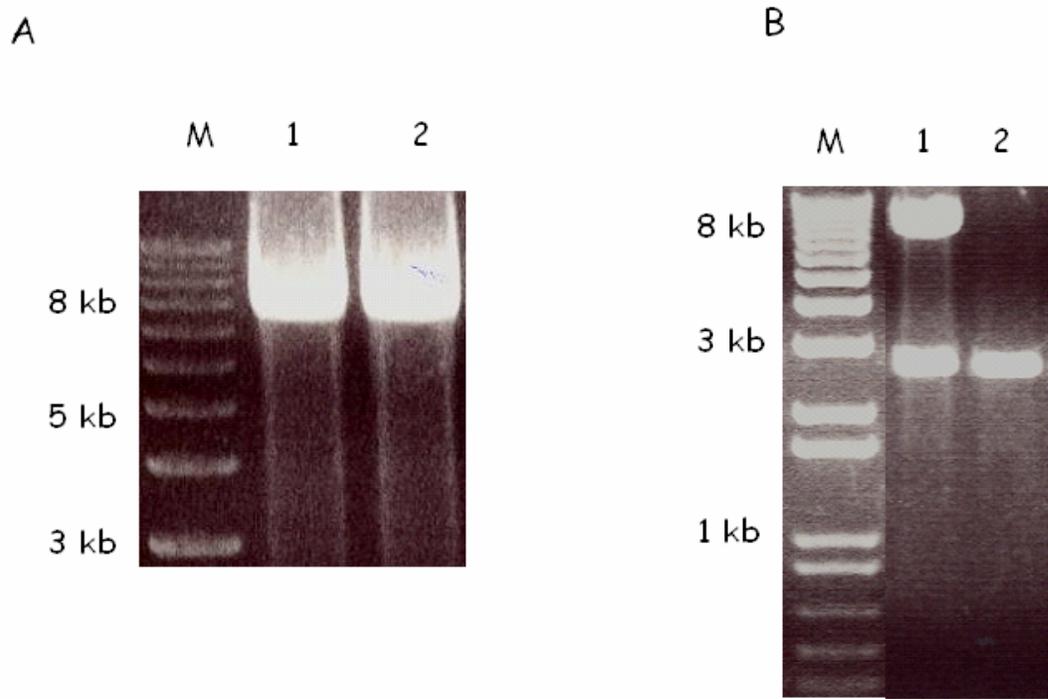


Figure 4-2-1 Amplification and cloning of *cgs*

PCR amplification of *cgs* (8199 bp) (A) and cloning into pNSGroE2His (B) lane1; plasmid plus insert digested with *Xba*I and *Bam*HI, lane2; control plasmid with no insert digested with *Xba*I and *Bam*HI.

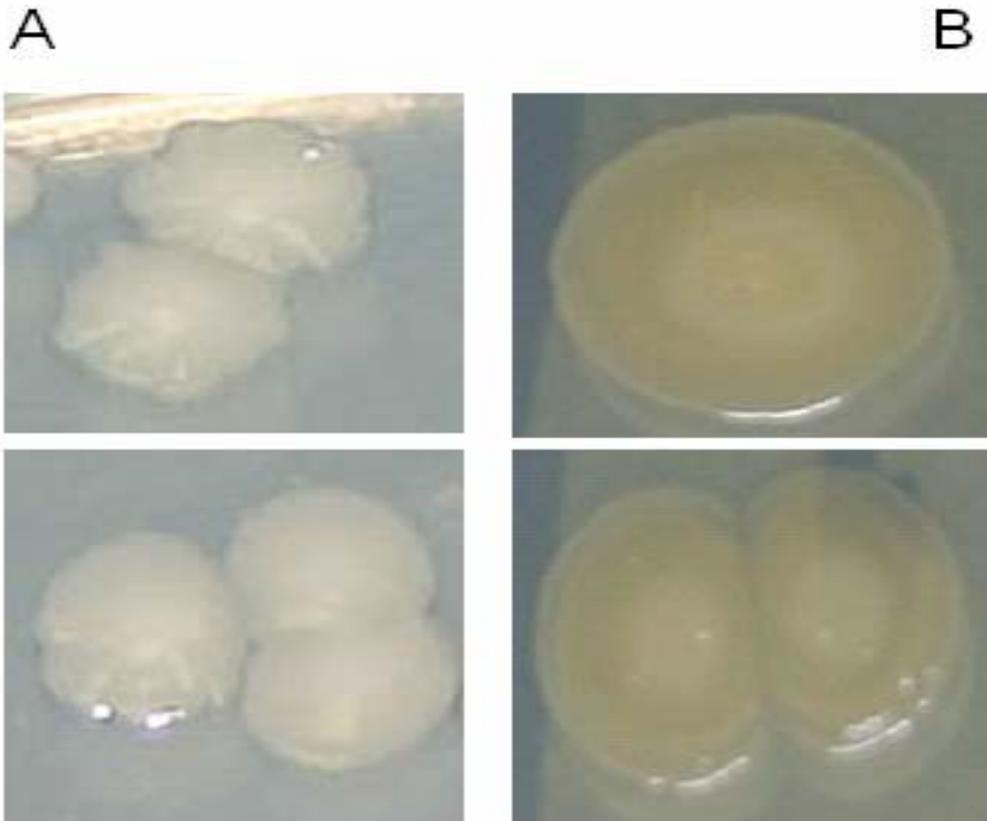


Figure 4-2-2: Macroscopic Morphology

Colonies of *O. anthropi* growing on TSA. A; control *O. anthropi* vector no insert, B; *O. anthropi/cgs*

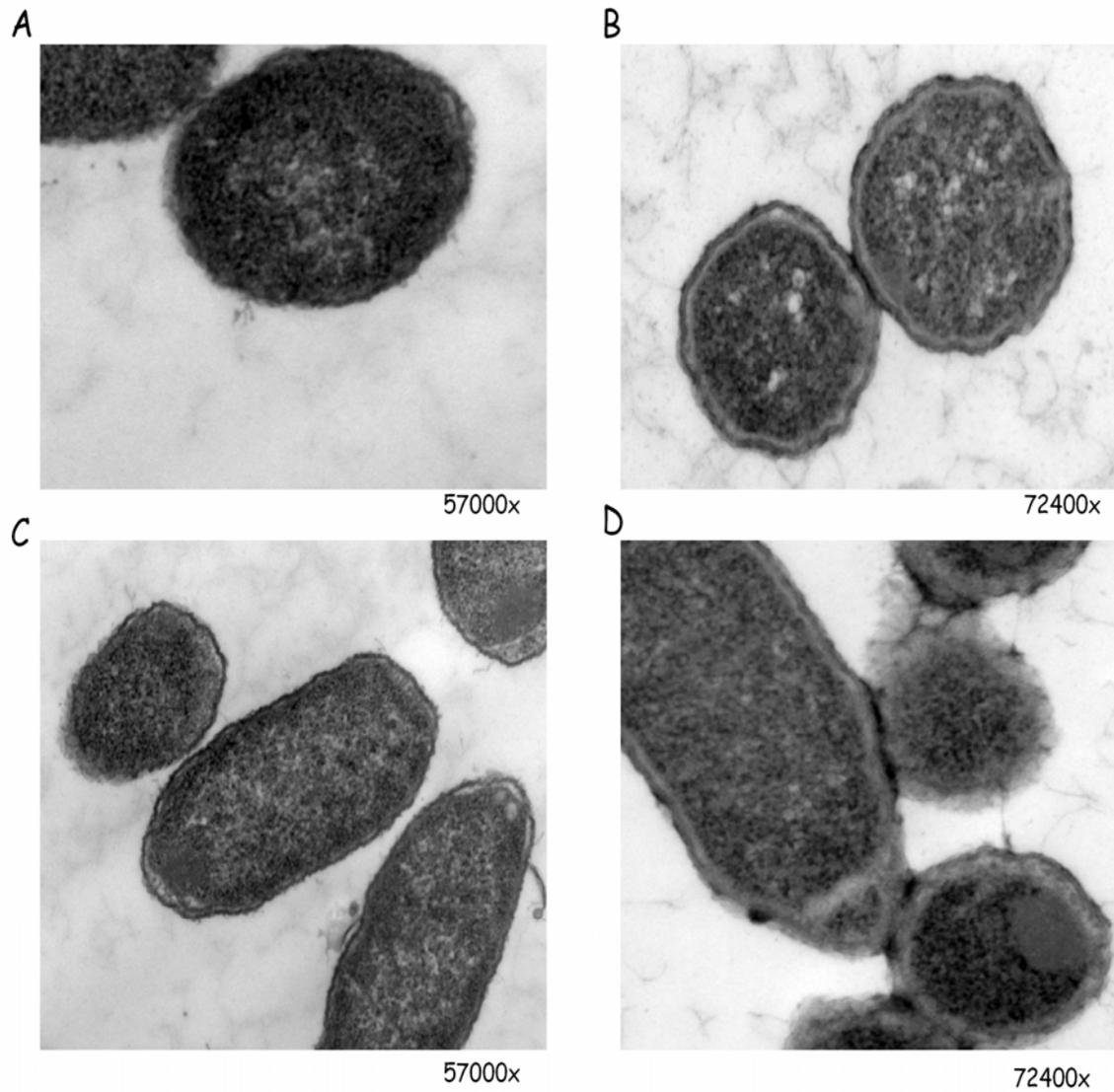


Figure 4-2-3: Cell morphology of *E. coli* as observed by electron microscopy. Control *E. coli* grown in TSB medium (A and C), recombinant *E. coli* expressing *B. suis cgs* (B and D).

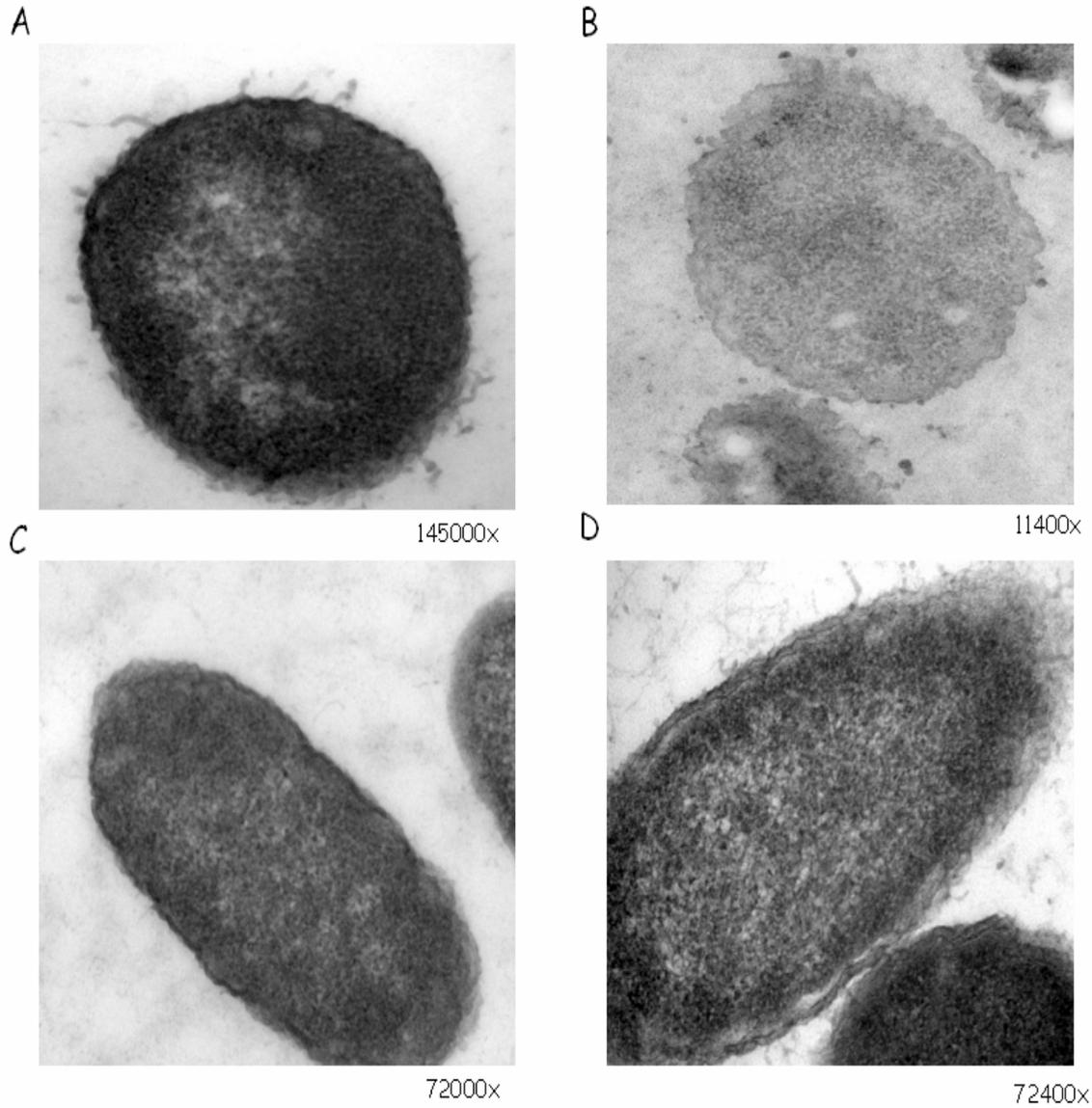


Figure 4-2-4: Cell morphology of *O. anthropi* as observed by electron microscopy. Early phase of growth control *O. anthropi* grown in TSB (A and C), *O. anthropi* expressing *B. suis cgs* (B and D).

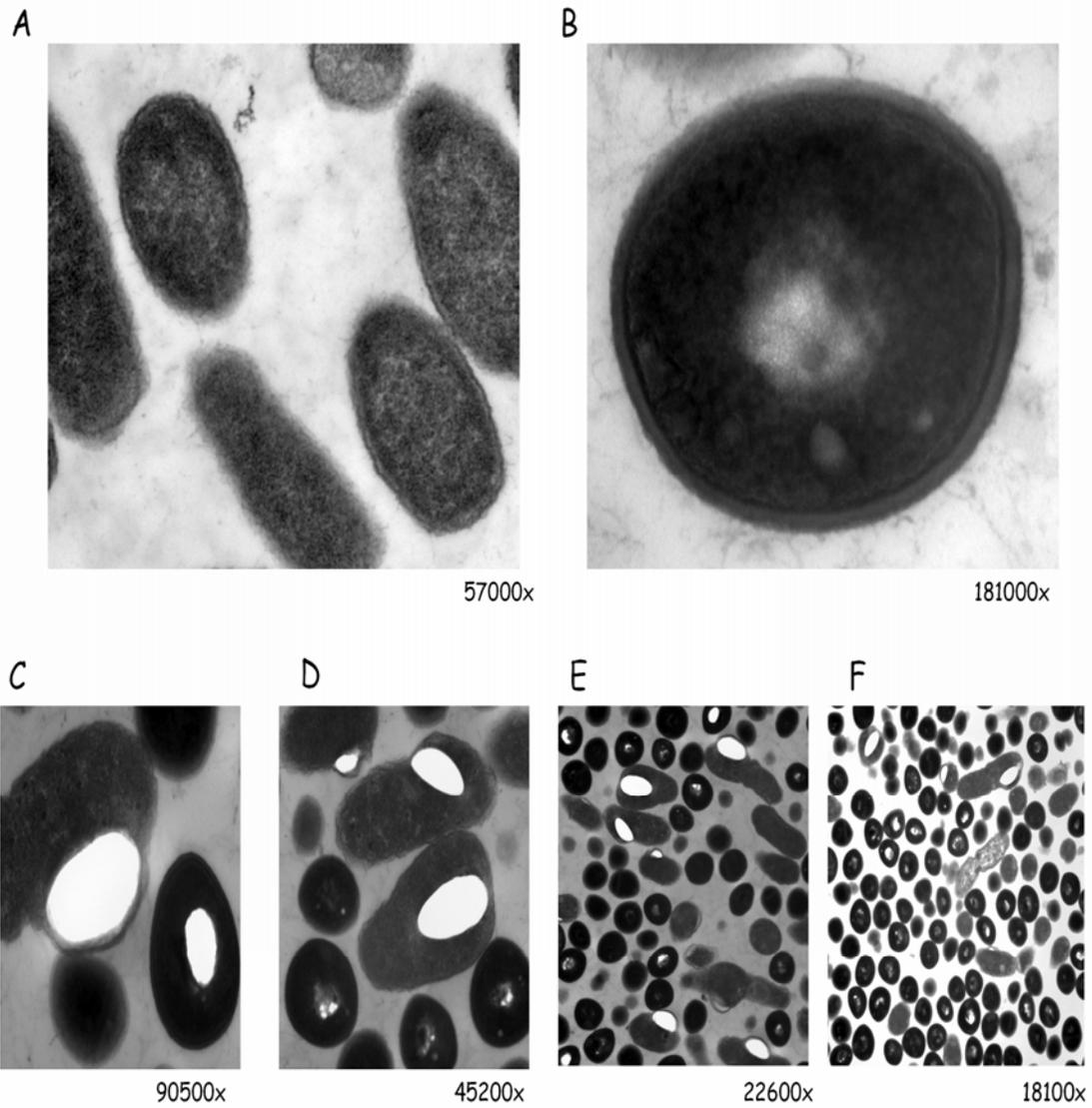


Figure 4-2-5: Cell morphology of *O. anthropi* as observed by electron microscopy. Mid log phase of growth control *O. anthropi* grown in TSB (A) and *O. anthropi* expressing *B. suis cgs* (B). late log phase *O. anthropi* expressing *B. suis cgs* (C, D, E and F)

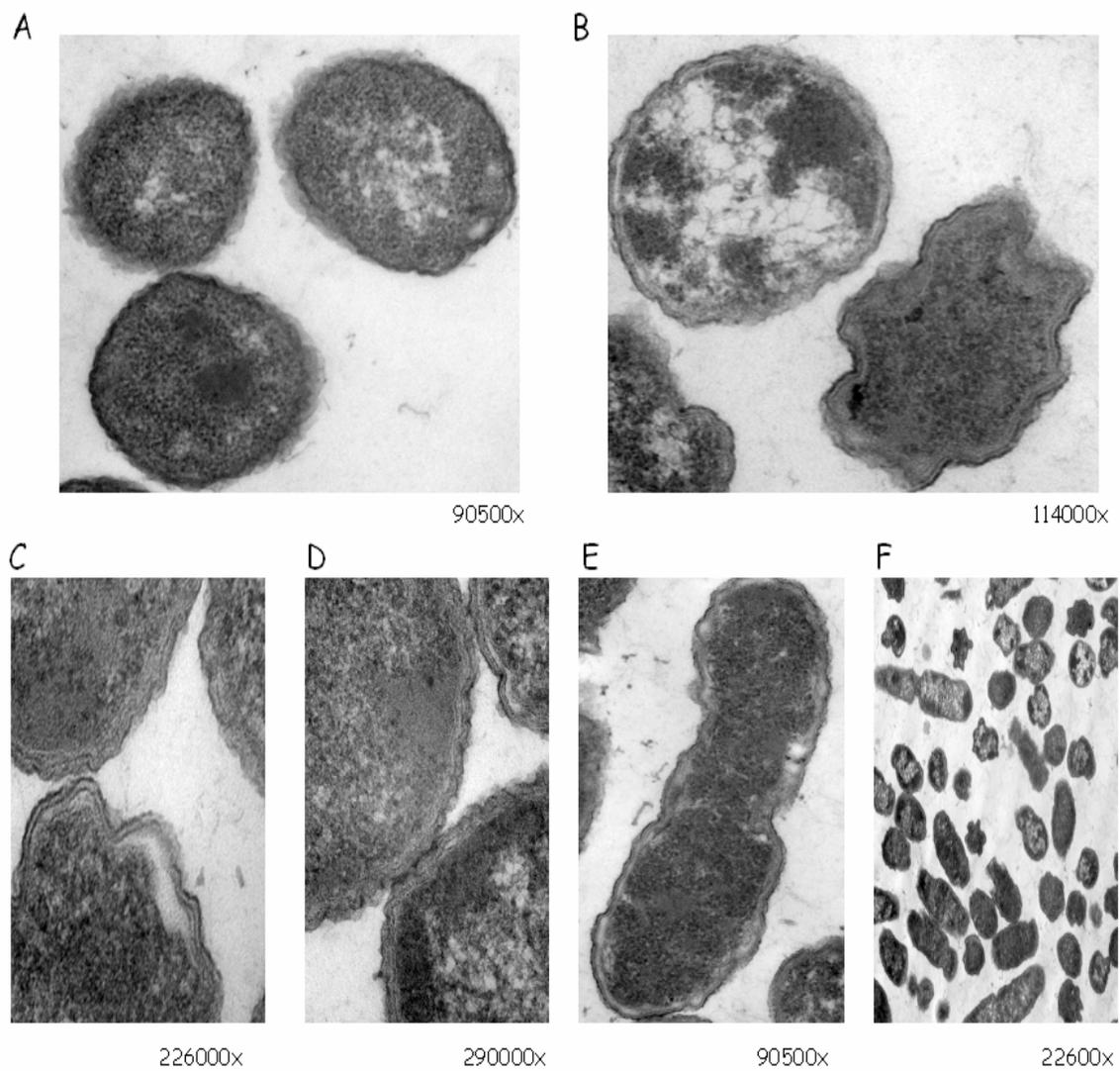


Figure 4-2-6: Cell morphology of *O. anthropi* grown in minimum medium as observed by electron microscopy.

Late log phase of growth control *O. anthropi* (A) and *O. anthropi* expressing *B. suis cgs* (B, C, D, E and F).

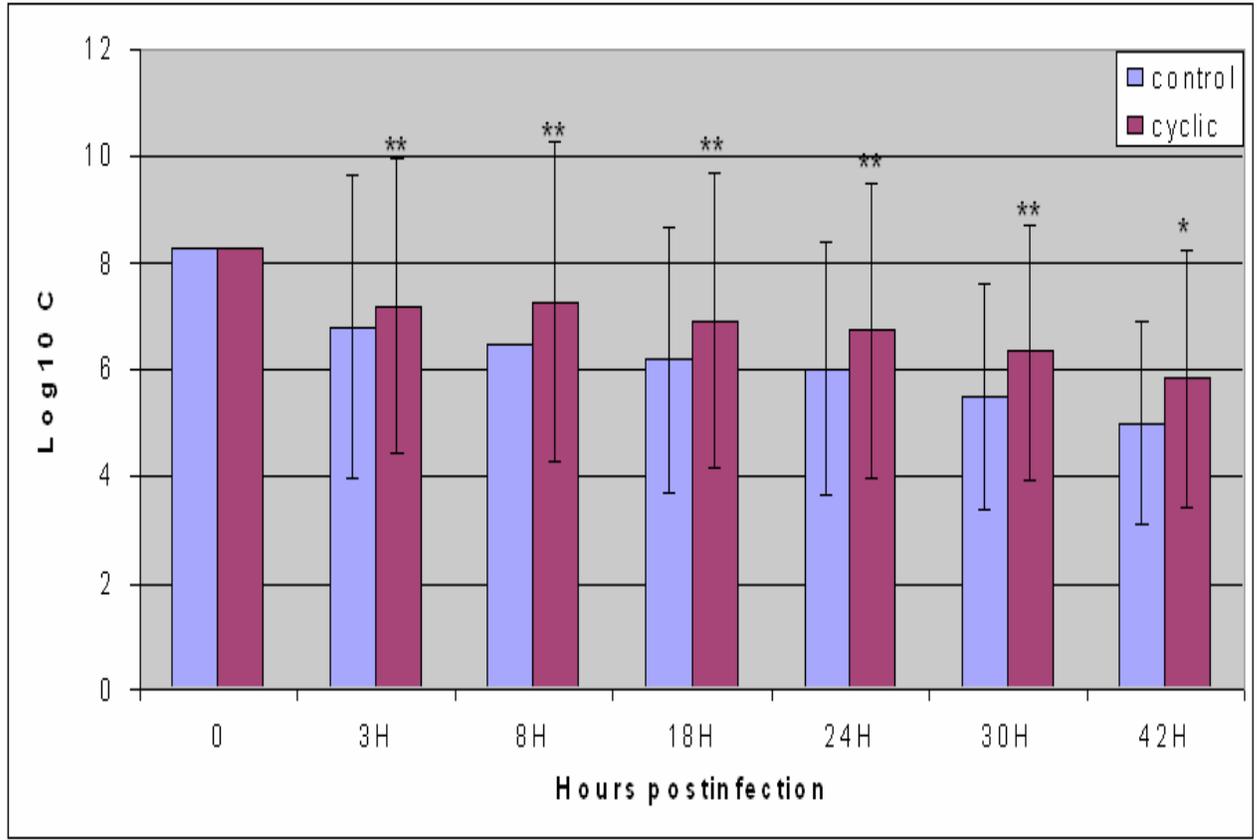


Figure 4-2-7

Survival of *E. coli* control and *E. coli* expressing *cgs* in J774 A.1 macrophages. *, $P < 0.05$ **, $P < 0.01$. Error bars represent the standard error of the mean

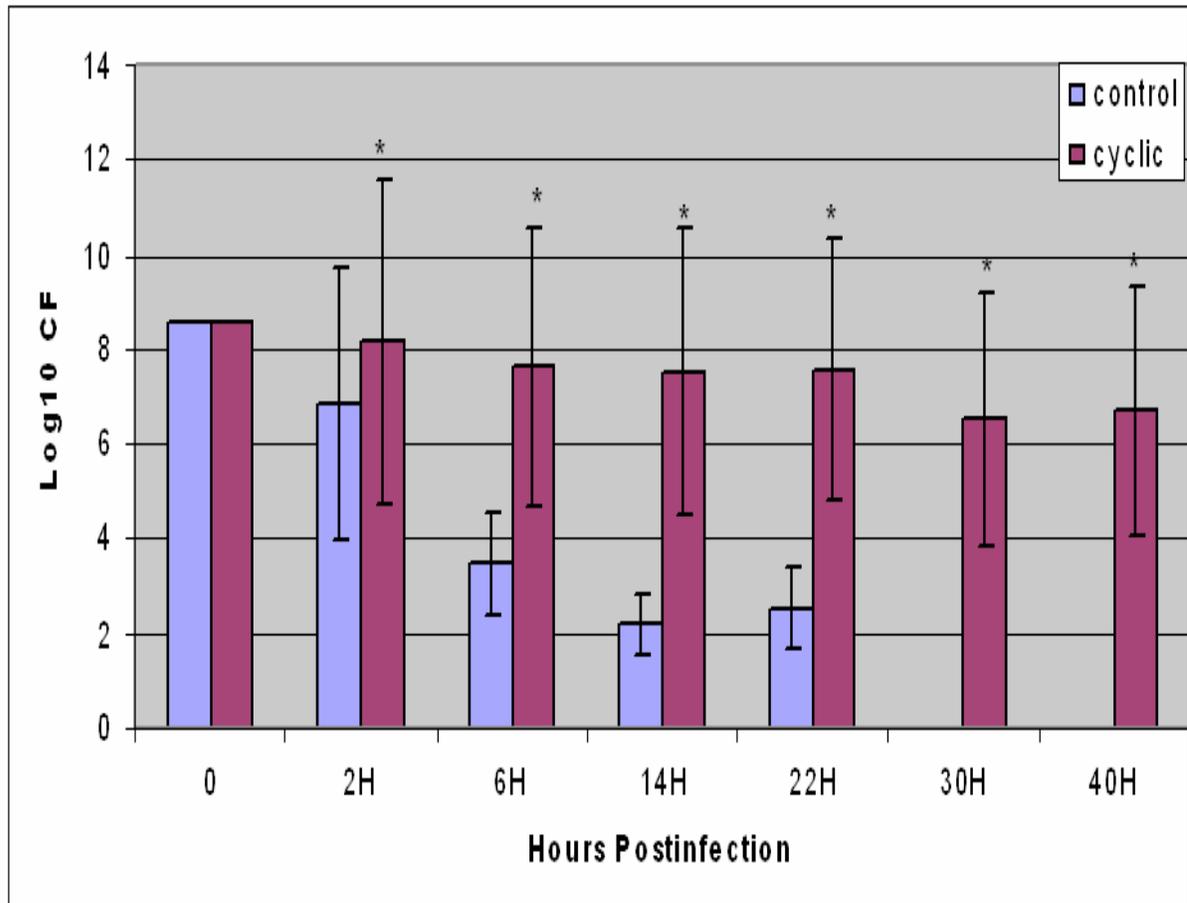


Figure 4-2-8

Survival of *O. anthropi* control and *O. anthropi* expressing *cgs* in J774 A.1 macrophages.

*, $P < 0.05$. Error bars represent the standard error of the mean

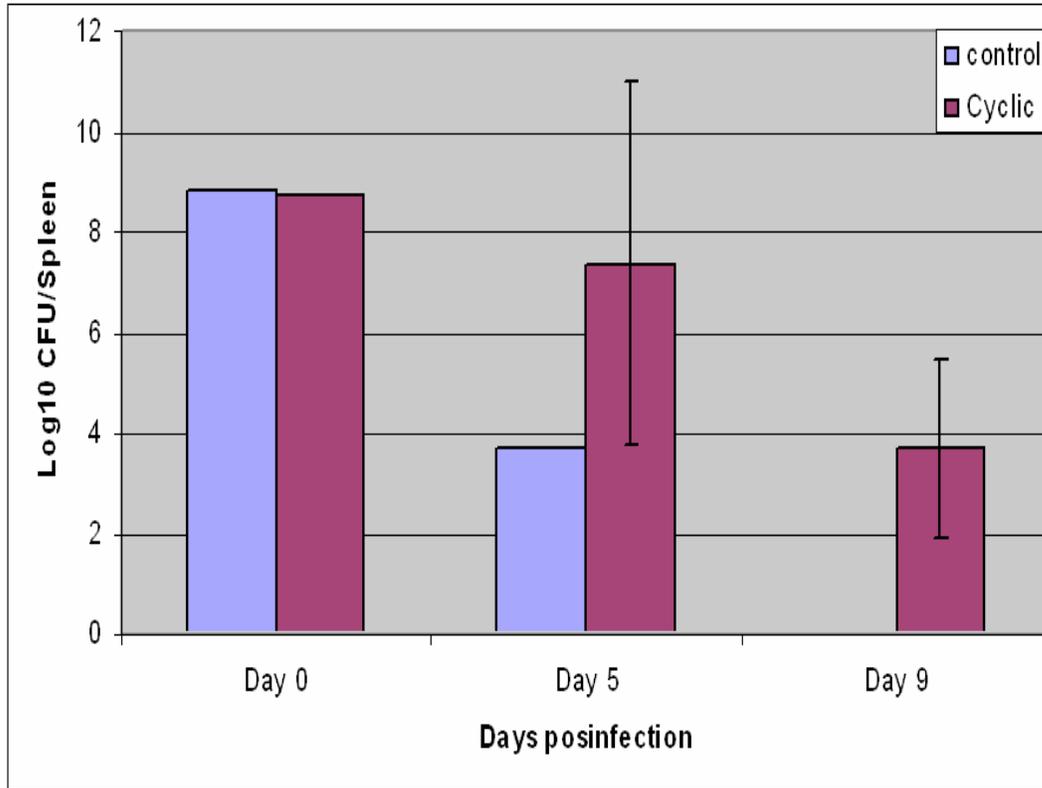


Figure 4-2-9: Spleen colonization by recombinant *O. anthropi* over-expressing *cgs*, and control at 5 and 9 days postinoculation. Columns indicate the average log₁₀ CFU colonization levels of spleen of 2 mice at each time point, and error bars indicate standard errors of the mean.

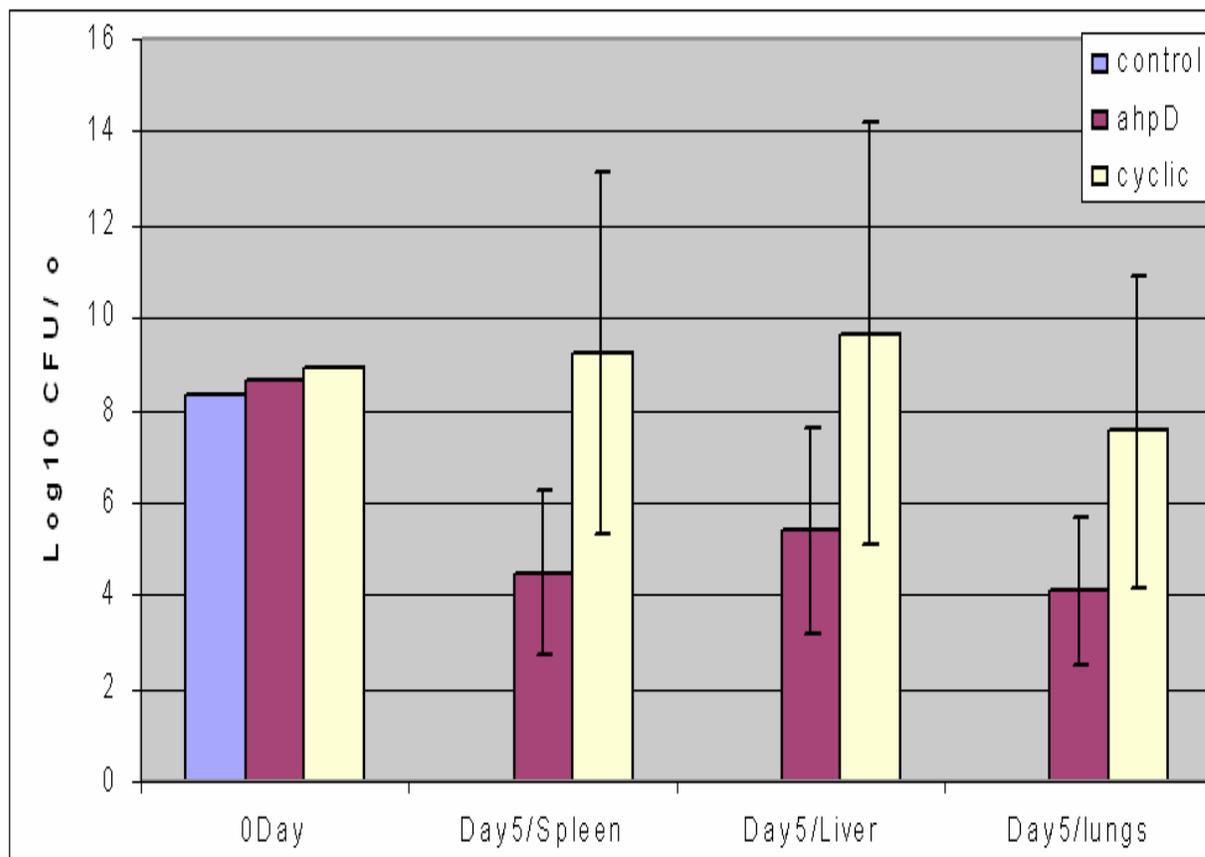


Figure 4-2-10: Spleen, liver and lungs colonization levels of recombinant *O. anthropi* over-expressing *cgs*, *O. anthropi* over-expressing *ahpD* and control at 5 and 8 days postinoculation. Columns indicate the average log₁₀ CFU colonization levels, and error bars indicate standard errors of the mean.

CHAPTER 5

Overall Summary and Discussion

Although species of *Brucella* were isolated and characterized almost 120 years ago and have been sequenced for almost 4 years, compared to other bacterial pathogen relatively little is known about the factors contributing to the persistence in the host and multiplication within phagocytic cells. The research described in this dissertation was aimed at establishment of an alternative method for studying *Brucella* putative virulence genes. The phylogenic relative of *Brucella*; *O. anthropi* strain 49237 was used as a gain of function model to facilitate study of *Brucella* putative virulence genes.

In this study we reported the construction of a series of expression vectors for *Brucella* and *Ochrobactrum* that can be used for heterologous gene expression, protein detection and purification. We characterized the transcriptional activity of several promoters in *O. anthropi* by measuring activity of the *lacZ* gene. We are the first to show a one-step detection and purification of recombinant green fluorescence protein (*gfp*) directly from *Brucella* and *O. anthropi*.

Beside the construction of more efficient expression vectors, we described new tools for enhancement of heterologous gene expression, protein detection and purification. We demonstrated for the first time that the creation of a double-His tag fusion that forms a RNA-stem loop in the mRNA encoding the N-terminus of the target protein was a novel single approach for enhancement of expression, purification and detection of recombinant protein in several microorganisms. We enhanced heterologous gene expression in *O. anthropi* up to 66-fold by placing an adenine (A)-rich upstream element (UP) between -38 and -59 of the core *trc* promoter. The high level of expression achieved by the UP element and the RNA-stem loop fusion tag facilitated detection and purification of heterologous proteins directly from *O. anthropi* and *Brucella*. We reported a rapid and simple method for preparation of highly efficient *O. anthropi* electrocompetent cells. The genetic techniques described herein should be applicable to the study of other significant organisms.

The expression vectors and the tools described in chapters 2 and 3 facilitated the cloning and expression of *Brucella* genes and further studies with protein purification and characterization.

The presumed importance of alkyl hydroperoxide reductases *ahpC* and *ahpD* genes and the contribution to intracellular survival of *Brucella* were studied by over-expressing them in *O. anthropi*. Although the deletion of *ahpC* and *ahpD* genes from *B. suis* did not attenuate *Brucella* in BALB/c mice clearance model, the importance of those two genes was proved by showing that the recombinant *O. anthropi* expressing *ahpC* and *ahpD* genes were able to resist *in vitro* killing by H₂O₂ and or cumene hydroperoxide and survive longer in macrophage J774 A.1 cell line. The control *O. anthropi* was cleared from BALB/c mice in five days while the recombinants were recovered from spleens, livers and lungs of infected mice up to eight days postinfection.

The over-expression of cyclic glucan synthetase of *B. suis* (*cgs*) in *O. anthropi* changed the microscopic and macroscopic appearance of the recombinants. The change in morphology was accompanied by significantly enhanced virulence of the recombinants *in vivo* (cell culture and mouse model).

In summary, novel vectors and methodologies for the genetic manipulation of *Ochrobactrum* have been developed that allow for the study of *Brucella* putative virulent genes in their close homologous host. *O. anthropi* as a gain of function model for studying putative virulence genes of intracellular pathogens in general and *Brucella* in particular proved to be very useful.

CURRICULUM VITAE

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EDUCATION

- **Doctor of Philosophy Candidate (PhD)**- Molecular Bacteriology/Molecular Cell Biology and Biotechnology option. Expected date of graduation (August 2006). Virginia Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University (Virginia Tech) Blacksburg, VA. Title: *Ochrobactrum anthropi*; a soil bacterium for the study of *Brucella* virulence. Advisor: Dr. Nammalwar Sriranganathan. GPA: 3.96
- **Master of Science (MSc)**- Virology/Zoonoses, 2000- College of Veterinary Medicine, Assiut University, Egypt Dissertation: Sero-epidemiological studies on Rift Valley Fever in Upper Egypt.
- **Doctor of Veterinary Medicine (BVSc / DVM)**, 1995 College of Veterinary Medicine, Assiut University, Egypt.

AWARDS & HONORS

- 1- Virginia Bioinformatics Institute (VBI) Scholarship Award April 26th 2006
- 2- Representative of Virginia-Maryland Regional College of Veterinary Medicine and Virginia Tech to the VA Council of Graduate Schools and VA legislators, March 29th 2006.
- 3- Second best presentation and research excellence in 16th Annual Research Symposium June 17th 2004, Virginia-Maryland Regional College of Veterinary Medicine.
- 4- Third best presentation and research excellence 56th Animal Disease Research Workers in the Southern States April 6th 2005, Blacksburg, VA
- 5- Graduate Teaching Assistantship from the College of Veterinary medicine, Virginia Tech, 2003-2006
- 6- Egyptian Ministry of Education Scholarship for Studying abroad 2001-2003 (channel system)
- 7- Employee Recognition Award, Sohag Governorate- Egypt 1998

- 8- Teaching and Research Assistantship from the College of Veterinary medicine, Assiut University, 1995-2001
- 9- Distinction & Third Rank Award, Faculty of Veterinary Medicine- Assiut University 1995.
- 10- Recognition Award, SEED Pharmaceutical company Egypt 1995

PRESENTATIONS/ABSTRACT:

REGIONAL:

1. **M. Seleem**, M. Ali, B. Mukhopadhyay, S. Witonsky, G. Schurig, S. Boyle and N. Sriranganathan. *Ochrobactrum anthropi*: a soil bacterium for studying *Brucella* virulence genes. Virginia-Maryland Regional College of Veterinary Medicine 18th Annual Research Symposium May 23rd 2006. Blacksburg, VA.
2. K. Waite, A. Coq, **M. Seleem**, T. Noble, N. Sriranganathan and S. Boyle. Generation of a FHV-1 Viral Vaccine Against Gonadotropin Releasing Hormone for Immunocontraception of Felines. Virginia-Maryland Regional College of Veterinary Medicine 18th Annual Research Symposium May 23rd 2006. Blacksburg, VA.
3. I. Sandal, **M. Seleem**, S. Elswaifi, N. Sriranganathan and T. Inzana. Efficient expression vectors for *Histophilus somnus*, Virginia-Maryland Regional College of Veterinary Medicine 18th Annual Research Symposium May 23rd 2006. Blacksburg, VA.
4. P. Rajasekaran, **M Seleem**, A. Contreras, N. Sriranganathan and S. Boyle. Use of an auxotrophic mutant of a brucellosis vaccine strain as a vector to express heterologous antigens. Virginia-Maryland Regional College of Veterinary Medicine 18th Annual Research Symposium May 23rd 2006. Blacksburg, VA.
5. **M Seleem**, M. Ali, P. Rajasekaran, S. Boyle and N. Sriranganathan. High-level heterologous gene expression in *Ochrobactrum anthropi* using A-rich UP element. The 22nd Annual Research Symposium, graduate student assembly at Virginia Tech. March 29th, 2006. Blacksburg, VA.
6. P. Rajasekaran, **M Seleem**, A. Contreras, N. Sriranganathan and S. Boyle. Attenuation characteristics of a vaccine candidate against bovine brucellosis. The 22nd Annual Research Symposium, graduate student assembly at Virginia Tech. March 29th, 2006. Blacksburg, VA.
7. **M. Seleem**, M. Ali, M. Abd Alazim, A. Contreras, S. Boyle, N. Sriranganathan. Tools for enhancement if gene expression in prokaryotes. The Second Annual Via Virginia College of Osteopathic Medicine (VCOM) Research Recognition Day Oct. 5 th, 2005. Blacksburg, VA.
8. P. Rajasekaran, A. Contreras, **M. Seleem**, N. Sriranganathan and S. Boyle. Expression of a heterologous antigen in a *Brucella abortus* mutant using a plasmid containing a non-antibiotic resistance gene as a marker. October 5th 2005. The 2nd Annual via Research Recognition Day, Edward via Virginia College of Osteopathic Medicine, Blacksburg, Virginia.
9. **M. Seleem**, M. Abd Alazim, A. Contreras, R. Vemulapalli, S. Boyle, G. Schurig, N. Sriranganathan. Gene Expression in *Brucella*; Past, Present and Future. Virginia-Maryland Regional College of Veterinary Medicine 17th Annual Research Symposium May 16th 2005. Blacksburg, VA.

10. P. Rajasekaran, A. Contreras, **M. Seleem**, N. Sriranganathan and S. Boyle. Complementation of a *Brucella abortus* mutant with a plasmid containing a non-antibiotic resistance gene marker. Virginia-Maryland Regional College of Veterinary Medicine 17th Annual Research Symposium May 16th 2005. Blacksburg, VA.
11. **M. Seleem**, R.Vemulapalli, S. M. Boyle, G. Schurig, N. Sriranganathan. Improved expression vectors for *Brucella* species. Virginia-Maryland Regional College of Veterinary Medicine 16th Annual Research Symposium June 17th 2004. Blacksburg, VA.
12. **M. Seleem**, S.M. Boyle, G.G. Schurig and N. Sriranganathan. Approaches to identify Pathogenicity Island in *Brucella*. Virginia-Maryland Regional College of Veterinary Medicine 15th Annual Research Symposium June 17th 2003. Blacksburg, VA.
13. **M. Seleem**, R.Vemulapalli, S. M. Boyle, G. Schurig, N. Sriranganathan. Improved expression vectors for *Brucella* and other related species Graduate Student Seminars. April 2003 Virginia-Maryland Regional College of Veterinary Medicine.

NATIONAL:

1. **M. Seleem**, M. Ali, S. Boyle, and N. Sriranganathan. How many genes does it take to change soil bacterium into a deadly pathogen? Annual 2006 CRWAD Meeting, December 3- 5, Chicago, Illinois.
2. I. Sandal, **M. Seleem**, S. Elswaifi, N. Sriranganathan and T. Inzana. Efficient expression vectors for *Histophilus somnus*, The Plasmid Biology 2006 conference, September 23-27, 2006, California.
3. K. Waite, T. Noble, **M. Seleem**, N. Sriranganathan, H. Baker and S. Boyle. Production of a tripartite fusion protein containing GnRh as a contraceptive antigen. Third International Symposium on Nonsurgical Contraceptive Methods for Cats and Dogs. November 9-12, 2006 - Alexandria Virginia, USA.
4. **M. Seleem**, R. Vemulapalli, S. Boyle, G. Schurig and N. Sriranganathan. Buy One Vaccine Get Three For Free. First Annual Graduate Student research Forum, March 29th 2006, Richmond, VA,
5. **M. Seleem**, M. Ali, M. Abd Alazim, S. Boyle, G. Schurig and N. Sriranganathan. Enhancement of gene expression. American Society of Microbiology (ASM Virginia Branch) Annual Meeting November 4th & 5th 2005, Norfolk, VA.
6. P. Rajasekaran, A. Contreras, **M. Seleem**, N. Sriranganathan and S. Boyle. Use of a non-conventional plasmid for heterologous gene expression in a *Brucella abortus* mutant inside macrophages. November, 2005. American Society of Microbiology, Virginia branch annual meeting, Norfolk, VA.
7. P. Rajasekaran, A. Contreras, **M. Seleem**, S. Boyle and N. Sriranganathan. Development of a plasmid vector containing a non-antibiotic resistance gene marker for *Brucella abortus*. 56th Animal Disease Research Workers in the Southern States April 6th 2005 Blacksburg, VA.
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9. **M. Seleem**, R. Vemulapalli, S. Boyle, G. Schurig, N. Sriranganathan. Improved expression vectors for *Brucella* species. Annual 2004 CRWAD Meeting November 2004 Chicago, Illinois.
10. A. Bandara, **M. Seleem**, C. Jordan, G. Schurig, D. Lindsay and N. Sriranganathan. *Brucella abortus* strain RB51 expressing antigens of *Toxoplasma gondii* as a potential dual vaccine. American Society of Tropical Medicine and Hygiene November 2004 Miami.
11. **M. Seleem**, R. Vemulapalli, S. M. Boyle, G. Schurig, N. Sriranganathan. Improved expression vectors for *Brucella* species 57th International Brucellosis Research Conference. November 2004 Chicago, Illinois.

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2. Bandara, **M. Seleem**, C. Jordan, D. Lindsay, G. Schurig, and N. Sriranganathan. Vaccination of Mice with *Brucella abortus* Strain RB51 Expressing Antigens of *Toxoplasma gondii*. IWOP-9 proceedings (International Workshop on Opportunistic Protists 2006) 2006. Lisbon, Portugal.
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6. N. Sriranganathan and **M. Seleem**. Accreditation of Veterinary Medical Education. April 26th, 2005, Qena, Egypt.
7. G. Schurig, R. Vemulapalli, A. Bandara, **M. Seleem**, S. Ramamoorthy, Sherry Poff, S. Boyle and N. Sriranganathan. Development of multivalent vaccines utilizing *Brucella abortus* RB51 as a vector. International symposium on Multilateral cooperation in Agriculture Biotechnology exchange in Pan-pacific Area November 8th 2004 Taiwan.

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3. **Seleem, M.N.**, A. Hussein, A. Ismail and E. Nafie (2002): Sero-epidemiological studies on Rift Valley Fever in some patients of fever hospitals in Upper Egypt. *J. Egypt. Vet. Med. Assoc.* 62, No. 6c: 261-271.

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4. **Seleem, M.N.**, M. Ali, S. Boyle, B. Mukhopadhyay, S. Witonsky, G. Schurig and N. Sriranganathan. Establishment of gene expression system in *Ochrobactrum* species. 2006 Oct. 72; No.10 "Applied and Environmental Microbiology".
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6. **Seleem, M.N.**, M. Ali, M. Abd Alazim, S. Boyle and N. Sriranganathan. Enhanced expression, detection and purification of recombinant protein using tandem fusion tags RNA stem loop." In Press 2006 " Journal of Applied Microbiology and Biotechnology.
7. **Seleem, M.N.**, S.M. Boyle and N. Sriranganathan. *Brucella*; a pathogen without classical virulence genes (Review). "Journal of Veterinary Microbiology"

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8. **Seleem, M.N.**, P. Rajasekaran, M. Ali, S.M. Boyle and N. Sriranganathan. Simple Method for Transformation of *Ochrobactrum anthropi*. "Journal of Microbiological Methods"
9. **Seleem, M.N.**, M. Ali, B. Mukhopadhyay, S. Witonsky, G. Schurig, S. Boyle and N. Sriranganathan. *Ochrobactrum anthropi*; a soil bacterium for studying *Brucella* virulence genes. "Molecular Microbiology.
10. **Seleem, M.N.**, M. Ali, S.M. Boyle and N. Sriranganathan. Reporter genes in *Ochrobactrum anthropi*. "Journal of Medical Microbiology"

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11. **Seleem, M.N.**, M. Ali, S. Boyle, and N. Sriranganathan. How many genes does it take to change soil bacterium into a deadly pathogen? In preparation.
12. **Seleem, M.N.**, R. Vemulapalli, S.M. Boyle, and N. Sriranganathan. In vivo expression of *Brucella* promoters. In preparation to be submitted to Journal of Microbiological Methods.
13. **Seleem, M.N.**, A. Contreras, A. Bandara, S. M. Boyle, G. Schurig, N. Sriranganathan. Purification and characterization of *Brucella suis* alkyl hydroperoxidases C&D. In preparation to be submitted to Journal of Bacteriology.
14. **Seleem, M.N.**, K. F. Key, M. Ali, E. Comer, X. J. Meng and N. Sriranganathan. Enhanced expression system for *Salmonella choleraesuis* wild type and vaccine strains. to be submitted to Gene.
15. **Seleem, M.N.**, I. Sandal, S. Elswaifi, T. Inzana and N. Sriranganathan. Efficient expression vectors for *Histophilus somnus*. Journal of Bacteriology.