Chapter 1

Introduction

Microorganisms belonging to the genus *Brucellae* are the etiological agents of brucellosis, an infectious disease that affects many animal species and can be transmitted to man (69). Six species are included in this genus according to their host preference, *B. melitensis* (goat), *B. suis* (swine), *B. abortus* (cattle), *B. canis* (dog), *B. ovis* (ram) and *B. neotomae* (desert mice). The first three species are of the smooth phenotype and pathogenic to humans in decreasing order of severity, making brucellosis a zoonotic disease. It appears that a seventh species exists and was isolated from sea mammals (52). Some *Brucella* species include several biovars. The *Brucella* species and their biovars can be differentiated according to their cultural, metabolic and antigenic properties, sensitivity to phages, pathogenicity characteristics and host preference. (68) Currently many PCR assays based on gene differences have been developed to detect or differentiate various *Brucella* strains (5,8,9,20,34,46,50,56,61, 68).

The *Brucellae* are small, nonmotile, nonsporulating, Gram negative coccobacilli or short rods (0.5 to 0.7um by 0.5 to 1.5um). (35) Growth occurs aerobically and often requires or is enhanced by 5-10% CO_2. The *Brucellae* are facultative intracellular bacteria that replicate within host macrophages (6). They are taxonomically placed in the alpha-2 subdivision of the class *Proteobacteria* and phylogenetically related to *Agrobacterium, Ochrobactrum,Phyllobacterium, and Rhizobium* (13).

The average size of the genome is 2.37×10^3 MDa with a DNA G+C content of 58-59 mol% (13). The genus itself is highly homogeneous with all members showing > 90% homology in DNA-DNA pairing studies, thus classifying *Brucella* as a monospecific genus (14). All *Brucella* species except *B. suis* biovar 3 have 2 chromosomes (27). Natural plasmids have not been detected in *Brucella*, although broad-host-range plasmids have been introduced into *Brucella* by electroporation (14).

Brucellosis has been classified as a zoonotic disease since the isolation of *Brucella melitensis* from military personal in Malta by Bruce and colleagues in 1887. It remains a major source of disease in humans and domesticated animals in many parts of the world (13). Typically, brucellosis is transmitted by the oral and/or respiratory routes (19). Venereal and congenital transmissions have also been documented as well as infection through damaged skin. After entry, *Brucella* survive and replicate in the reticuloendothelial system of local lymph nodes and then migrate to the spleen, lymph nodes, the mammary gland and the reproductive tract (46); particularly in the pregnant uterus which in some animal species, has high concentration of erythritol (43,57). Infection will result in undulant fever in humans and in animals, abortions, still birth, retained placenta, sterility, lymphoplasmacytic mastitis and tissue granuloma are the most common observations (14).

Although *Brucellae* are sensitive to many antibiotics in vitro, only a few work in vivo. For animals, treatment is very expensive, of unproved value and is not practical. On the other hand, prevention of brucellosis by vaccination is and has played a key role in eradication programs. Several vaccines have been used in animals, such as *B. abortus* strain 19 and strain RB51 in cattle and *B. melitensis* strain Rev 1 in goat and sheep. Live vaccines as well as killed and fractionated ones have been developed. However, live vaccines have proven to induce better protective immunity as is the case with many diseases caused by facultative intrtacellular parasites (55).

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B. abortus vaccine strain 19 is a smooth strain containing lipopolysacchride (LPS). Although rather effective as a vaccine, this strain has a distinct disadvantage because it will induce antibodies to the LPS O-chain in serum and milk particularly if given in inappropriate doses. These antibodies interfere with the diagnosis of the disease since all diagnostic assays are based on the detection of O antibodies induced by the infection (15,53-55). For this reason, strain 19 has been replaced by strain RB51.

B. abortus stain RB51 is now the official vaccine for bovine brucellosis used in the USA. This strain was obtained through the successive passage of *B. abortus* virulent strain 2308 on media containing penicillin and rifampin (55). Strain RB51 is a highly attenuated, rough *Brucella* strain essentially devoid of O-chain. Minimal amount of O-chain may exist in RB51 but are insufficient to induce antibodies (54). Its roughness is very stable in vitro and in vivo in various species of animals (55,58,59). One clear advantage of strain RB51 over strain 19 is that RB51 cannot induce any detectable antibody responses against O-chain of LPS even when given in multiple injections for boosting purpose (55). Because O-chain antibodies are commonly used to detect brucellosis in field test, the use of strain RB51 dramatically reduces false positive and greatly benefits brucellosis eradication programs.

The antigen that dominates the antibody response in *Brucella* is the O-chain of LPS. In the smooth phase, the S-LPS comprises a lipid A; distinctive fatty acid; a core region, and an O chain (13). The structure of the LPS of rough strain is similar to that of the S-LPS except that the O-chain is either absent or reduced to a few residues. The O-chain is a protective antigen in some animal species and the protective effect is antibody mediated (1,40, 64).

Brucella major outer membrane proteins (OMPs) were initially identified by selective extraction techniques and classified according to their molecular mass as group 1 (88-94 kDa), group 2 (35-39 kDa), and group 3 (25-31 kDa) OMPs (69). OMPs appear to be poor immunogens in animals. However, some of them, such as Omp31, can stimulate protective antibody against some rough *Brucella* (41). Deletion or disruption of some OMPs, like Omp25, may attenuate *Brucella* (10,16,17). An 18 kDa lipoprotein (Omp19) is present on the surface of *Brucella*. This lipoprotein can induce antibody production in infected mice, sheep, goats, dogs, and humans (33). This protein can also induce a CMI

in the mouse model. Deletion of the 18 kDa lipoprotein from *B. abortus* alters the outer membrane property of *B. abortus* and decreases the virulence of *B. abortus* in mice (62). However, a recombinant vaccinia virus expressing this protein does not protect against virulent *Brucella* infection and disruption of this gene in strain RB51 does not affect either the vaccine strain's protective capability or its in vivo attenuation (63). These data suggest that 18 kDa lipoprotein plays no role in protective immunity.

Brucella Cu/Zn SOD is a *Brucella* protective periplasmic antigen. Purified *Brucella* Cu/Zn SOD (3) or SOD synthetic peptides (60) provide protection against virulent *B. abortus* strain 2308 infection in mice. Vaccination of mice with live *E. coli* expressing the *Brucella* Cu/Zn SOD indicated a protective role for this antigen against *Brucella* infection (45). However, mice vaccinated with *B. abortus* strain RB51 have no detectable antibody and selected cell activated immunity (CMI) responses against Cu/Zn SOD suggesting that there is no significant role for this protein in protection conferred by RB51. In contrast to RB51, recombinant RB51 overexpressing Cu/Zn SOD provides better protection against strain 2308 challenge and mice vaccinated with this strain develops Cu/Zn SOD specific antibody and IFN-γ, producing CMI responses in mice without altering the attenuation of the vaccine (65). *Ochrobactrum anthropi*, a close relative to *Brucella*, can also express *Brucella* Cu/Zn SOD as a foreign protein. After switching the mouse immune responses to a Th1 type by injection of CpG, recombinant *O. anthropi* expressing Cu/Zn SOD can provide significant protection in mice against virulent *B. abortus* 2308 challenge (22,23).

The last category of the immunogenic Brucella proteins are the cytoplasmic and ribosomal proteins. Heat shock proteins (Hsp) are cytoplasmic proteins. GroEL and GroES are *Brucella* Hsp that are involved in the folding, assembly, and transport of factors necessary for growth under stress conditions (37). GroEL is expressed at higher levels under stress stimuli. GroEL was identified as a highly immunogenic antigen in many microbial pathogens (29). Cattle and mice vaccinated or infected with Brucella developed antibody responses to GroEL (51). GroEL alone, expressed by recombinant vaccinia virus or used as a DNA vaccine can induce IFN-γ production and Th-1 type immune responses in the mice model (44), however, no protection against virulent *Brucella* was provided by recombinant vaccines (4,7,35).

Both 18 kDa protein and SOD protein have signal sequences at their N-terminal end, which are responsible for their localization to the outer membrane and periplasmic regions respectively. Signal sequences play a key role in targeting nascent proteins in both eukaryotes and prokaryotes. They usually reside in the N-terminal portion of the protein and are cleaved from the precursor proteins by a membrane bound signal peptidase after targeting the proteins. Signal sequences can also be located within a protein or at its C-terminal end (38).

The CMI response plays an important role in protection against brucellosis like most other intracellular bacteria infections (13). *B. abortus* induces a Th1 type immune

responses (2). IFN-γ, as well as some other Th1 type cytokines, is produced; these in turn activate macrophage and up-regulate their bactericidal ability. Specific cytotoxic T lymphocytes (CTLs) are also found in *B. abortus* infected and vaccinated mice. They probably play a crucial role in protective CMI responses to *B. abortus* (24).

In the design of vaccines against intracellular bacterial infections such as brucellosis, three major parameters have to be considered. First, the actual presence and quantity of protective antigen(s), such as *Brucella* Cu/Zn SOD, is important; second, appropriate intracellular localization of the bacteria is needed in order to stimulate correct antigen processing and MHC mediated presentation; and finally, an appropriate antigen display by the vaccines is important since this can markedly influence the kinetics of T cell activation (25,30).

In the infected host, *B .abortus* replicate within the endosome of phagocytic cells and non phagocytic cells (14,46). The processed peptides are primarily presented to MHC class II restricted CD4 T cells (30). *B.abortus* strain 2308 can also transit through autophagic pathway and replicate in the endoplasmic reticulum (ER) (48). The processed peptides are then presented to CD8 cells through MHC I pathway (30). It has been showed that purposely release of *B. suis* from the phagosome to the cytoplamic region of macrophage results in the loss of the ability of *B. suis* to replicate within the macrophage. This kind of direct release of brucellae or their antigen to the cytoplasm of macrophage may be a promising approach for the development of new types of vaccine strains (31).

Concerning antigen display, certain intracellular bacteria are not killed within resting macrophage (MP). They persist in a dormant stage or are metabolically active and replicate. Only after activation by T cells does macrophage acquire the capacity to kill their intracellular predators. Proteins within the bacterial cytoplasma are unavailable to antigen processing until bacteria are killed and processed. Hence they fail to induce an early T cell response. In contrast, proteins secreted by bacteria inside host cells can be captured by MHC molecules and subsequently presented to T lymphocytes. Some cell wall associated proteins (or glycolipids) may be cleaved from the bacterial surface while the bacteria are still living and hence may also be processed and presented to T lymphocytes independently from bacterial destruction by macrophages. Because vaccine-induced T cells must mobilize antimicrobial effector mechanisms immediately after contact with the invading pathogen, secreted proteins appear to be the best candidates for protective antigens (21,25,28).

Green fluorescent protein (GFP) is found in the bioluminesent jelleyfish *Aequorea victoria.* GFP yields a bright green fluorescence when stimulated by blue or UV light. (11) It is a novel reporter gene system and is widely used in both eukaryotic and prokaryotic systems for protein localization, gene expression monitoring and viability assessing (47). GFP has a molecular mass of 27kDa, functions as a monomer. Its 3D structure is known to attain relatively uncomplicated β-can structure. Also, GFP emits green light following excitation of an internal fluorophore composed of a Ser-Tyr-Gly

sequence near the proteins amino terminus (18). As GFP antibodies are commercially available, the above characteristics of GFP are very useful to study protein localization that can be affected by different signal sequences.

Based on the presented information, one could propose the following hypothesis:
1. Brucella antigens can be expressed by *B. abortus* strain RB51 at different locations by using specific signal sequences (i.e. SOD protein and 18 kDa lipoprotein signal sequences),
2. Expression of *Brucella* antigen by *B. abortus* strain RB51 at different locations can lead to different antibody and/or CMI responses.

In order to test this general hypothesis, this dissertation had two main objectives:
a. Determine if the GFP is expressed at specific cellular localization in B. abortus strain RB51 when expressed with *Brucella* SOD or 18kDa lipoprotein signal sequences,
b. Determin if the antibody response to GFP is different depending on its different localizaion.

If "a" is positive, specific signal sequences of *Brucella* can be used to localize antigens within different compartment for vaccine design. If "b" is positive, signal sequences can be used to engender the most appropriate immune responses for protection. It needs to be kept in mind that a negative here does not prove any effect on CMI. Measurements of CMI responses are out of the scope of this thesis.

Chapter 2

Materials and Methods

Plasmids and Reagents

pGFPuv vector, recombinant Green Fluorescent Proteins (GFP) protein, living color a.v peptide antibody and living color a.v. monoclonal antibody were purchased from Clontech (Palo Alto, CA). Plamid pCR2.1-TOPO was purchased form Invitrogen (Carlsbad, CA). Plasmids pBBR1MCS and pBBgroE were already available in our laboratory (63) Western blot substrate 4-CN/DAB was purchased from PIERCE (Rockford, IL). All chemicals were from either Sigma-Aldrich (St Louis, MO) or Fisher Scientific (Suwanee, GA) unless stated otherwise.

Bacterial strains and growth condition

Brucella abortus strain RB51 was obtained from Dr. Gerhardt Schurig's (Virginia Tech) culture collection. *E. coli* strain DH5α and Top10 (GIBCO Carlsbad,CA) were used as host strains for cloning. All bacteria were grown in Trypitic soy broth (TSB) or on Tryptic soy agar (TSA) plates. Chloramphenicol at 30ug/ml was added to the broth or agar while culturing bacteria containing the broad-host-range plasmid pBBR1MCS. Kanamycin at 50ug/ml or ampicillin at 100ug/ml was added to broth or agar while culturing bacteria containing plasmid pCR2.1-TOPO (Invitrogen Carlsbad, CA). All bacteria were grown at 37°C unless stated.

PCR amplification

The gene of GFP was amplified via PCR from pGFPuv vector. A primer pair consisting of a forward and a reverse primer was designed according to the nucleotide sequence provided by CLONTECH, and a restriction site (*EcoR* V for forward primer and *Xba* I for reverse primer) was engineered into the forward primer to facilitate the further cloning. Ready-To-Go PCR beads (Pharmacia Biotech) were used for the PCR. Amplification was performed in an Omni Gene thermocycler (Hybaid, Franklin, Mass.) at 95°C for 5min, followed by 30 cycles that each included 1min denaturation at 95°C, 1 min of annealing at 54°C, and 1min of extension at 72°C, and finished with a final extension at 72°C for 5 min.

The promoter and signal sequence of 18 kDa lipoprotein and *Brucella* Cu/Zn SOD protein were amplified via PCR from *B. abortus* strain 2308 genome DNA. Primers were designed according to the nucleotide sequence (Gene Bank No L27997 for 18 kDa lipoprotein and SOD sequence available in the laboratory). A restriction site (*EcoR* V) was engineered into reverse primers to facilitate the further cloning. The forward and reverse primers were used to amplify the whole promoter and signal sequence region of

18 kDa lipoprotein or SOD protein. Amplification procedure was similar to the one used for GFP gene with the exception that the annealing temperature was 60°C.

The signal sequence-GFP fusions were amplified from plasmid pT18psGFP or pTSpsGFP. The F' primers include the ribosome binding site (RBS) so that the amplified fragments contain RBS, signal sequence of 18 kDa lipoprotein or SOD protein and the open reading frame of GFP gene. A restriction site (*BamH* I) was engineered in to the F' primer to facilitate the further cloning. Amplification procedure is similar to the one used for GFP gene with the exception that the annealing temperature was 54°C.

Primer name	Primer sequence	Comments
GFPF	5'-GAT ATC ATG AGT AAA GGA GAA GAA CTT-3'	Primers GFPF and GFPR were used to amplify GFP gene
GFPR	5'-TCT AGA TTA TTT GTA GAG CTC ATC CA-3	
18F	5'-GAA TTC CGA CCC GCG CGC-3'	Primer 18F and 18R were used to amplify promoter and signal sequence of 18 kDa lipoprotein; 18F' and GFPR were used to amplify the 18 kDa signal sequence–GFP fusion construct
18R	5'-GAT ATC GCA CCC GGC CAG GAC AAT GGC-3'	
18F'	5'-AGA TCT ATG GAG AAC CTG ATG GGA AT-3'	
SODF	5'-ATC ATG TGA ATG ATC GAT AA-3'	Primer SODF and SODR were used to amplify promoter and signal sequence of Brucella Cu/Zn SOD protein; SODF' and GFPR were used to amplify the SOD signal sequence –GFP fusion construct
SODR	5'- GAT ATC TGC GAA AGC CGG AAA AGC-3'	
SODF'	5'-AGA TCT GGA GAA GTG ATG AAG TCC-3'	

Construction of recombinant plasmids for GFP expression

The PCR products were seperated on 1% agrose gels with EtBr. After electrophoresis, the fragment of correct size was cut from the gel under the UV light. After gel extraction, the fragments were ligated to pCR2.1 vector to produce pTGFP, pT18ps and pTSps . Then the GFP fragment was excised from pTGFP with *EcoR* V and *Xba* I and subcloned into pT18ps and pTSps. A fragment containing GFP and signal sequences of either 18kDa protein or *sod*C were amplified from pT18psGFP or pTSpsGFP by specific primers. PCR products were ligated with pCR2.1-TOPO vector using TOPO TA Cloning System to get pT18sGFP and pTSsGFP. 18sGFP and SsGFP fragments were exised by

*Bam*II and *Xba* I and subcloned into pBBgroE vector to get pBBg18sGFP and pBBgSsGFP, respectively. The whole procedure is depicited in Fig A.

The GFP fragment was digested from pTGFP with *Spe* I and *Xba* I and ligated to pBBgroE (digested with *Spe* I and *Xba* I too). The resulting plasmid was designated as pBBgGFP.

pT18sGFP, pTSsGFP, pT18psGFP, pTSpsGFP and pTGFP were sequenced to confirm the cloning of the GFP gene in frame with *Brucella* 18 kDa lipoprotein and Cu/Zn SOD protein signal sequences.

Generation of recombinant RB51 strains expressing GFP

pBBgroE, pBBgGFP, pBBg18sGFP, pBBgSsGFP were then isolated from *E. coli* and electroporated into *B. abortus* strain RB51 according to a standard protocol (40). In summary, a single colony of *B. abortus* strain RB51 was inoculated into 10ml TSB media. The culture grew at 37°C with shaking at 180rpm for 30-33 hrs. 2ml of the culture was transferred into 380 ml TSB media into a 1L flask with side arm for measuring Klett units using a flask with side arm. Culture was incubated at 37°C with shaking at 180 rpm until a density of 70-75 Klett units. Then the culture is chilled on ice for 15-30 min and transferred into 250mL prechilled, sterile centrifuge bottles. Cells were centrifuged at 5000×g for 6 minute at 4°C. The pellet was resuspended in 380mL of cold distilled water. Repeated the washing step once and pellet was finally resuspended in cold dH_2O to reach the final volume of 500ul. Cells were transferred to a prechilled microcentrifuge tube, plasmid added at a ratio of 1-5 µl of 0.5 µg DNA/µl to 100ul of cell suspension, mixed well, and the cell-DNA mixture placed on ice for up to 30min. 100µl cell-DNA mixture was added between electrodes separated by 1mm and pulsed at 625V for 10ms. After that, the cells were transferred to 0.9mL of TSB plus 10mM $MgCl_2$ and 10mM $MgSO_4$ and incubated at 37°C for 24 h with shaking at 180rpm. The culture was plated on TSA plates with 15ug/ml chloramphenicol. Colonies appeared after a week and were transferred to TSA plates with 30ug/ml chloramphenicol. Colonies containing the plasmids were selected on TSA plates with 15ug/ml chloramphenicol. The expression of GFP by these recombinant RB51 strains at 37 and 42°C was confirmed by Western blot analysis with anti-GFP peptide or monoclonal antibodies.

Western Blot

To screen for the expression of GFP, the recombinant *E. coli* or *B. abortus* RB51 strains were cultured to OD_{600}1.0. 1ml *E. coli* cultures or 3ml *Brucella* cultures were pelleted by centrifugate at 12000 rpm for 1 min, washed with 1ml 0.1M Tris-HCl (pH 8.0), and then resuspended in 100 µl 0.1M Tris-HCl (pH8.0) and 100µl Lammeli buffer. The mixture was heat in boiling water for 5 min to release the protein and then centrifuged at 6000rpm for 5min. The supernatants were analyzed for protein expression by separating in a 12.5% SDS-PAGE, followed by transfer to 0.2µm nitrocellulose. The membrane was probed with anti-GFP antibody as primary antibody and anti-rabbit or anti-mouse IgG coupled to

hydrogen peroxidase as secondary antibody. The serological reaction was visualized by incubation in a developing solution consisting 4-CN/DAB. The development of color reaction was stopped after 5 min by placing the membrane in distilled water.

Immunoabsorption

Immnoabsorption was used to detect the surface expression of GFP in *E. coli*/pBBg18psGFP and B. abortus RB51/pBBg18sGFP. The OD value (at 600 nm) of overnight cultures of *E. coli* strain DH5α containing pBBGFP, pBB18psGFP and of pBBSpsGFP and *B. abortus* RB51 cultures containing pBBgGFP, pBBgSsGFP and pBBg18sGFP were measured. OD values of each culture were adjusted to the same OD in order to achieve the same amount of cells for the experiment.

Equal volumn of OD adjusted *E. coli* strain DH5α culture containing pBBGFP, pBB18psGFP and pBBSpsGFP and the *B. abortus* strain RB51 cultures containing pBBgGFP, pBBgSsGFP and pBBg18sGFP were centrifuged and the pellet was resuspended in 1ml TBS with different dilution of anti-GFP peptide antibody (1:100, 1:200, 1:300, 1:400, 1:500) and incubated at room temperature overnight. The mixture was then centrifuged and the supernatant was used as the absorbed antibody, and goat anti-rabbit IgG(1:1000) was used to detect the GFP by Western blotting.

Selective extraction of periplasmic proteins of recombinant RB51 strains

1×10^{10} CFU of *B. abortus* RB51/pBBgGFP, pBBg18sGFP or pBBgSsGFP were suspended in 10ml dH_2O and then frozen in -80°C freezer and then thawed at room temperature. The freeze-thaw procedure was repeated 3 times and the mixture was centrifuged at 4°C, 6000×g for 5 minutes. Both the pellet and the supernatant were saved. Later, the supernatant proteins were concentrated by TCA (trichloride acid) precipitation (12). Western blotting was performed to check for the presence of GFP in both supernatant and pellet proteins. It was assumed that the supernatant would contain the periplasmic proteins and therefore, it was also tested for the presence of *Brucella* GroEL protein since its presence would be an indication that cytoplamic proteins leaked into the periplasmic part during the freeze-thaw.

Immunoelectron Microscopy

B. abortus strain RB51 transformed with either pBBgroE, pBBgGFP, pBBg18sGFP, or pBBgSsGFP was grown in the TSB and washed twice in PBS using centrifugation at 2400g for 15min. The bacteria were fixed in 1ml of 2.5%(vol/vol) glutaradehyde in PBS overnight at 4C, and samples were set in 2%(wt/vol) agar and washed in PBS. The agarose plugs were then dehydrated via stepwise alcohol series, embedded in acrylic resin LR white in gelatin capsule, and baked at 55°C for 24 h under vacuum. Ultrathin sections were obtained by cutting the embedded materials and deposited on 200-mesh

nickel grids. Immunogold labeling of the grid was performed according to the published procedure (62) with some modification. The grids were blocked with PBST+BSA for 30 min, and then incubated in diluted primary antibody (mouse anti-GFP monoclonal antibody, 1:1000 diluted in PBST+2%BSA) for 2h at room temperature. Grids were washed three times with PBST, then incubated for 2h in a 1:5 dilution of goat anti-mouse IgG conjugated with 10nm gold particles. Grids were washed three times with PBST and once with ddH2O. The grids were stained with 4% uranyl acetate in water and 0.4% lead citrate in 0.1M NaOH. Then the grids were observed with a transmission electron microscope. (JEOL 100CXII) *B. abortus* strain RB51 containing pBBgroE alone was used as a control for non-specific staining between RB51 and gold-conjugated anti-IgG.

Survival of recombinant *B.abortus* strain RB51 expressing GFP in mice

Groups of 5 female BALB/c mice (Charles River Laboratory, MA) each were injected intraperitoneal (i.p.) with 5×10^8 CFU of either *B. abortus* strain RB51, RB51/pBBgGFP, RB51/pBBgSsGFP, RB51/pBBg18sGFP. Four weeks post injection, Two mice from each group were killed by CO_2 asphyxiation. Their spleens were collected and the bacteria CFUs per individual spleens were determined by plating the serial dilutions of the spleen homogenates on TSA plates as well as on chloramphenicol containing TSA plates. Six weeks post injection, the other three mice in each group were killed and their spleen homogenates were plated for detecting bacteria CFUs.

Immunization of mice

Groups of five female BALB/c mice (Charles River Laboratory, MA) were injected i.p with 5×10^8 CFU of either *B. abortus* strain RB51, RB51/pBBgGFP, RB51/pBBgSsGFP, RB51/pBBg18sGFP. Before injection, two mice from each group were bled and the sera were used as negative control. Three weeks later, all mice were bled and the sera were tested for the presence of anti-GFP and anti-RB51 antibodies by Western blot analysis. two days after bleeding, mice were boosted i.p. with 1×10^7 CFU of either *B. abortus* strain RB51, RB51/pBBgGFP, RB51/pBBgSsGFP, RB51/pBBg18sGFP. Two weeks after the boost, mice were bled again and the sera were tested for the presence of anti-GFP and anti-RB51 antibody by Western blot analysis. Two days after the second bleeding, mice were each injected i.p. with 10µg GFP in saline and then killed two weeks later and sera were obtained as a source of mouse anti-GFP peptide antibody.

ELISA

ELISA was performed according to the protocol provided by CLONTECH (Palo Alto, CA). Purified recombinant GFP was absorbed to wells of polystyrene plates (NUNC, Maxisorp) at a concentration of 0.25 µg GFP/well in 100µl bicarbonate buffer (pH9.6). After incubation at room temperature for 8hrs, plates were blocked with 2%

bovine serum albumin (BSA) in phosphate buffered saline (PBS) (pH7.4) at 4°C overnight. Mice serum samples at 1:100 dilution in blocking buffer were added to wells in duplicate and incubated for 1hr at room temperature. The plates were washed three times with PBS containing 0.05% Tween-20. Goat anti-mouse horseradish peroxidase conjugated (ICN Pharmaceuticals, Inc) antisera were added for 1hr at room temperature, plates were washed five times. 100µl TMB substrate solution (KPL, Gaithersburg, MD) was added and incubated in dark for 10 min. The reaction was stopped by adding 100µl/ well of 0.18M sulfuric acid and the absorbance of the developed color was measured at 450nm.

Chapter 3

Results

Nomenclature used in the thesis

The construction nomenclature utilized in this study can be explained as follows: pBBg18sGFP-- pBB denotes plasmid pBBR1MCS; g denotes *Brucella* groE promoter; 18s denotes *Brucella* protein from which the signal sequences is derived, in this case 18 kDa lipoprotein; s denotes presence of signal sequences; and GFP denotes the protein that the signal sequence is fused to.

pBB18psGFP—pBB denotes plasmid pBBR1MCS; 18ps denotes *Brucella* protein from which both promoter and signal sequence are derived, in this case 18 kDa lipoprotein; p denotes promoter and s denotes signal sequence; GFP denotes the protein that is expressed

Plasmids	Promoter	Origin of Signal sequence
pBBgGFP	GroE promoter	No signal sequence
pBBg18sGFP		18kDa lipoprotein signal sequence
pBBgSsGFP		SOD signal sequence
pBBSpsGFP	SOD promoter	SOD signal sequence
pBB18psGFP	18 kDa lipoprotein promoter	18kDa lipoprotein signal sequence

Construction of pBBg18sGFP, pBBgSsGFP and pBBgGFP plasmids

Successful amplifications by PCR were achieved for the coding sequence of GFP, 18kDa protein gene promoter and signal sequence (18ps), and sodC gene promoter and signal sequence (Sps) (Fig 1). They amplified DNA fragments were cloned into pCR2.1 vector. The resulting plasmids, pTGFP, pT18ps and pTSps were tested for the correct orientation of the cloned fragments. A fragment containing the whole GFP encoding sequence was excised from pTGFP and subcloned into pT18ps and pTSps. The resulting plasmids were designated pT18psGFP and pTSpsGFP, respectively. A fragment containing GFP gene and nucleotide sequences encoding signal peptides of either 18kDa

protein or SOD was amplified from pT18psGFP or pTSpsGFP using specific primers (Fig 2). The resulting PCR product was cloned into pCR2.1-TOPO vector to get pT18sGFP and pTSsGFP. The inserts of pT18sGFP and pTSsGFP were subsequently subcloned into pBBgroE vector to obtain pBBg18sGFP and pBBgSsGFP, respectively.

After the successful construction of pBBgGFP, pBBg18sGFP and pBBgSsGFP, the expression of GFP was assessed in recombinant *E. coli* strains by Western blot analysis. But very low expression was observed in *E. coli*/pBBg18sGFP and *E. coli*/pBBgSsGFP using both peptide and monoclonal (Fig 3) anti-GFP antibody after culturing bacteria at either 37 or 42°C.

The 18psGFP and SpsGFP fragments were excised from pT18psGFP and pTSpsGFP and ligated into pBBR1MCS vector to obtain expression of GFP by either the 18kDa protein gene promoter or *sodC* gene promoter. In *E. coli* harboring pBB18psGFP and pBBSpsGFP, the expression of GFP was detected by Western blotting using both peptide and monoclonal (Fig 4) anti-GFP antibody; the expression of GFP was detected at either 37 or 42°C.

Generation of recombinant RB51 strains expressing GFP

When pBBgGFP, pBBg18sGFP and pBBgSsGFP were electroporated into *B. abortus* RB51, the expression of GFP in the resulting recombinant RB51 strains were clearly detected by Western blotting with either peptide or monoclonal (Fig 5) anti-GFP antibody at both 37 and 42°C. These recombinant strains were also tested with goat anti-RB51 sera to make sure that they were strain RB51 (data not shown).

Immunoabsorption (Fig 6)

To check the outer membrane localization of the GFP in E.coli/pBB18psGFP, immunoabsorption was performed with all three recombinant strains expressing GFP. The unabsorbed anti-GFP antibodies contribute to the presence of GFP band on Western blot. Using *E. coli*/pBB18psGFP, the GFP band detected in Western blot by the absorbed antibody began to decrease in intensity using absorbed sera of 1:200 dilution and totally disappeared using it at 1:400 dilution. While using *E. coli/* pBBGFP and *E. coli/* pBBSpsGFP, no decreasing density of GFP band was found at any dilutions, indicating that they do not express GFP on the surface.

Similar results were obtained in recombinant strain RB51. RB51/pBBg18sGFP absorbed anti-GFP antibody. The GFP band detected in Western blot by the absorbed antibody gradually decreased in intensity from using the absorbed sera at 1:200 dilution to using it at 1:400 but never totally disappeared. This result showed that GFP protein in strain RB51/pBBg18sGFP located on the surface of recombinant RB51.

Periplasmic location of GFP in *B. abortus* RB51/pBBgSsGFP

Freeze-thawing of the three GFP expressing RB51 strains successfully exposed the periplamic proteins. Western blot analysis demonstrated the periplamic location of GFP in RB51/pBBgSsGFP under the leading of SOD signal sequence, while RB51/pBBgGFP and RB51/pBBg18sGFP showed no periplamic GFP expression. (Fig 8) The presence of *Brucella* GroEL in periplamic extract was also checked since its presence would be an indication that cytoplamic proteins leaked into the periplasmic part. No higher amount GroEL protein was found in the periplasmic extract of RB51/pBBgSsGFP than that of RB51/pBBgGFP and RB51/pBB18sGFP. (data not shown)

Immunoelectron Microscopy

To visualize the localization of GFP in different RB51 strains, immuno-electron microscopy was performed using specific anti-GFP antibody and gold conjugated anti-mouse IgG. *B. abortus* RB51/pBBgroE exposed only to gold conjugated anti-mouse Ig G revealed no cross-reaction. Immunostaining was performed with different concentration of mouse anti-GFP monoclonal antibody (1^{st} antibody) and gold conjugated anti-mouse IgG (2^{nd} antibody) dilutions to establish the best combination of the 1^{st} and 2^{nd} antibody. The immunostaining was performed with 1:1000 dilution of 1^{st} antibody and 1:5 dilution of 2^{nd} antibody. *B. abortus* RB51/pBBgGFP, expressed the GFP eventually in the cytoplamic region as the gold particles showed as dark round spots inside the cell. RB51/pBBg18sGFP demonstrated gold particles on the surface of the cell, indicating the expression of GFP was on the surface of the RB51 as expected from the immunoabsorption results. (Fig 7)

Immunization of mice

All recombinant strains expressing GFP were cleared from the mouse spleen by the 4^{th} week similar to mice injected with strain RB51 alone. So expression of GFP did not increase the in vivo survival of *B. abortus* RB51.

Mice failed to develop any anti-GFP antibody by 3 weeks post injection and 2 weeks post boost by Western blot analysis. After 2 weeks post boost with 10µg GFP, anti-GFP antibody was found in all mice (even the mice injected with RB51 alone) by Western blot analysis. ELISA was performed to quantitatively compare the anti-GFP antibody production by different GFP expressing RB51 strains. No significant difference was found in the amount of anti-GFP antibody produced by mice injected with strain RB51, RB51/pBBgGFP, RB51/pBBg18sGFP and pBBgSsGFP by ANOVA analysis (Excel).

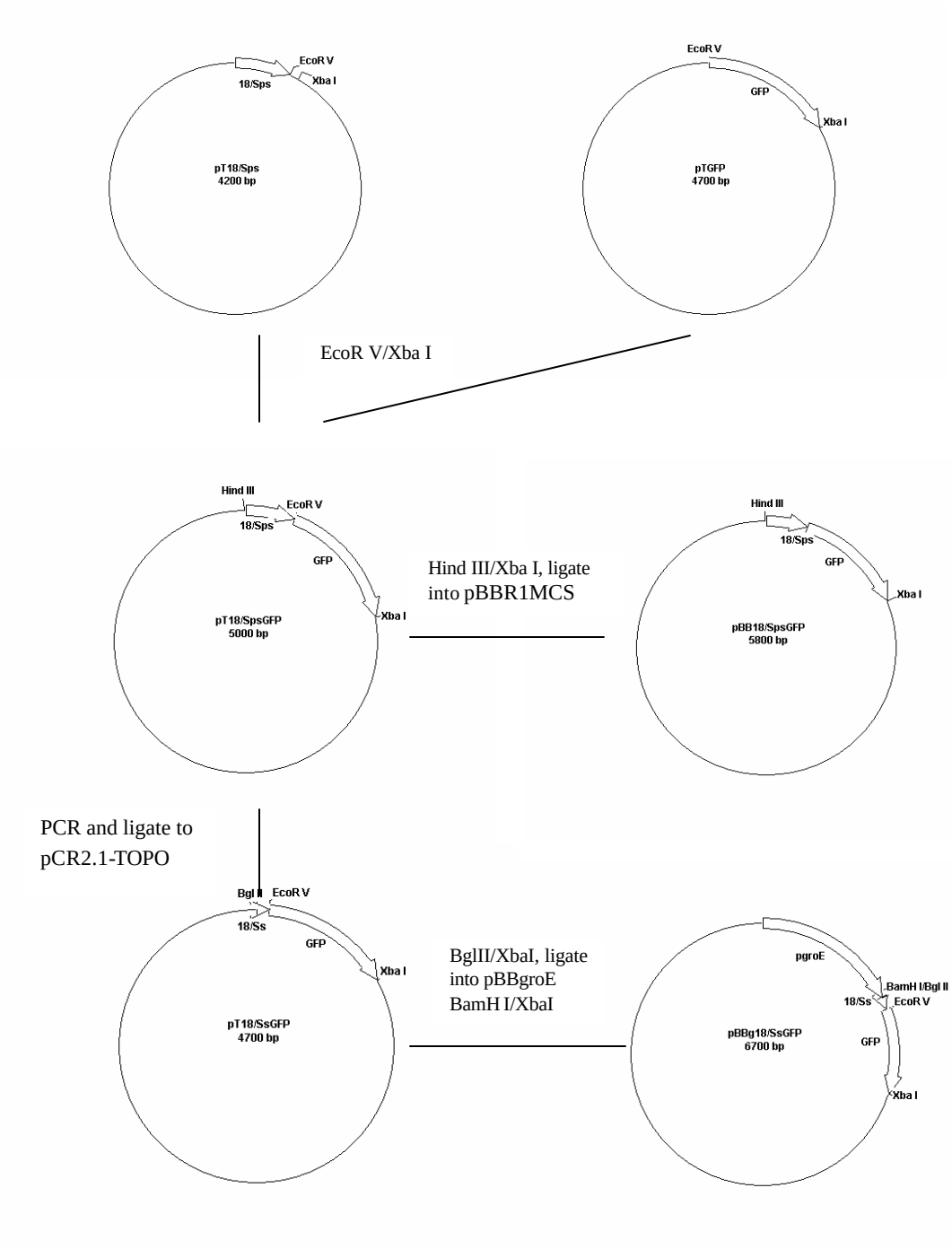

Fig A. Flow chart depicting the construction of plasmids for GFP expression.

Chapter 4

Discussion

Green fluorescent protein gene (gfp) has often been used as a reporter gene for study of protein localization, gene expression monitoring and signal transduction (47). Several investigators have used the GFP gene in *Brucella* work as a reporter gene for protein expression monitoring (31) or presentation of Brucella proteins in macrophage (42). Since *Brucella* is able to express GFP and the gene has been used as a reporter gene in *Brucella*, we decided to use the GFP gene in our study to assess the localization of a protein joined to a specific signal sequence.

The gene coding green fluorescent protein (gfp) was successfully cloned as fusions to the signal sequences of the 18 kDa protein and SOD protein. Expression of GFP was driven by the groE promoter since previous result indicated that the groE promoter was upregulated in *B. abortus* when this strain was under heat stress or inside host macrophage (67). DNA sequencing showed the correct orientation and in frame of both the signal sequence and gfp gene.

When expressing recombinant GFP in *E. coli* DH5α strain, very low level GFP expression was found in either *E. coli*/pBBg18sGFP or *E. coli*/pBBgSsGFP by Western blot analysis although this was not the case when using RB51. This phenomenon persisted even when the bacterial cultures were stressed at 42°C to upregulate the groE promoter. It is possible that, fusion of GFP with 18 kDa lipoprotein or the SOD signal sequence interferes with the correct folding of GFP protein since the GFP protein loses its fluorescence when it is located in the periplasmic region or the outer membrane (ref). This conformational change of the GFP protein may change its epitopes in a manner which is not recognized by anti-GFP antibodies which in turn results in no detectable GFP expression levels in *E. coli*/pBBg18sGFP and *E. coli*/pBBgSsGFP using western blot nanalysis.

When plasmid pBBg18sGFP, pBBgSsGFP and pBBgGFP were electroporated into *B. abortus* strain RB51, GFP expression was very clearly observed by Western blot analysis contrasting with the result found in *E. coli.* When the culture temperature was increased to 42°C, GFP expression was not clearly increased even though groE promoter was shown to be upregulated at higher temperature as described before. (67)

The amount of GFP expressed in *B. abortus* RB51 strain was considered low. Ponceau stain S and Coomassie Blue R-250 couldn't reveal the GFP band on the gel. Only western blot analysis can detect the GFP expression. Western blot analysis showed that 1/10 of an OD2.0 OD reading (at 600nm) recombinant RB51 culture expressed less than 50 µg of GFP. Immunoelectron microscopy also suggested a low level expression of GFP in recombinant RB51. This is consistent with the work of other researcher who

determined that expression level of some heterologous protein in *B. abortus* RB51 is low (66,67, personal communication with Andrea Contreras). There are several possible explanations for this low level of GFP expression in *B. abortus* RB51. First, the GFP protein gene we used is optimized for efficient expression in *E. coli*. The codon usage in *E. coli* and *B. abortus* is somewhat different and this can affect the level of expression, making it low. If this is the problem, GFP protein gene can be changed to a more *Brucella* friendly codon usage. Second, groE promoter may not be a strong promoter for heterologous protein expression in strain RB51, which could be part of the reason for low expression. Exchanging groE promoter to a stronger promoter may increase the expression level of GFP in *Brucella*.

Although the level of GFP expression in recombinant RB51 was not analyzed quantitatively, the consistent usage of the *Brucella* groE promoter in all GFP expressing plasmids ensure that the same amount of GFP should be expressed in similaramo unt of recombinant *E. coli* or *B. abortus* RB51 cultures. Thus, when we immunized mice with same colony forming units of different GFP expressing strains, the amount of GFP given to each mouse is considered the same. Differences in the immune response should therefore reflect difference in the localization of GFP.

Immuno-electron microscopy (EM) observation indicated the presence of GFP protein in *B. abortus* strain RB51/pBBgGFP and RB51/pBBg18sGFP. Strain RB51/pBBgGFP showed GFP expression only in cytoplasmic region. This is consistent with the fact that there were no signal sequences added at N-terminus of GFP protein. Strain RB51/pBBg18sGFP expressed GFP not only inside the cytoplasmic area, but also on its surface. This is consistent with the observation of other researchers. (25). We were not able to demonstrate the periplamic localization of GFP in RB51/pBBgSsGFP although GFP was observed in the cytoplasm. It is possible that the periplasmic region of RB51 is too narrow and the method used is not sensitive enough to clearly detect or locate small amount of the protein within this space. Freeze-thaw experiment of viable strains aimed at leaking periplasmic protein from *B. abortus* RB51 constructs containing GFP indicated that GFP was expressed in the periplasmic area in RB51/pBBgSsGFP but not in the other two strains. The same technique did not release GroE protein which is not located in the periplasmic space.

When mice were immunized with RB51, RB51/pBBgGFP, RB51/pBBg18sGFP and RB51/pBBgSsGFP, no induction of anti-GFP antibodies was observed in Western blot analysis 3 weeks post primary injection and 2 weeks post boosting. A potential explanation for this observation may be that the amount of GFP expressed by recombinant RB51 strains was too low to induce a humoral immune response. Also, the mice strain used in this study-BALB/C- may play a role in the absence of response. It has been demonstrated that BALB/c mice immunized with GFP DNA based vaccines do not produce any antibodies to GFP (26).

In order to see if the mice were "sensitized" by the injection of the recombinant *B. abortus* RB51 strains to GFP protein, all mice were given 10 μg GFP 2 weeks post last strain injection. Two weeks after this GFP injection, anti-GFP antibodies were induced in all mice serum samples, including the ones injected with RB51 alone, by Western blot analysis. We also found that goat48 serum (goat anti-RB51 serum) can recognize GFP and anti-GFP polyclonal antibody can pick up a couple of bands in whole RB51 cell antigen by Western blot analysis. ELISA result also showed no difference in the level of antibody production after GFP injection suggesting that the mice did not recognize the GFP antigen by the previous injection. The exact implication of this finding in the immune response to GFP after the described immunization is unclear. With the present data, we cannot reach any conclusion about the relationship between antibody production and location of the GFP protein in RB51.

The successful expression of GFP in *B. abortus* RB51 in these experiment supports the previous work indicating that this strain RB51 can be a vector for expressing heterologous protein (66,67). By adding appropriate signal sequences, GFP is successfully transported to the periplasmic region or outer membrane of strain RB51. Since signal peptides can ordinarily be presented to MHC class I molecules (38), the addition of them to the heterologous antigens may also help the immune response to such antigens

No significant level of anti-GFP antibodies were detected in mice vaccinated with the GFP expressing RB51 strains, suggesting that expression of heterologous proteins needs to be above a certain amount for the induction of detectable, humoral immune responses. (67). Since no significant differences were found in the anti-GFP antibody production in mice vaccinated with the GFP expressing RB51 stains, we could not draw a conclusion regarding the localization of GFP on the immune response to it. Since RB51 is a strong inducer of a Th-1-biased CMI, checking indicators of Th-1 response, such as IFN-γ production, may reveal some differences in immune responses to GFP based on location of its expression.

Chapter 5

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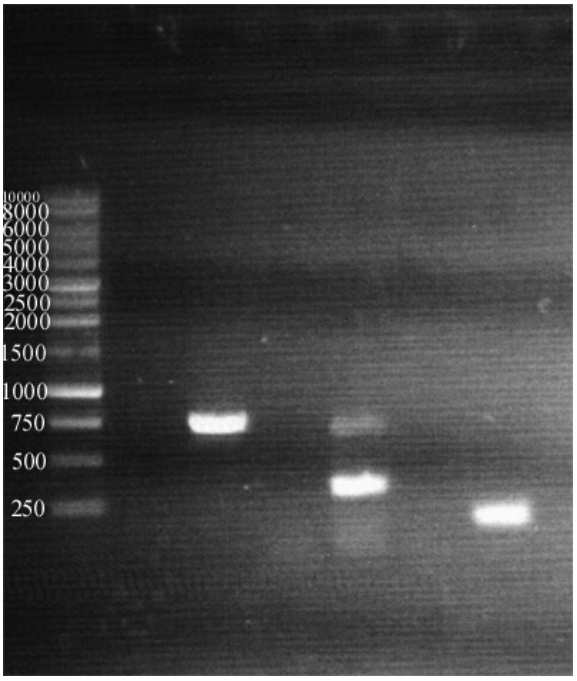


Fig.1 Results of PCR reactions for green fluorescent protein gene (gfp), 18 kDa protein promoter and signal sequence region (18ps) and SOD protein promoter and signal sequence region (Sps). Lane1, Marker; Lane2, negative control for gfp; Lane3, gfp (750 bp); Lane4, negative control for 18ps; Lane 5, 18ps (300bp); Lane6, negative control for Sps; Lane7, Sps (250bp)

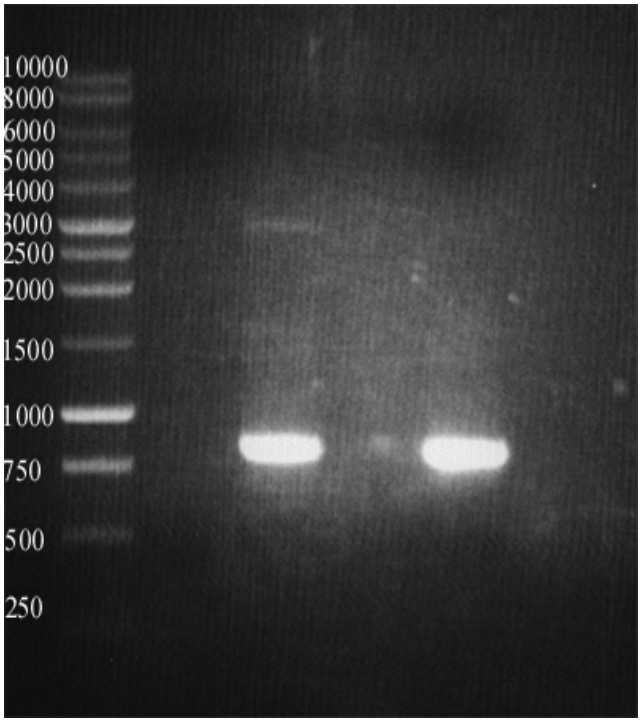
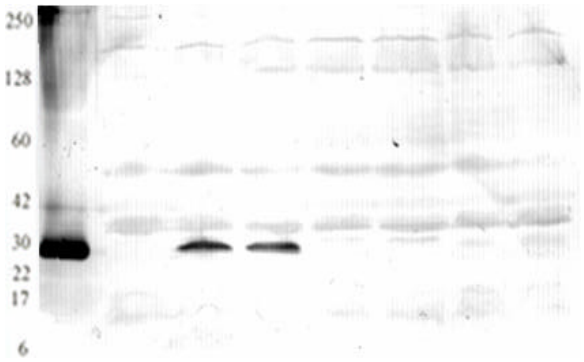
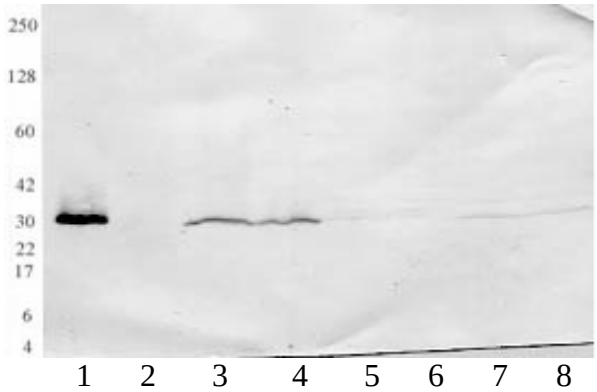


Fig.2 Results of PCR reactions for GFP fused with either 18 kDa protein signal sequence (18sGFP) or SOD signal sequence (SsGFP). Lane1, Marker; Lane2, negative control for 18sGFP; Lane3, 18sGFP; Lane4, negative control for SsGFP; Lane5, SsGFP

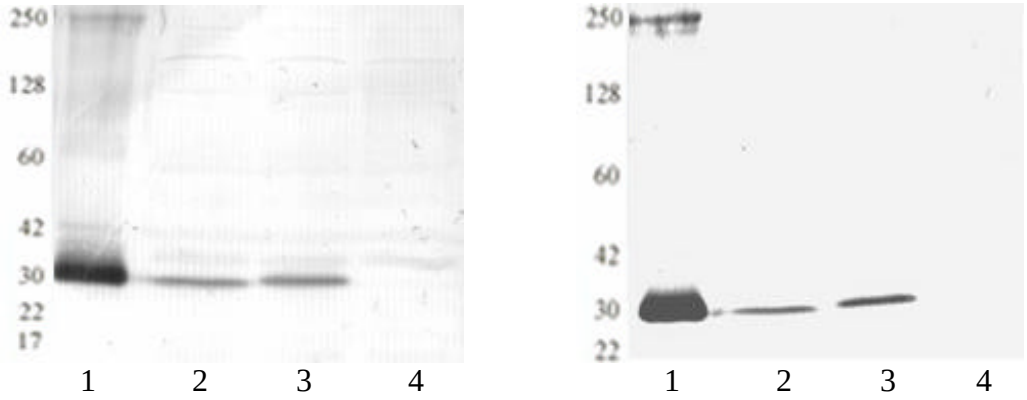


(A)



(B)

Fig. 3 Western blot analysis of expression of GFP protein by recombinant *E. coli* strain DH5α using anti-GFP peptide (A) and monoclonal (B) antibody. Lane1, Purified recombinant GFP protein; Lane2, *E. coli*/pBBgroE; Lane3, *E. coli*/pBBgGFP37°C; Lane4, *E. coli*/PBBgGFP42°C; Lane5, *E. coli*/pBBg18sGFP37°C; Lane6, *E.coli*/pBBg18sGFP42°C; Lane7, *E. coli*/ pBBgSsGFP37°C; Lane8, *E. coli*/pBBgSsGFP42°C..

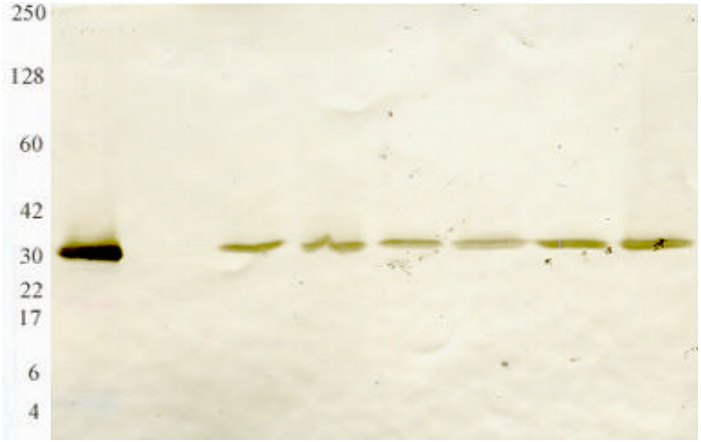


(A) (B)

Fig. 4 Western blot analysis of expression of GFP protein by recombinant *E.coli* strain DH5a using anti-GFP peptide (A) and monoclonal (B) antibody. Lane1, Purified recombinant GFP protein; Lane2, *E.coli*/pBB18psGFP; Lane3, *E.coli*/pBBSpsGFP; Lane4, *E.col* /PBBR1MCS.

1 2 3 4 5 6 7 8

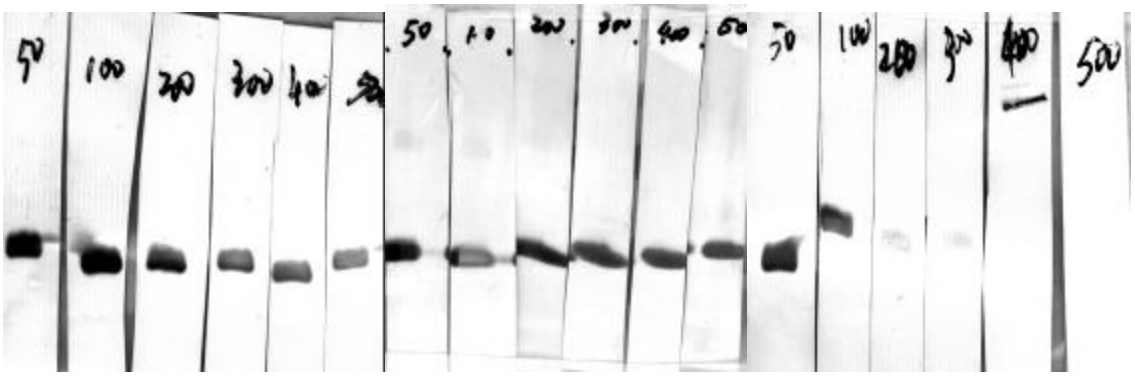
(A)



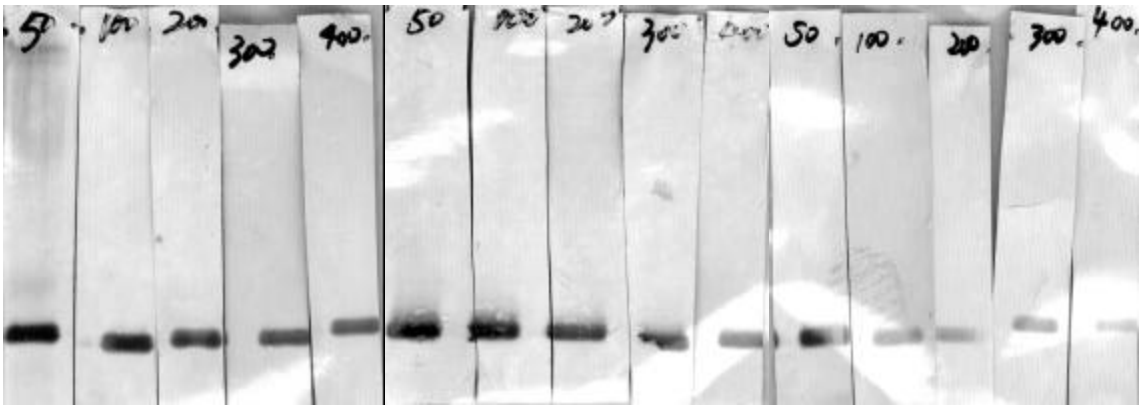
1 2 3 4 5 6 7 8

(B)

Fig 5 Western blot analysis of expression of GFP protein by recombinant B.abortus strain RB51 using anti-GFP peptide (A) and monoclonal (B) antibody. Lane1, Purified recombinant GFP protein; Lane2, RB51 Lane3, RB51/pBBgGFP37°C; Lane4, RB51/PBBgGFP42°C; Lane5, RB51/pBBg18sGFP37°C; Lane6, RB51/pBBg18sGFP42°C; Lane7, RB51/pBBgSsGFP37°C; Lane8, RB51/pBBgSsGFP42°C.

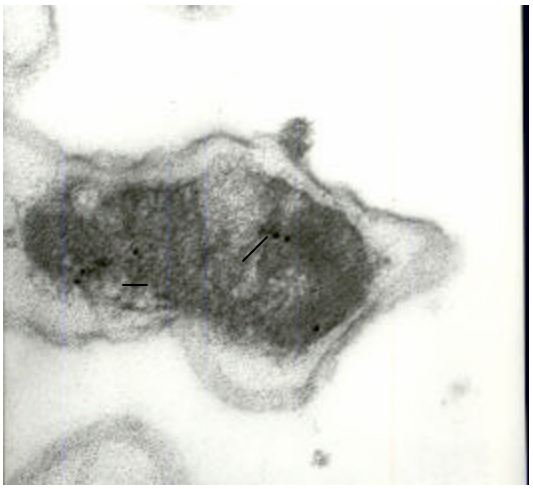


E/pBBGFP E/pBBSpsGFP E/pBB18psGFP

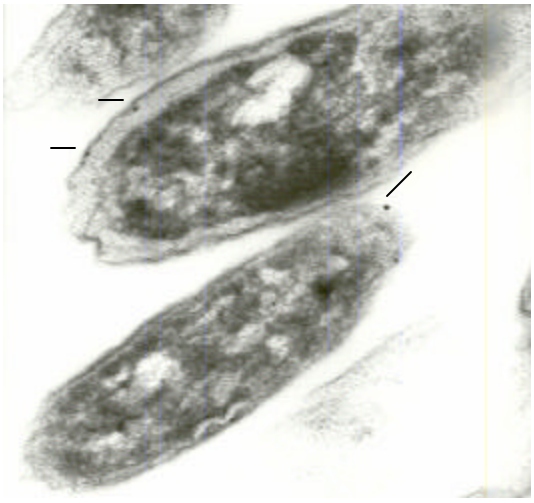


RB51/pBBgGFP RB51/pBBgSsGFP RB51/pBBg18sGFP

Fig 6 Immunoabsorption of GFP expressing *E. coli* strains and *B. abortus* RB51 strains



RB51/pBBgGFP



RB51/pBBg18sGFP

Fig 7 Immunoelectron microscopy of *B. abortus* strain RB51 expressing GFP. The dark black round dots indicate the location of GFP expression.

Fig 8. Western blot analysis of the periplasmic extractions from *B. abortus* RB51/pBBgGFP, RB51/pBBg18sGFP and RB51/pBBgSsGFP by anti-GFP monoclonal antibody. Lane 1, purified recombinant GFP; lane 2, RB51/pBBgGFP; lane 3, RB51/pBBg18sGFP; lane 4, RB51/pBBgSsGFP.

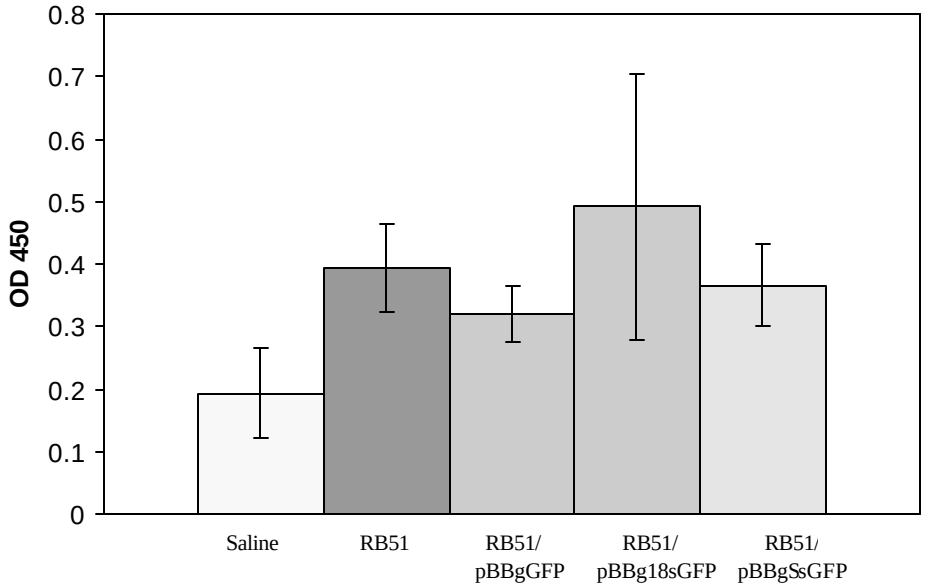


Fig 9 Antibody responses to green fluorescent protein (GFP) stimulated by saline, *B. abortus* strain RB51, RB51/pBBgGFP, RB51/pBBg18sGFP, or RB51/pBBgSsGFP in mice.

Vita

Hailan (Helen) Liu was born in 1976 in Anshan, Liaoning Province, P. R. China, daughter of Changlu Liu and Shuqin Jia. She attended Anshan High School in Anshan , Liaoning Province, P. R. China. She received her college education in Biochemistry Department of Nanjing University from 1994-1998. After graduation, she worked two years in Shanghai Research Center of Biotechnology, Chinese Academy of Sciences in Shanghai. Since August 2000, she has been worked as a master candidate in Veterinary Medical Sciences at the Virginia-Maryland Regional College of Veterinary Medicine of Virginia Polytechnic Institute and State University, Blacksburg, VA.