A STUDY ON *BRUCELLA ABORTUS'S TRP*E GENE

By

BOBBY ALFONSO GRAMLING, JR.

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Langston University

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Thesis Approved:

Richard C Essenberg

Thesis Advisor

Robert L Matt

Ulrich Melcher

Timothy D. Pettitone

Dean of the Graduate College

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NOMENCLATURE

amp	ampicillin
bp	base pair
cam	chloramphenical
cm	centimeter
DNA	deoxyribnucleic acid
hr	hour
kan	kanamycin
kb	kilobase
µl	microliter
µg	microgram
ml	mililiter
NCBI	National Center for Biotechnology Information
PCR	polymerase chain reaction
TB	terrific broth
YENB	yeast extract and nutrient broth

Chapter I

Introduction

Contraction of *Brucella abortus*

There are six recognized species of *Brucella*: DNA hybridization studies suggest that *Brucellae* are part of a monospecific genus. It has been suggested that they be grouped as biovars of the single species *Brucella melitensis* (Michaux-Charachon et al., 2002). *Brucella abortus* causes abortion in cattle, *Brucella melitensis* infects sheep and goats, *Brucella ovis* parasitizes rams. *Brucella canis*, *Brucella suis* and *Brucella neotomae* are pathogenic to dogs, pigs, and rats respectively. The classification is based on differences in pathogenicity and host preference (Michaux-Charachon et al., 1997). *Brucella* belong to the α-2 group of proteobacteria and have close phlyogentic relationships with *Agrobacterium*, *Rickettsia*, *Rhizobium*, *Bartonella*, *Ochrobactrum* and *Rhodobacter* (Teixeira-Gomes et al., 2000). Most of the α-2 proteobacteria are intracellular symbionts or pathogens of plants and animals. *Brucella abortus* is an aerobic, gram-negative coccobacillus that causes a zoonosis in bovines known as brucellosis. *B. abortus* has a special tropism for reproductive organs and in cattle brucellosis is primarily a disease of the female . *B. abortus* localizes in the udder, uterus, lymph nodes, placenta, fetal tissue and mammary tissue. Erythritol encourages the growth of *B. abortus* and is found in cattle placentas; this leads to the aborting of a fetus and an infection of the mammary gland (Lin and Ficht, 1995). In bulls the organism localizes in the testicles and causes inflammation of the testicles, which leads to sterility (Lin and Fitch, 1995). The disease is spread among cattle when they consume contaminated forages or lick calves or aborted fetuses from infected cattle. In humans, *B. abortus*

causes undulant fever defined by headaches, intermittent fever, joint and bone pain, along with various other symptoms (Hoover et al., 1999). The bacteria may enter the body through cuts, mucous membranes, and the digestive or respiratory tracts. The disease is transmitted to humans by direct contact with infected animals, carcasses, or by ingestion of unpasteurized milk or milk products (Borrok, 1998).

The spread of brucellosis is controlled in developed countries by livestock testing, vaccination, and slaughter programs. The disease is a serious problem in parts of Africa, Middle East, Latin America, Asia and the Mediterranean, because it causes severe economic loss (Boschiroli et al., 2001).

Macrophage Killing Mechanisms

An immune response involves recognition of the foreign material or pathogen and mounting a reaction to eliminate it. There are two different types of immune responses: innate (non-adaptive) and adaptive immune responses. The innate response does not change on repeated exposure to the same pathogen, whereas the adaptive response improves each time with the same pathogen. The adaptive immune response is highly specific for a pathogen because the immune system remembers the pathogen and can mount a more rapid and effective response with each encounter preventing it from causing disease later (Roitt et al., 1998). Phagocytes like monocytes, macrophages, and polymorphonuclear neutrophils play an important role in the innate immune response by engulfing and killing pathogens. Lymphocytes have a significant role in adaptive immune responses because they can distinguish individual pathogens anywhere in the body. Lymphocytes fall into two categories: T lymphocytes (T cells) and B lymphocytes

(B cells). T cells have a variety of functions ranging from the development of B cells and antibody production, to helping phagocytes destroy internalized pathogens, and recognizing infected cells and destroying them. B cells fight extracellular pathogens by releasing antibodies, which bind to specific targets on the pathogen, called the antigen (Roitt et al., 1998). Antigens can be toxins that the pathogen makes or a molecule on its surface. There are some interactions between the phagocytes and the lymphocytes. Phagocytes can take up an antigen and present it to T cells in a form they can recognize, a process termed antigen presentation. As a result, T cells release cytokines that activate phagocytes to destroy the foreign material they have engulfed. Also the antibodies produced by B cells are used by phagocytes to identify pathogens more effectively.

B. abortus is a facultative intracellular pathogen capable of surviving inside macrophages and other professional phagocytes (Arenas et al., 2000). Macrophages derive from bone marrow stem cells which, after differentiation to blood monocytes, finally settle in the tissue as mature macrophages. The function of macrophages is to engulf foreign materials and destroy it by a process termed phagocytosis. During phagocytosis the foreign material is surrounded by the membrane of the macrophage, and is internalized in a plasma membrane derived organelle (called a phagosome) in the cytoplasm of the macrophage (Desjardins et al., 1997). Macrophage cells have developed several mechanisms to kill pathogens once they are engulfed. These killing mechanisms can be put into two categories: oxygen dependent and oxygen independent killing mechanisms.

Oxygen dependent killing mechanisms involve an enzyme in the phagosome membrane, which reduces oxygen to superoxide anion (O_2^-), a reactive oxygen

intermediate (ROI). This can give rise to hydroxyl radicals (•OH), singlet oxygen, and hydrogen peroxide (H_2O_2); all are toxic and can be secreted into the phagosome. A second mechanism, if lysosome fusion occurs, is that myeloperoxidase may enter the phagosome. Myeloperoxidase acts on peroxides in the presence of halides (preferably iodide). Additional toxic oxidants like hypohalite (HIO,HClO) are then generated. Also, there is the nitric oxide pathway, where inducible nitric oxide synthase combines oxygen with the guanidino of L-arginine with tetrahydrobiopterin as a cofactor, making nitric oxide toxic for the pathogen (Roitt et al., 1998). Some oxygen independent mechanisms are the acidification of pathogen-containing phagosomes to a harmful, low pH, and the fusion of phagosomes with lysosomes to form phagolysosomes (Tjelle et al., 2000)). The lysosomes are full of hydrolytic enzymes for particle digestion that are active at a very low pH.

Intracellular Survival In Phagocytic Cells

Just as phagocytic cells developed various ways of killing pathogens, intracellular pathogens have developed various methods to offset these host cell assaults, leading to survival and multiplication in macrophages (Arenas et al., 2000). *B. abortus* has found ways to survive under extreme conditions inside macrophages and interfere with macrophage killing mechanisms. Evidence shows that following phagocytosis by macrophages, *B. abortus* resides in the phagosome, where the bacteria multiply and remain enclosed during infection (Harmon et al., 1988). Once foreign material is in the phagosome the phagosome undergoes a maturation process where it changes into a phagolysosome. The maturation process is a series of fusion events with early and late

endosomes as well as lysosomes that modify the composition of the membrane and its contents. The environment of phagosomes is a malicious one characterized by a low pH (pH 4.0 to 4.5), limited nutrition, and low oxygen tension (Ko et al., 2000). Changes to the normal process of phagosome maturation have been outlined for several pathogens such as *Mycobacterium*, *Legionella*, *Chlamydia*, *Salmonella* and *Listeria* (Pizarro-Cerda et al., 1998). *Brucella* inhibits the phagosome-lysosome fusion; results indicate that *Brucella* significantly delays fusion with preformed lysosomes and prevents the interaction with newly formed endosomes (Porte et al., 1999). Generally, the maturation of newly formed phagosomes to phagolysomes is a fast process. The postponed maturation seen for *Brucella* may be significant in preventing degradation and allowing time for the bacteria to express genes for intracellular survival. To survive in the phagosome of macrophages, *Brucella* needs particular ways to deal with the low pH, restricted nutrition, and low oxygen tension (Porte et al., 1999). Studies using two-dimensional polyacrylamide gel electrophoresis (PAGE) have shown that *B. abortus* is able to change its protein synthesis and gene expression patterns when subjected to specific environmental and stress conditions which may mimic the environment in macrophages during intracellular growth (Rafie-Koplin et al., 1998). A more in depth study of each protein induced during macrophage infection could possibly shed some light on how *B. abortus* and other intracellular pathogens are able to survive in such a hostile environment.

Virulence Factors

Brucella are able to survive and replicate within macrophages and bypass the microbial killing mechanisms of the cell. This leads to the hypothesis that *Brucella*

virulence is related to the ability to multiply inside the host cells. The specific mechanisms and virulence factors that allow *Brucella* to deal with the intracellular environment of phagocytic cells are poorly understood. It was first thought that erythritol was necessary for *B. abortus* virulence but mutants in erythritol catabolism were able to remain virulent (Sola-Landa et al., 1998). Several studies have shown that DnaK, HtrA, Cu-Zn superoxide dismutase (SOD) and RecA are possibly involved in virulence (Teixeira-Gomes et al., 2000). DnaK is a heat shock protein that is essential for intracellular survival and replication of pathogens. High temperature requirement A (HtrA) is a member of stress proteins that are serine proteases which function by degrading damaged proteins before they can accumulate to toxic levels in the cell. Copper-Zinc superoxide dismutase [Cu-Zn]-SOD is a member of the metalloproteins that catalyze the dismutation of the highly reactive superoxide radical anion to hydrogen peroxide and oxygen. RecA plays a role in the SOS response with UV repair in addition to its role in recombination repair. *Brucella* deletion mutants for these proteins showed reduced survival rates during the first days of infection but they were able to establish chronic infections (Teixeira-Gomes et al., 2000). Also an interruption in the *cyd*B gene, that is part of the *cyd*AB operon encoding cytochrome bd oxidase, which catalyzes an alternate terminal electron transport step in bacterial respiration, by Transposon5 (Tn5) mutagenesis showed attenuated intracellular survival and virulence in the mouse model of infection (Endley et al., 2001). A modification of the classical transposon mutagenesis, called signature-tagged transposon mutagenesis (STM), has the advantage in that a large number of mutants can be screened for attenuation at one time. In transposon mutagenesis each individual mutant has to be screened to identify an attenuated

phenotype (Foulongne et al., 2000). The STM technique has turned up a lot of mutants that are involved in the virulence of *Brucella*. Mutations in genes that are involved in basic metabolism like: *aro*C, encoding chorismate synhthase, the final enzyme in the biosynthesis pathway of chorismate; *pgi*, coding phosphoglucose isomerase, which converts glucose-6-phosphate to fructose-6-phosphate; *gpt*, encodes hypoxanthine-guanine phosphotransferase, an enzyme involved in nucleotide biosynthesis; *pyc*, codes for pyruvate carboxylase that catalyzes the ATP-driven formation of oxaloacetate from pyruvate and HCO_3^-; *gln*D coding for a uridylyl transferase, which controls the concentration of glutamine in host cells. The mutants were attenuated in both macrophages and HeLa cells (Boschiroli et al., 2001). Signature-tagged transposon mutagenesis also displayed mutants in genes that are included in transport, secretion, stress/detoxification, regulation, and in the bacterial envelope (Boschiroli et al., 2001). Mutants that had insertions in genes involved in the VirB type IV secretion system were unable to establish infections in the mouse model (Boschiroli et al., 2002). Type IV secretion systems are a family of multiprotein complexes that serve to secrete macromolecules. Examples of systems playing a role in pathogen virulence are the VirB system in *A. tumefaciens* involved in DNA transfer of the Ti plasmid. The Ptl system of *Bordetella pertussis* secretes the pertussis toxin. The Dot/Icm type IV system of *Legionella pneumophila* exports the macromolecules, which affect the maturation of the phagosome by allowing the bacteria to, developed intracellularly. In *Helicobacter* pylori, the proteins encoded by genes of the *cag* pathogenicity island translocates an effector molecule which activates the NF-κB signaling pathway.(Foulongne et al., 2000). Smooth lipoploysaccharide (LPS) is an important factor in the virulence of *Brucella* because

rough mutants are usually attenuated or nonvirulent. Smooth strains of *Brucella* contain an O antigen as part of their LPS structure. The O antigen of *Brucella* is a homopolymer of 4,6-dideoxy-4-formamido-α-D-mannopyranose (N-formyl perosamine). Insertions in the *man*B gene encoding phosphomannomutase, an enzyme involved in the synthesis of perosamine, converting mannose-6-phosphate to mannose-1-phosphate, were unable to synthesize the O antigen (Foulongne et al., 2000). Another study shows that a knockout mutant for the *hem*H gene, a member of the ferrochelatase family, of *B. abortus* demonstrated the mutant was unable to survive inside murine J774 macrophages, in human HeLa cells, and did not cause virulence in BALB/c mice (Almiron et al., 2001). Recent studies identified *exs*A, a gene encoding an ATP-binding cassette (ABC) transporter of *B. abortus* strain 2308. ABC transporters use the free energy of ATP hydrolysis to pump substances across the membrane against a concentration gradient into or out of cells. A deletion mutant was obtained by gene replacement and there was a decreased survival in mice with the *exs*A mutant when compared to the wild-type strain 2308 (Rosinha et al., 2002). This shows that *exs*A is involved in virulence during *Brucella* infection. All of these studies are useful in the process of trying to identify *Brucella* genes that are critical for virulence.

Auxotropic mutants

In the 1950's, Bacon and coworkers established the link between bacterial metabolism and virulence, by mutagenizing a virulent strain of *Bacterium typhosum* and showing that certain mutants including those requiring leucine, purines, or para-amino benzoic acid for growth, were less virulent for mice when compared to the wild type

genes (Hondalus et al., 2000). Because of these early experiments, several groups have reported auxotropic mutants in *Salmonella*, *Legionella*, *Shigella* and *Corynebacterium* to be attenuated for growth in vitro within macrophages or in animals. *Salmonella* (*aro*A), *Cornybacterium* (*aro*Q), and *Shigella* (*aroA* & *aro*D) had mutations in genes for the common aromatic amino acid biosynthesis pathway, with the end product being chorismate, the precursor to tyrosine, tryptophan, and phenylalanine (Hondalus et al., 2000). Legionella mutants were deficient in the synthesis of tryptophan and thymidine. A study in *Mycobacterium tuberculosis* showed a leucine auxotroph, constructed by insertional disruption of *leu*D, which encodes isopropyl malate isomerase, an essential enzyme for leucine biosynthesis. The results of this work demonstrated that *M. tuberculosis* with a deletion mutation in *leu*D cannot multiply in either macrophages or mice (Hondalus et al., 2000). *Mycobacterium tuberculosis*, also had mutations resulting in avirulence in the trpD (tryptophan) and proC (proline) genes (Smith et al., 2001). In *B. melitensis*, the purEK operon has been isolated and sequenced. Deletions were made in the *pur*E and *pur*K genes. The mutation in the *pur*E gene led that mutant to require exogenous purines for growth and had attenuated replication within human monocyte-derived macrophages (MDM)(Drazek et al., 1995). Mutations in the *B. suis aro*C gene, encoding chorismate synthase, the final enzyme in the synthesis of chorismate, was highly attenuated in tissue culture (THP1 macrophages and HeLa cells) and murine virulence models (Foulongne et al., 2001). All of these auxotropic mutants would be potential candidates for vaccines because live vaccines are more efficient and provide better protection against intracellular pathogens.

Vaccines

Vaccination with live *B. abortus* strains (S19 & RB51) has been effective in preventing *B.abortus* infections and abortions in cattle. Live vaccines for intracellular organisms are usually more efficient and consist of attenuated variants of a particular pathogen which have lost the ability to cause clinical disease but are still able to induce an immune response in the host (Vemulapalli et al., 1999). Strain 19 (S19) is a naturally occurring smooth attenuated strain of *B. abortus* and was the vaccine used for cattle brucellosis. *B. abortus* strains having a smooth phenotype contain a surface exposed O polysaccharide chain (O antigen) as part of their lipopolysaccharide (LPS) structure (Vemulapalli et al., 2000a). Research has shown the smooth LPS in *B. abortus* is a virulence factor because rough strains that were derived from smooth virulent strains were attenuated and only smooth *B. abortus* strains could replicate in macrophages (Vemulapalli et al.,2000b). Since S19 is a smooth strain of *B. abortus* it contains the O side chain that is responsible for an immunodominant antibody response after vaccination. S19 has been helpful in the prevention of brucellosis but there are some problems with its use like: it is too virulent for human use; there is the occasional abortion in pregnant animals; and there is difficulty in differentiating between vaccinated and infected animals (He et al., 2002). Rough strains like RB51 do not have the O antigen in their LPS, and thus became the official strain for vaccination against cattle brucellosis. RB51 is a stable rough mutant derived from its parent strain 2308, a smooth and virulent strain of *B. abortus* (Vemulapalli et al., 2000a). RB51, S19, and *B. melitensis* Rev 1 are being used to control brucellosis in animals but there is no safe vaccine available to humans. To actually control brucellosis a vaccine is needed that will be

noninfectious to humans and effective in stimulating an immune response in humans and animals. Several groups are trying different techniques to develop a better Brucella vaccine such as development of subunit vaccines, the utilization of vaccinia virus as vector, over expression of protective homologous antigens, and attenuating certain strains by deletion or insertion of specific genes.

Why tryptophan

Microbes, living in an intracellular location, must develop mechanisms to access available nutrients or extinction will ultimately occur. Little is known about the nutrients available in the phagolysosomes. McCullough & Deel in 1943 and Gerhardt & Wilson in 1948 both showed that *Brucella* was able to synthesize tryptophan by its ability to grow on minimal media. Whether tryptophan is present in the phagolysosome or if *Brucella* needs tryptophan for its intracellular survival, has yet to be determined. Macrophages are known to posses the tryptophan degrading enzyme indoleamine 2,3 dioxygenase (IDO), which catalyzes the initial and rate limiting step in the metabolism of tryptophan along the kynurenine pathway (Munn et al., 1999). This enzyme is induced by interferon-gamma (IFN-gamma). IDO's role is thought to function as a host defense mechanism, inhibiting the replication of intracellular pathogens, by depleting the supply of tryptophan (Mellor et al., 1999). Anthranilate synthase is the first enzyme of the tryptophan pathway (Fig.1). The active enzyme is encoded by an aggregate of two genes *trpE* (component I) and *trp*D (component II). Tryptophan inhibits the activity of anthranilate synthase and anthranilate phosphoribosyl transferase activity of the aggregate. Component I has been purified from *E. coli* and *S. typhimurium*, has a molecular weight around 60,000 and contains the binding site for chorismate. Component I alone cannot catalyze the

formation of anthranilate using glutamine as the nitrogen source, but it can form anthranilate with reduced efficiency using ammonia in place of glutamine. Component II has been purified from *S. typhimurium* and also has a molecular weight about 60,000. It contains two activities, first a glutamidotransferase activity, that is required to activate component I in the anthranilate synthase reaction. The second activity converts anthranilate to anthranilate-5-phosphoribosyl pyrophosphate, termed anthranilate phosphoribosyl transferase (Neidhardt et al., 1996). In certain species anthranilate synthase is the product of the *trp*E(G) complex. This is the case for *Rhizobium meliloti*, a plant symbiont to *B. abortus* (Bae et al, 1990).

There are no published reports on *B. abortus* having an amino acid uptake system but there are known amino acid sequences for *B. melitensis* and *B. suis* that show many potential transporters, including several for amino acids. The *Brucella* species show over 90% homology in DNA-DNA hybridization assays (Wang et al., 2001), since the species are closely related, one could make an educated guess about sequences that are in *B. abortus*. That is why I am investigating whether tryptophan is necessary for intracellular survival inside macrophage cells. I tried to starve *Brucella* cells of tryptophan, an essential amino acid, by disrupting the *trp*E gene, which encodes the enzyme anthranilate synthase, the first enzyme in the synthesis of tryptophan. I used the auxotrophic mutant approach based on research done on several intracellular pathogens previously described that share similar characteristics with *B.abortus*. Those studies were used as a model for my research.

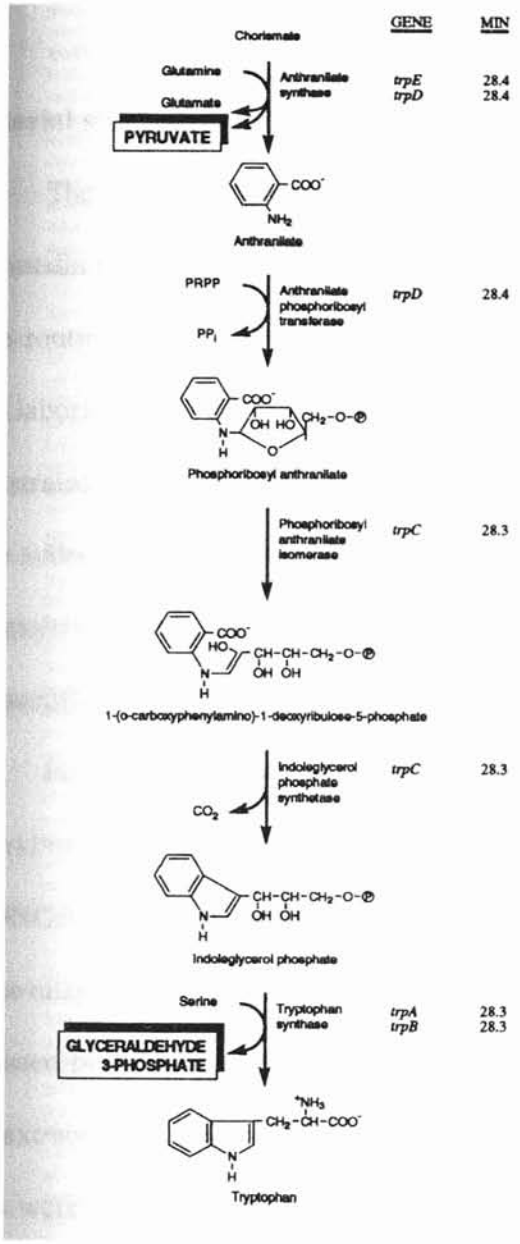

Figure 1. Terminal pathway of tryptophan biosynthesis

Chapter II

Materials and Methods

Bacterial strains, plasmids, media and growth conditions

The bacterial strains and plasmids used in this study are listed in Tables 1 and 2. The media for growing *B. abortus* and *E. coli* strains are listed in Table 3. *E. coli* strains were routinely cultured at 37°C overnight. *Brucella* cells are grown and handled in a BL3 laboratory at 37°C under an atmosphere containing 5% CO_2 for 4 to 6 days. For *E. coli* strains, 50 μg/ml ampicillin, 30 μg/ml kanamycin and 30 μg/ml chloramphenical were added as required, while *Brucella* strains required 100 μg/ml ampicillin, 60 μg/ml kanamycin and 30 μg/ml chloramphenical.

Preparation and manipulation of DNA

Plasmid DNA was purified by the QIAprep Spin Miniprep Kit Protocol (Qiagen). Restriction endonucleases, *Bam*HI, *Sau*3AI, *Bgl*I, *Pst*I, *Eco*RI, *Sty*I, *T4* DNA ligase and ELONGASE Enzyme Mix were purchased from Gibco BRL, Promega and Invitrogen. These enzymes were used under conditions specified by their manufacturers unless noted. Digested plasmids were separated by electrophoresis on 0.8% low melting agarose gels and extracted by the QIAquick Gel Extraction Kit Protocol (Qiagen). Competent *E. coli* cells were made and transformed (Heat-Shock) by the method of Inoue et al., 1990. Standard techniques were used for electrophoresis and molecular cloning.

Nucleotide sequencing

pRE271 was transformed into competent *E. coli* cells and purified by methods previously described (Inoue et al., 1990). The nucleotide sequences were determined by the Oklahoma State University Recombinant DNA/Protein Resource Facility. Gaps were

filled in by primers designed from the known sequence using the software package Oligo 4.0 (National Biosciences) and were synthesized by the Oklahoma State University Recombinant DNA/Protein Resource Facility. The DNA sequences were aligned using the Genetics Computer Group (GCG) Fragment Assembly system, which is a series of programs that help assemble overlapping sequences. ORFs were determined using Mac Vector (Oxford Molecular). A NCBI Blast search determined that the sequence matched similar sequences, which encode for the enzyme, anthranilate synthase.

Construction of suicide plasmids

Homologous recombination was used in an attempt to replace the endogenous chromosomal copy of the *trpE* gene with an inactivated copy of the gene. pBluescript has a ColE1 origin so it is a suicide plasmid because it cannot replicate in *Brucella*. pRE271, which has the pBluescript vector contains an ampicillin resistance (*bla*) and *trpE* gene, and was digested with *Sty*I which left an 894 base pair deletion (cuts at positions 1857 & 2751) in the *trpE* gene (Fig 2). The digested plasmid was separated by electrophoresis on a 0.8% agarose gel and the appropriate band was extracted as previously described (Qiagen). Then the plasmid was ligated back to itself with 1.5 units of *T4* DNA ligase (pBAG), transformed into competent *E. coli* cells, and a Qiagen kit was used to isolate the plasmid. pBAG was then partially digested with a 1:100 dilution of 10 units of *Sau*3AI for 12 minutes and for 13 minutes (pLAG), the digested plasmid (pLAG) was separated by electrophoresis on a 0.8% agarose gel and the appropriate band was extracted as previously described (Qiagen). pUM24 (Reid and Collmer, 1987) was digested with 5 units of *BamHI* and was separated by electrophoresis on a 0.8% agarose gel and the appropriate band (the 3.8 kb fragment, *sac*B-kanamycin resistance cassette)

was extracted as previously described (Qiagen) and was ligated to pLAG (pMAG) with 1.5 units of *T4* DNA ligase (Fig 3 & 4). *BamHI* and *Sau*3AI leave the same ends after digestion. The purpose of the partial digestion was to try and get the *sac*B-kanamycin cassette inserted into the *trpE* sequence of pRE271. pRE271 was partially digested with a 1:100 dilution of 10 units of *Sau3*AI for 3 minutes (pLAGI), the digested plasmid (pLAGI) was separated by electrophoresis on a 0.8% agarose gel and the appropriate band was extracted as previously described (Qiagen). The extracted plasmid vector was ligated to the *sac*B-kanamycin cassette from pUM24 digested with 5 units *Bam*HI (pJAG) (Fig 5).

Complementation using JA200 cells

pBAG, pJAG, and pMAG were transformed into JA200 cells (*trpE* minus strain of *E.coli*) and plated on LM plates containing ampicillin. The next day the cells were replicated onto minimal plates containing glucose, threonine, leucine and vitamin B1, followed by everything mentioned plus tryptophan and then back onto a LM plate containing ampicillin (Fig 6). Growth was scored after two days by comparing the two minimal plates in the same orientation and looking at the colonies to determine which plates the cells were able to grow on.

Preparation of competent *Brucella* cells

Wild type *Brucella* cells (2308) were grown in 5ml YENB (Yeast Extract and Nutrient Broth) media for 33 h at 37°C with shaking. Then 2.5 ml of the overnight culture was added to 50 ml of fresh YENB media and the mixture was placed in a 37°C shaker until the Klett reading was between 70 and 80. The cells were harvested by chilling the flask on ice for 5 min and spinning the cells at 4000 x g for 10 min at 4°C.

The medium was removed and discarded, then the pellet was washed twice by resuspending in 5 ml of cold water and centrifuging at 4000 x g for 10 min at 4°C. The supernatant was again removed and discarded. Then the pellet was resuspended in 1 ml of cold 10% glycerol and centrifuged and the supernatant removed and discarded like before. The cells were resuspended to a final volume 200-300 μl of cold 10% glycerol (Sharma and Schimke, 1996).

Electroporation of suicide plasmids into *B. abortus*

50 μl of competent *Brucella* cells were placed in a microfuge tube and 1.5-2.1 μg of DNA was added. This was mixed and placed on ice for 1 min. The cells and DNA were transferred to a 0.1 cm electroporation cuvette. The cuvette was put in its holder and was attached to the power unit. The voltage was set to 1250 and the resistance to R6 or R7, then it was pulsed. The cuvette was immediately removed and the cells washed out with 1 ml SOC-B and placed in a sterile microfuge tube. The microfuge tubes were open and incubated at 37°C, 5% CO_2 for 5 minutes. After the 5 minutes the tubes were closed and they continued incubating for 1 h. Then the cells were centrifuged for 5 min in a Millipore microfuge. The supernatant was removed and decontaminated. The pellet was resuspended in 0.2 ml SOC-B and plated on 2 SOC-B plates (0. 1ml each) with no antibiotic. The plates incubated overnight at 37°C, 5% CO_2. The next day the growth was removed and resuspended in 0.2 ml tryptose. This was spread on 2-4 tryptose plates with selective antibiotics (Fig 7) and incubated at 37°C, 5% CO_2 for 4-6 days (Lai et al., 1990).

Killing *B. abortus* cells with methanol

In order to remove *B. abortus* cells from the BL3 lab for DNA extraction to check if the mutated gene is present in *B. abortus*, the cells have to be killed. First the cells are grown in 3 ml of tryptose broth overnight at 37°C. Next the cells are transferred to a sterile microfuge tube and are spun for 5 min, the supernatant was removed and decontaminated. The pellet was resuspended in 0.1-0.2 ml tryptose broth and 1-2 ml of methanol was added. This was incubated at room temperature for 5 min and then the cells were spun for 5 min, the supernatant was removed and decontaminated. Again, the cells were resuspended in 0.1-0.2 ml tryptose broth and 1-2 ml of methanol was added. Then the cells were incubated at room temperature for 5 min and were centrifuged for 5 min, the supernatant was removed and decontaminated. The cells were resuspended in 0.1-0.2 ml tryptose broth and the viability of killed cells was checked by spreading 10 µl on tryptose plates and growing for 2 days at 37°C, 5% CO_2.

Genomic DNA extraction of *B. abortus*

Once the cells were killed the genomic DNA from *B. abortus* was extracted as described except that the proteinase K digestion was carried out overnight at 37° instead of 1 hr (Wilson, 1994).

PCR

Based on pRE271 sequence, primers were designed from the program Oligo 4.0 to amplify the part of the *trpE* gene insert containing the *Sty*I sites. The forward primer was 5'CGGCTGGCTTCMAAGTTAGGG and the reverse primer 5'GGAAACGAAAGAGTTTGGGTAG. PCR was performed using Elongase following the protocol from Invitrogen and the amplification conditions were 94°C for 30 sec,

followed by 40 cycles of 94°C for 30 sec, 55°C for 30, and 68°C for 5 min. The amplified PCR products were separated by electrophoresis on 0.8% agarose gels.

Southern blot

A Southern blot was performed on the genomic DNA of *B. abortus* to determine if the samples actually had the *trpE* gene present and to detect if the *sac*B-kanamycin cassette was also present. The transfer of DNA to the membrane was followed as described in the S&S TurboBlotter and Blotting Assembly for Alkaline and Neutral Transfer. The probe was labeled with photobiotin (Forester et al., 1985) and detected by the Southern-Light & Southern-Star Chemiluminescent Detection System (Tropix).

Strains	Characteristics	Source or reference
E.coli		
XL-1 Blue	rec A1 endA1 gyrA96 thi-1 *hsd*R17 supE44 rel A1 lac[F' proAB lacI^{q} ZΔM15 Tn10(Tet^{r})	Stratagene
JA200	F^{+}/ thr-1 leu B6 Δtrp E63 recA56 thi-1 ara-14 lacYl gal K2 gal T22 xyl-5 mtl-1 λV-supE44	*E. coli* Genetic Stock Center (# 6059)
B. abortus		
2308	wild type strain	OADDL

OADDL, Oklahoma Animal Disease Diagnostics Laboratory, Oklahoma State University, Oklahoma

Table 1. Bacterial Strains used in this study

Plasmids	Characteristics	Source or reference
pBluescript SK+	cloning vector, ColE1 origin, amp^r	Stratagene
pUM24	*sac*B-kan^r cassette	Reid and Collmer,1987
pRE271	pBluescript with trpE gene	RCE
pBBR 1MCS	cam^r, can replicate in Brucella	Kovach et al., 1994
pBAG	pRE271 with a 894 bp deletion in the trpE gene	This work
pLAG	pBAG partially digested with Sau3AI for 12 & 13 min	This work
pLAGI	pRE271 partially digested with Sau3AI for 3 min	This work
$pMAG_{12\ \&\ 13\ min}$	pUM24 digested with BamHI for the sacB-kanamycin cassette ligated to $pLAG_{12\ \&\ 13\ min}$	This work
$pJAG_{3\ min}$	*sac*B-kanamycin cassette from pUM24 ligated to $pLAGI_{3\ min}$	This work

RCE, Richard C. Essenberg

Table 2. Plasmids used in this study

Media for growing *E. coli.*	Reference
LM	Hanahan, 1983
TB	Tartof and Hobbs, 1987
Media for growing *B. abortus*	
Tryptose Broth	Difco
SOC-B	Lai et al., 1990
YENB	Sharma and Schimke, 1996
Minimal media with carbon sources at 0.1%	McCullough and Dick 1943

Table 3. Media used in this study

A.

B.

pRE271 + *Sty*I=

Figure 2. Construction of pBAG
red = *trp*E, red with black diagonal lines = *trp*E with deletion, yellow = pBluescript vector, green = amp^{r}A.pRE271 has a ampicillin resistance and a t*rp*E gene. **B.** pRE271 digested with *Sty*I leaves a 894 base pair deletion (cuts at positions 1857 & 2751) in the *trp*E gene.

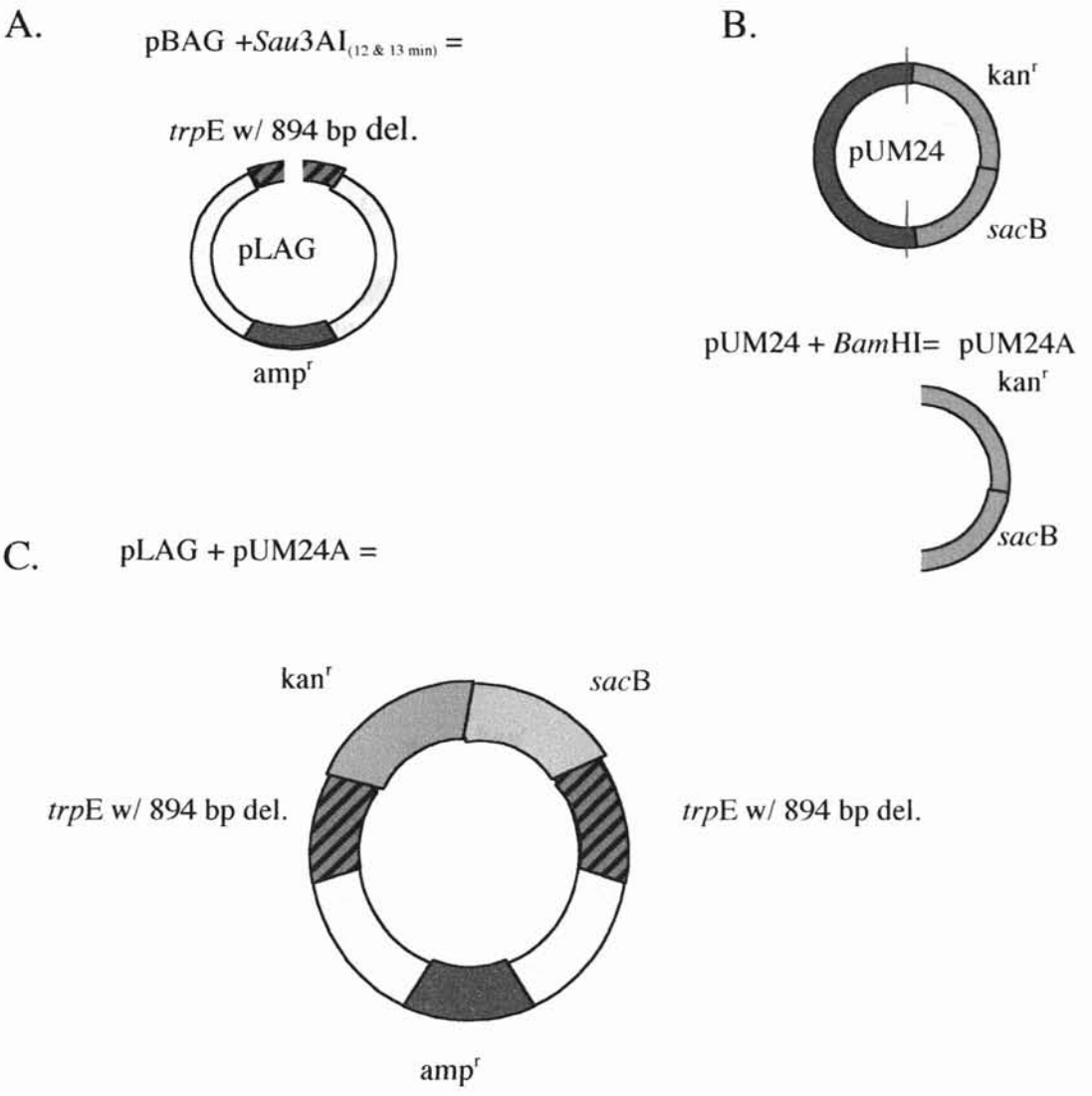

Figure 3. The ideal construction of pMAG$_{(12 \& 13 \text{ min})}$
red = *trp*E, red with black diagonal lines = *trp*E with deletion, yellow = pBluescript vector, green = amp^r, aqua = kan^r, orange = *sac*B
A. pBAG partially digested with *Sau*3AI for 12 & 13 minutes (pLAG). **B.** pUM24 digested with *Bam*HI the *sac*B-kanamycin resistance cassette. **C.** *sac*B-kanamycin resistance cassette from pUM24 ligated to the pLAG $_{(12 \& 13 \text{ min})}$.

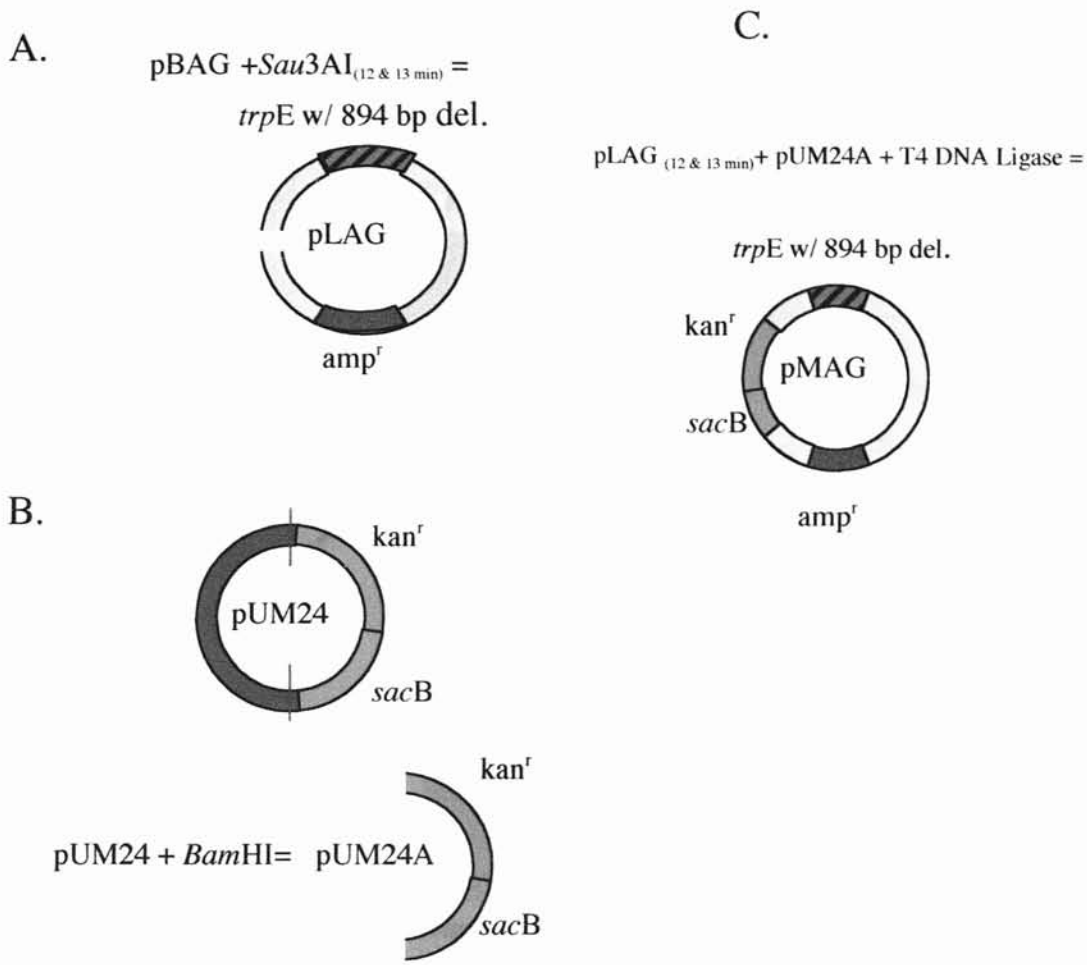

Figure 4. Construction of pMAG$_{(12 \& 13 \text{ min})}$
red = *trp*E, red with black diagonal lines = *trp*E with deletion, yellow = pBluescript vector, green = amp^r, aqua = kan^r, orange = *sac*B
A. pBAG partially digested with *Sau*3AI for 12 & 13 minutes (pLAG). **B.** pUM24 digested with *Bam*HI, which leaves the *sac*B-kanamycin resistance cassette. **C.** pLAG $_{(12 \& 13 \text{ min})}$ ligated to the *sac*B-kanamycin resistance cassette from pUM24.

Complementation using JA200 cells

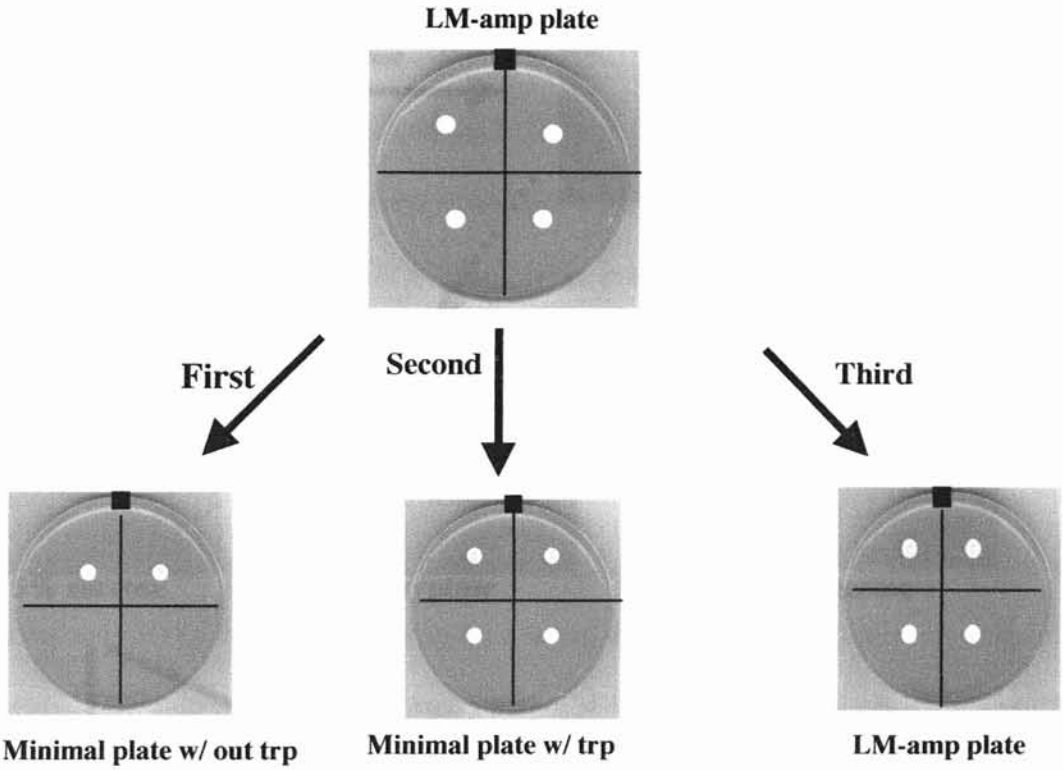

Figure 6. Diagram of complementation using JA200 cells

The black square is for orientation, which is important when replicating from plate to plate and the circles represent colonies.

pRE271, pJAG, and pMAG were transformed into JA200 cells (*trp*E⁻ strain of *E.coli*) and plated on LM-amp plates. **First** the LM-amp plate is replicated onto a minimal plate with out trp. **Second** the LM-amp plate is replicated onto a minimal plate with trp. **Third** the LM-amp is replicated onto a LM-amp to verify the replication is working. Growth was scored after two days by comparing the plates in the same orientation to determine which plates the colonies were able to grow on. No growth is expected on minimal plates w/out trp. So the bottom two colonies would be chosen since they were unable to grow w/out trp.

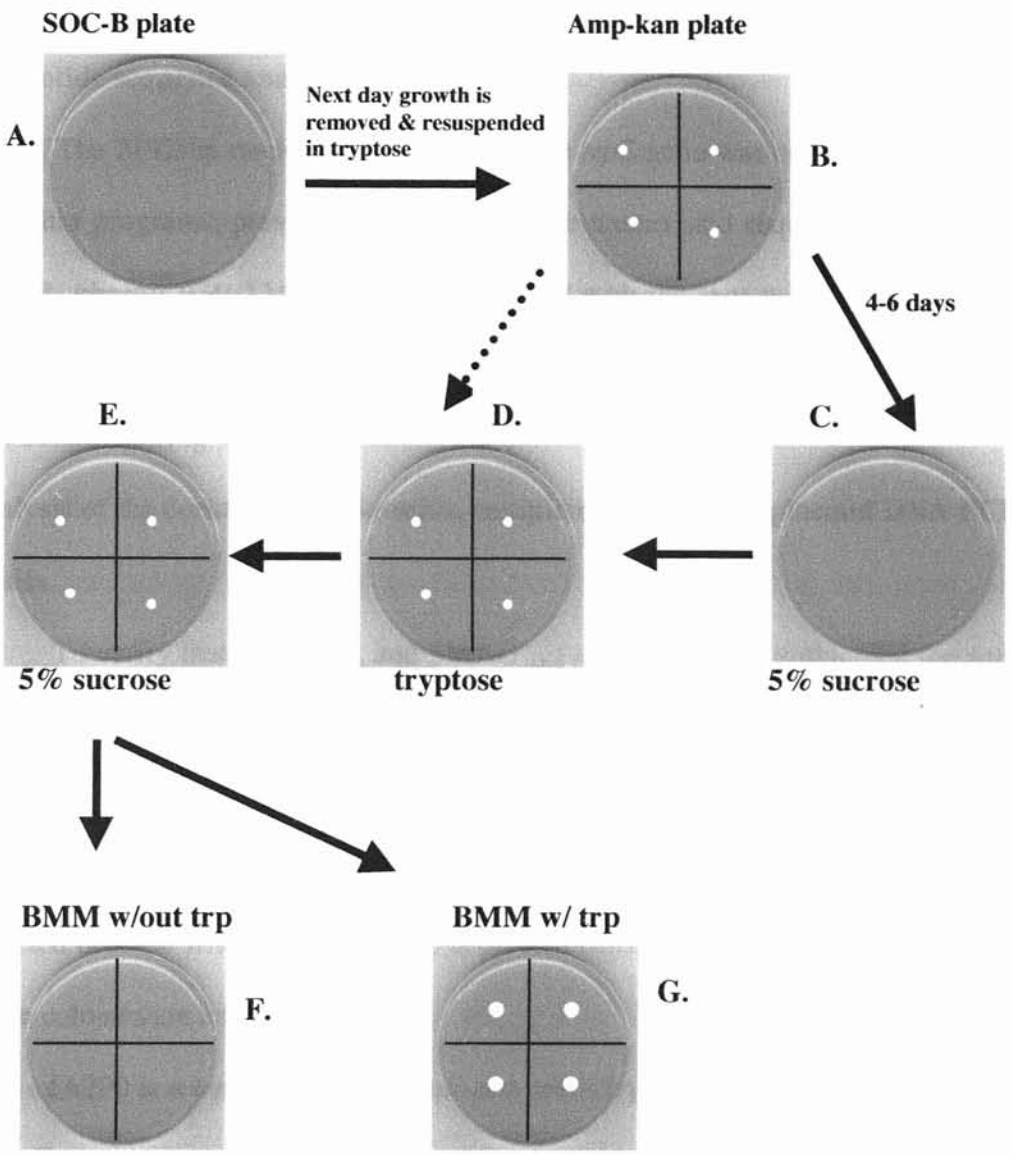

Figure 7. Diagram of electroporation

Circles represent colonies.

A. pBBR1MCS, pJAG, and pMAG were electroporated into *Brucella* cells and plated on SOC-B plates. **B.** The next day the growth was removed & resuspended in tryptose and spread onto an amp-kan plate and incubated at for 4-6 days at 37°C, 5% CO_2, only colonies that underwent the first crossover will grow. **C.** Then the colonies are streaked onto a 5% sucrose plate and no colonies should grow because the *sac*B gene. **D.** The amp^r-kan^{r}suc^s colonies are now streaked onto a tryptose plate, which allows the second crossover event to take place, during this event the plasmid vector is lost. **E.** The colonies are now streaked onto a 5% sucrose plate and this time the colonies should grow because the *sac*B gene is lost. **F.** The suc^r colonies are streaked onto BMM w/out trp and **(G)** BMM w/ trp. Growth is expected on plates w/ trp but no growth on plates w/out trp. The colonies that cannot grow on plates w/out trp are selected.

Chapter III

Results

Nucleotide sequence analysis of the *trp*E gene

The 2192-bp nucleotide sequence of the *trp*E gene was confirmed by a series of computer programs, previously mentioned. The amino acid sequence of the *trp*E gene from *B. abortus* (GI: 13487153) was compared with the sequences of *Azotobacter* (GI: 23105933), *Rhizobium* (GI: 15966140) (Fig 8), *B. suis* (GI: 23502434) and *B. melitensis* (GI: 17986732)(Fig 9).

Analysis of the construction plasmids, complementation and plasmid DNA PCR results

To verify that pJAG $_{3min}$ and pMAG $_{12 \& 13 min}$ successfully obtained the *sac*B-kan cassette, they were transformed using competent *E. coli* cells and plated on amp-kan plates. Single amp^r-kan^r colonies were compared by streaking them onto 5% sucrose amp-kan plates and amp-kan plates. There was little growth on the 5% sucrose plates as expected because when the *sac*B gene is present cells die in the presence of 5% sucrose. So the colonies are amp^r-kan^r-suc^s.

JA200 is a *trp*E$^-$ strain of *E. coli* and needs tryptophan in the medium to grow or the trpE gene can complement it or it can survive with a transformation with pRE271. So a transformation with a deletion or insertion in the *trp*E gene will fail to complement. The purpose of replicating the cells onto the minimal plates without trp, then with trp was to make sure that the deletion in the gene inactivated *trp*E. pRE271 and pJAG $_{3min}$ grew on both plates with or without tryptophan as expected. pMAG $_{12 \& 13 min}$ did not grow on plates without tryptophan because the plasmid has an 894bp deletion in the *trp*E gene.

B. abortus 2308 (wild type), pRE271, pJAG $_{3min}$,and pMAG $_{12 \& 13 min}$ were compared to make sure that the *trp*E gene was present. Primers were designed based on the pRE271 sequence to amplify the region of the *trp*E gene that would be deleted. 2308 (wild type), pRE271, and pJAG $_{3min}$ had a band that was 2.2 kb and pMAG $_{12 \& 13 min}$ which has a 894 bp deletion had a band that was 1.4 kb (Fig. 10 & 11). The plasmids as well as the wild type *B. abortus* strain have the *trp*E gene present but the one in pMAG $_{12 \& 13 min}$ has a 894 bp deletion.

Electroporation of suicide plasmids into *B. abortus*

Three suicide plasmids were constructed based on pRE271 and pUM24. For a positive control pBBR1MCS (Kovach et al., 1994) was used because of its ability to replicate in *Brucella*. pJAG $_{3min}$ was also used as a positive control since there was no deletion or insertion of the *sac*B-kanamycin cassette in the *trp*E gene. *B. abortus* competent cells with no DNA were used as the negative control and no colonies appeared on SOC-B plates. pMAG $_{12 \& 13 min}$ has a deletion in the *trp*E gene. Our procedure for making mutants is a two-step gene replacement (Fig. 12). pJAG $_{3min}$,and pMAG $_{12 \& 13 min}$ are suicide plasmids that are electroporated into *Brucella* cells, and the primary recombinants are selected on amp-kan plates. Since the plasmid can not replicate in *Brucella*, any amp-kan resistant clones are a result of the integration of the plasmid into the chromosome by a single-crossover event, via the homology between the wildtype chromosomal gene and the disrupted plasmid-borne gene. The plasmid also has the *sac*B gene from pUM24, which causes sensitivity to sucrose. So the clones that are amp and kan resistant are streaked onto plates with 5% sucrose and no clones should grow (suc ^{s}). Now the clones have a duplication of the target gene: one wild type chromosomal gene

and the disrupted plasmid-borne gene. Now the suc^s clones are streaked on tryptose plates to allow a second recombination event to occur between the duplicated regions. During this event the plasmid vector is lost with the *sac*B, amp, and kan genes, leaving behind either the wild type or mutant gene, depending on where the crossover takes place (it is possible that the recombination and integration will reconstitute a functional gene). The gene replacement technique has about a 50% probability of producing the mutant gene. To select for these suc^r clones they are streaked onto plates with 5% sucrose to kill any clones that did not undergo the second recombination event. These suc^r clones are screened for the mutant phenotype by plating on *Brucella* Minimal Media (BMM) with and without trp. Clones that grew on BMM plates with trp but not on BMM plates without trp were selected. The BMM plates were checked after 4 days and stayed no longer than a week in the incubator at 37°C, 5% CO_2.

During the first trial of electroporations 147 clones from pJAG $_{3min}$ met the criteria of undergoing both of the crossover events, so they were streaked onto BMM plates with and with out trp and checked after four days. Of the 147 clones two had slow growth on plates without trp, meaning when they were checked during the fourth day of incubation there was no growth but two days later the clones started to grow. The rest of the clones had grown on both plates after four days. The electroporation was unsuccessful for pMAG $_{12\&13\ min}$, no colonies at all on selective antibiotic plates.

The second trial yielded forty-two colonies that were streaked onto BMM plates with and without trp. Two colonies had no growth after four days on plates without trp, six had slow growth on plates without trp, and four grew on plates without trp but no growth on plates with trp (this is the opposite of what was expected).

For the rest of the electroporations the colonies were amp^r and kan^r which meant that a single crossover event took place but they were suc^r in the presence of 5% sucrose. They should have been suc^s because they only lose the plasmid vector during the second crossover event, which takes out the amp, kan, and *sac*B genes. The amp^r-kan^r-suc^r colonies were restreaked onto plates containing amp and kan and the single colonies were streaked onto plates containing 5% sucrose and again the colonies were suc^r. 151 amp^r-kan^r-suc^r colonies were randomly selected, 8 $pJAG_{3min}$ and 143 $pMAG_{12\&13min}$ (70 pMAG $_{12\ min}$ and 73 pMAG $_{13\ min}$ there is no significant difference between pMAG $_{12\&13\ min}$ because one plasmid was digested for one more minute with *Sau*3A1) and tested on BMM plates with and without trp. None of them were trp$^-$. Their genomic DNA was isolated and was subjected to PCR and Southern blot analysis.

PCR and Southern blot analysis on *B. abortus's* genomic DNA

A total of 161 colonies (10 pJAG and 151 pMAG) had their genomic DNA extracted and analyzed by PCR. Thirty-three colonies had both the wild type and deletion band present (Fig 13), 117 had just the wild type band (Fig 14), and 11 showed no band in the PCR. None contained just the deletion band.

During the first Southern blot the genomic DNA from the two clones ($12_{A\rightarrow C}$ and $12_{A\rightarrow I}$) that failed to grow without trytophane (but had both the wild type and deletion PCR band present), pRE271, and pUM24 were digested with *Pst*I. The probe pRE271, which contains the wild type trpE gene, was digested with *Bgl*I. $12_{A\rightarrow C}$ and $12_{A\rightarrow I}$ both have one band that hybridized with the probe (Fig 15). This was expected because the genomic DNA has incorporated both the wild type and deleted *trp*E sequence in its genome. For the second Southern blot (Fig. 16) $12_{A\rightarrow C}$, $12_{A\rightarrow I}$, and pUM24 were digested

with *Pst*I. pUM24 was used as the probe and it was also digested with *Pst*I. $12_{A\rightarrow C}$ and $12_{A\rightarrow I}$ had three bands that hybridized with the probe: the 2.6 kb band from the *sac*B, the 1.6 kb band from kan that make up the 3.8 kb *sac*B-kanamycin cassette from pUM24 and the last band which is around 3.8 kb, which may be the plasmid vector. These two clones met the phenotypic criteria of undergoing both crossover events so they should have lost the *sac*B-kanamycin cassette and the vector but the results of the Southern blot prove otherwise.

Six clones that were amp^r-kan^r-suc^r that contained the wild type and PCR deletion band were digested with *Pst*I and probed with pRE271 digested with *Bgl*I. In Figure 17, these six clones have multiple bands, which are expected because the strain is still amp^r and kan^r, which means that the plasmid did not get removed during the second recombination event. In the last Southern blot the six clones and the probe pUM24 were digested with *Pst*I and there were no bands present. The clones are suc^r and the only way that is possible is if the *sac*B gene is not present or not active. The results of the Southern blot confirmed this (Fig 18) but the genomic DNA should have kan, amp, and the vector since the clones had undergone the first crossover event. Which means there should have been adequate sequences to allow the probe to hybridize.

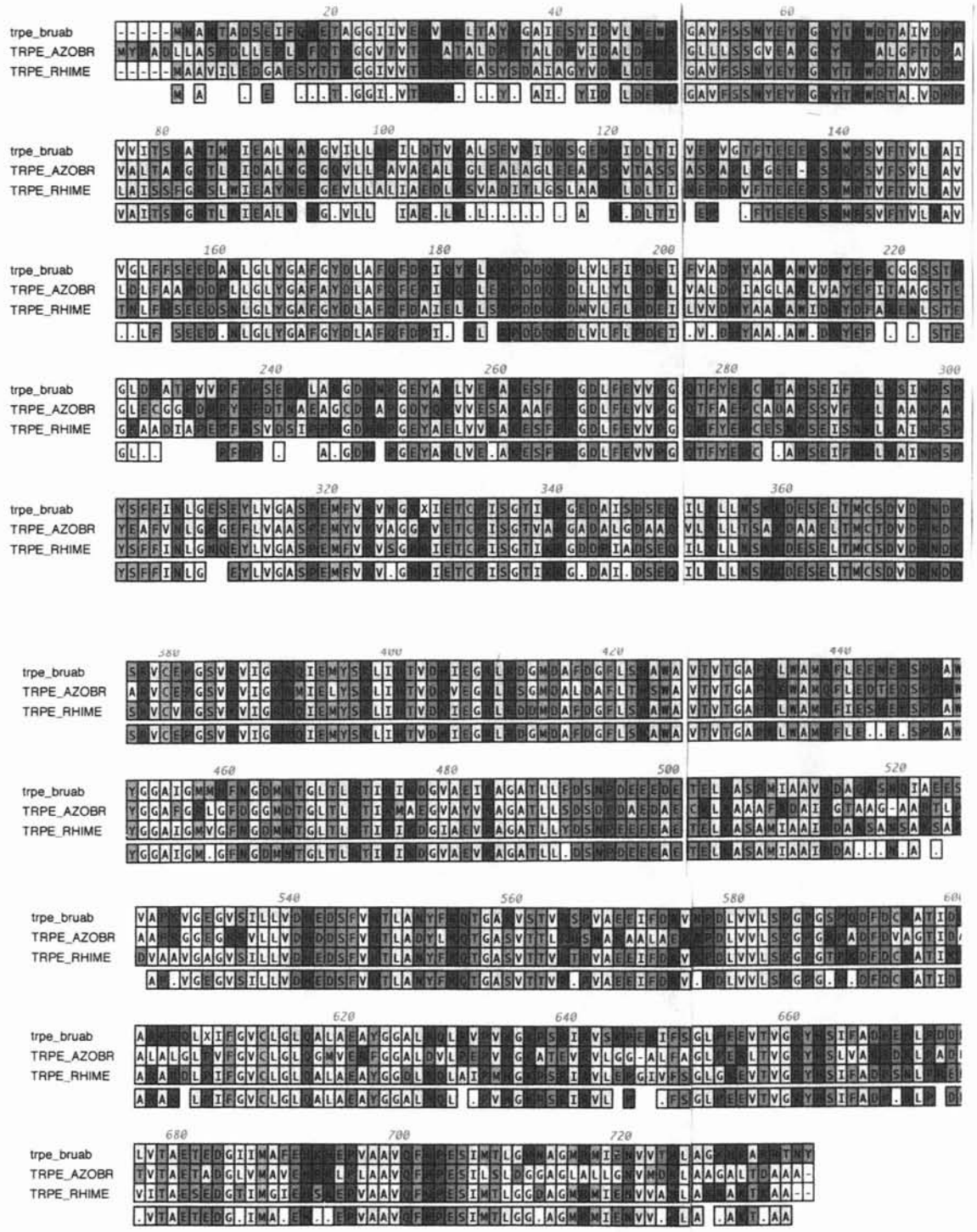

Figure 8. Comparison of the deduced amino acid sequemce of *B. abortus's trp*E protein with *Aztobacter* and *Rhizobium*
The fourth row is the consensus sequence

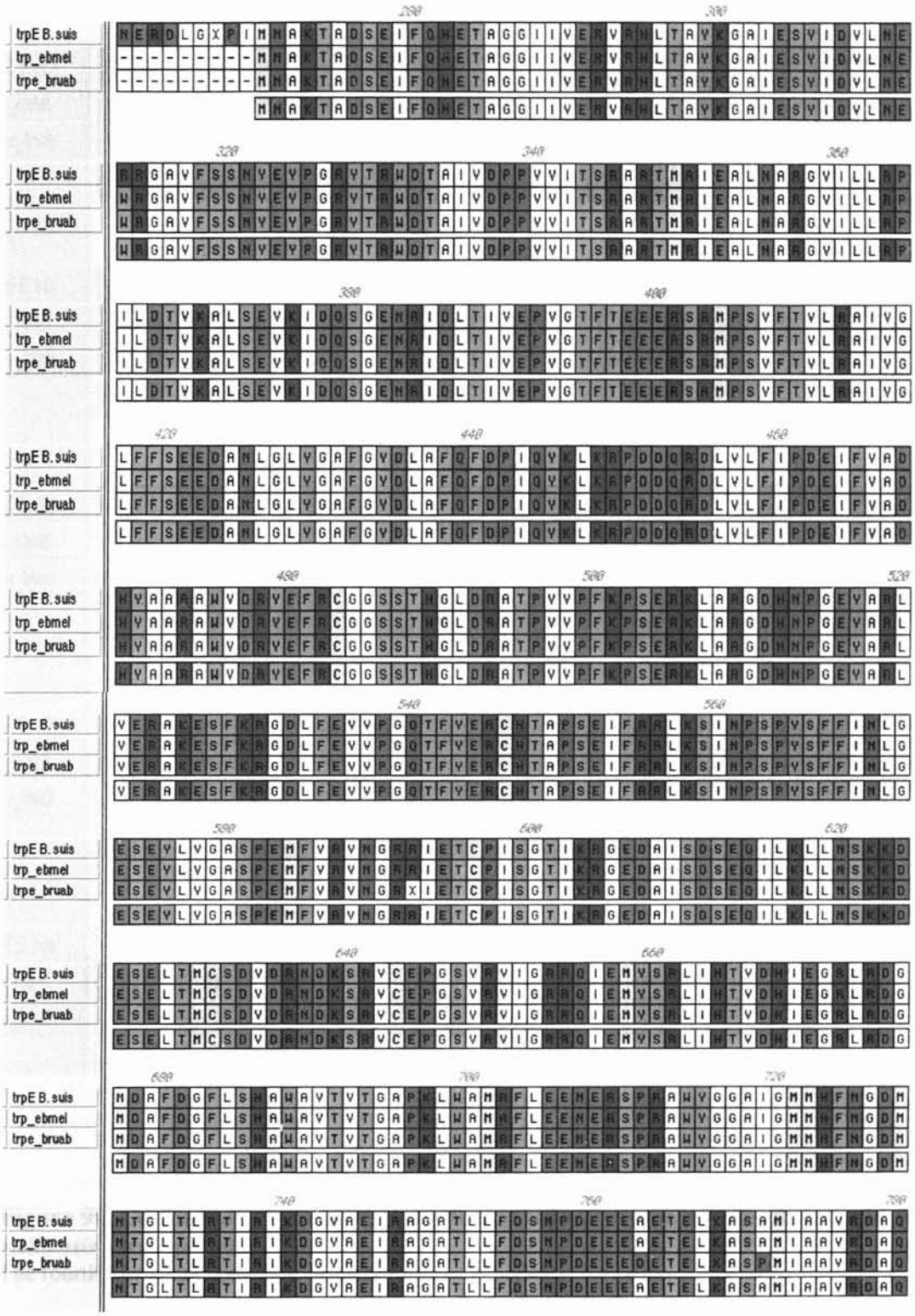

Figure 9a. Comparison of the deduced amino acid sequences of *B. abortus*, *B. suis*, and *B. melitensis's* trpE protein.

The fourth row is the consensus sequence

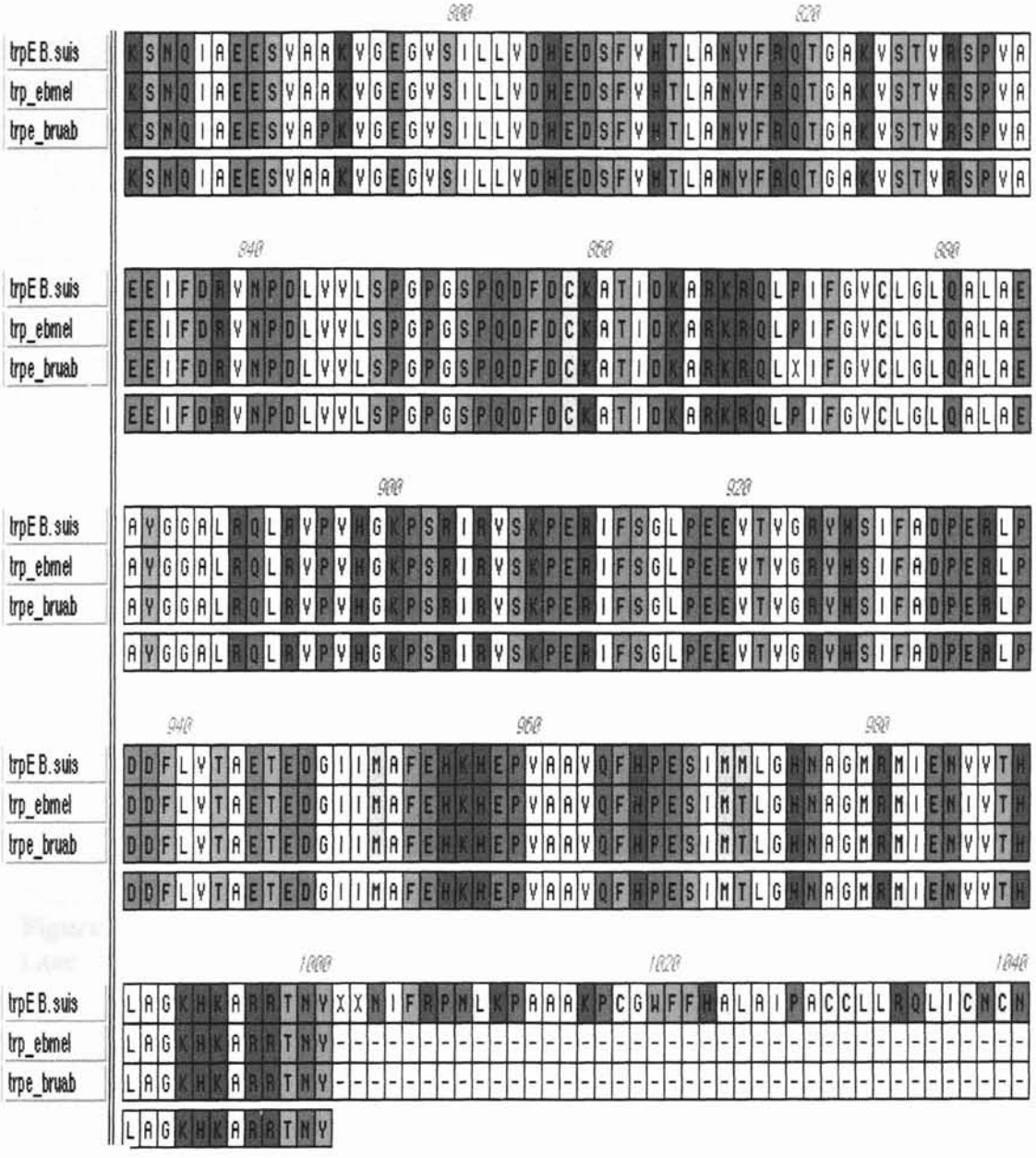

Figure 9b. Comparison of the deduced amino acid sequences of *B. abortus*, *B. suis*, and *B. melitensis's* trpE protein.
The fourth row is the consensus sequence

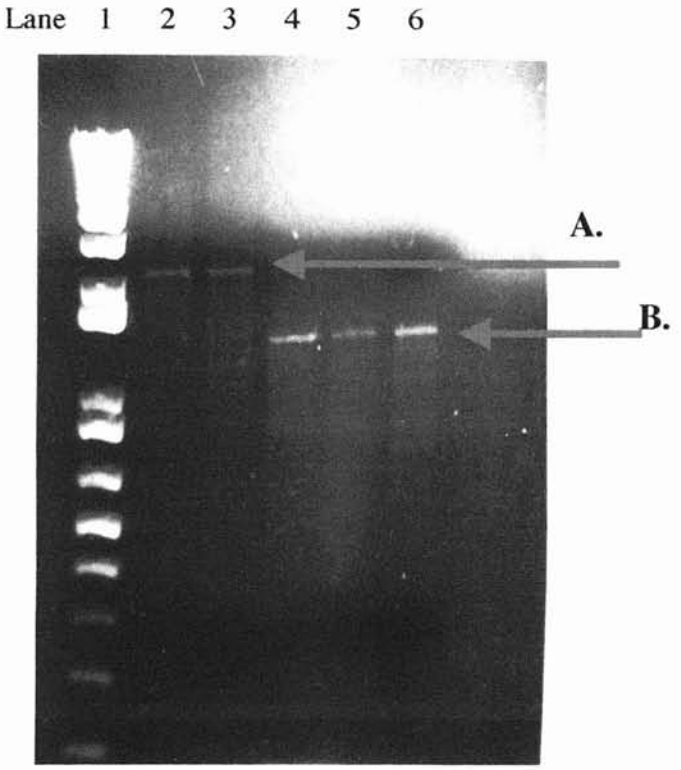

Figure 10. Comparison of the *trp*E insert insert pRE271, pJAG, and pMAG

Lane (1) $1Kb^{+}$
(2) pRE271
(3) pJAG
(4) $pJAG_{3B}$
(5) $pMAG_{12\ min}$
(6) $pMAG_{13\ min}$

A. Lanes 2 & 3 contain the wild type band

B. Lanes 4-6 have the deletion band present.

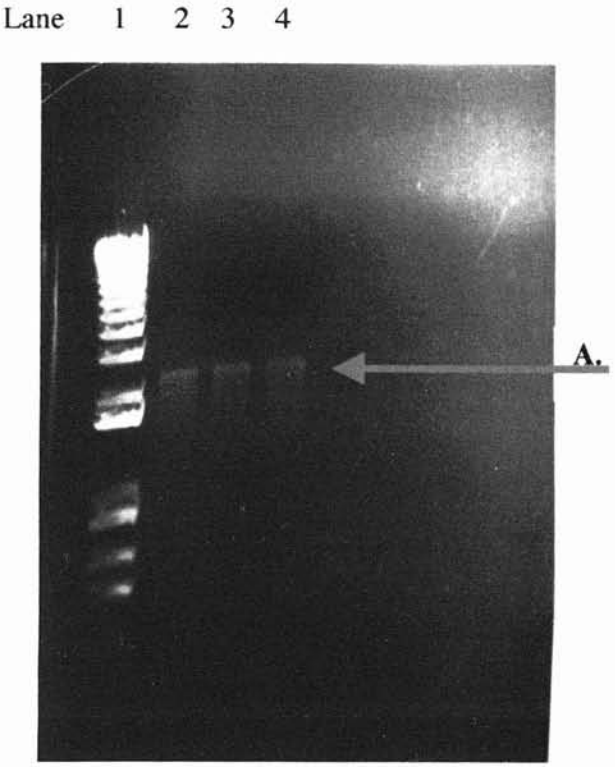

Figure 11. Comparison of *trp*E insert wild type *B. abortus* strain 2308 and pRE271
Lane (1) 1 Kb^{+}
(2) 1:10 dilution 2308
(3) 1:100 dilution 2308
(4) 1:100 dilution pRE271
A. Wild type *trp*E band

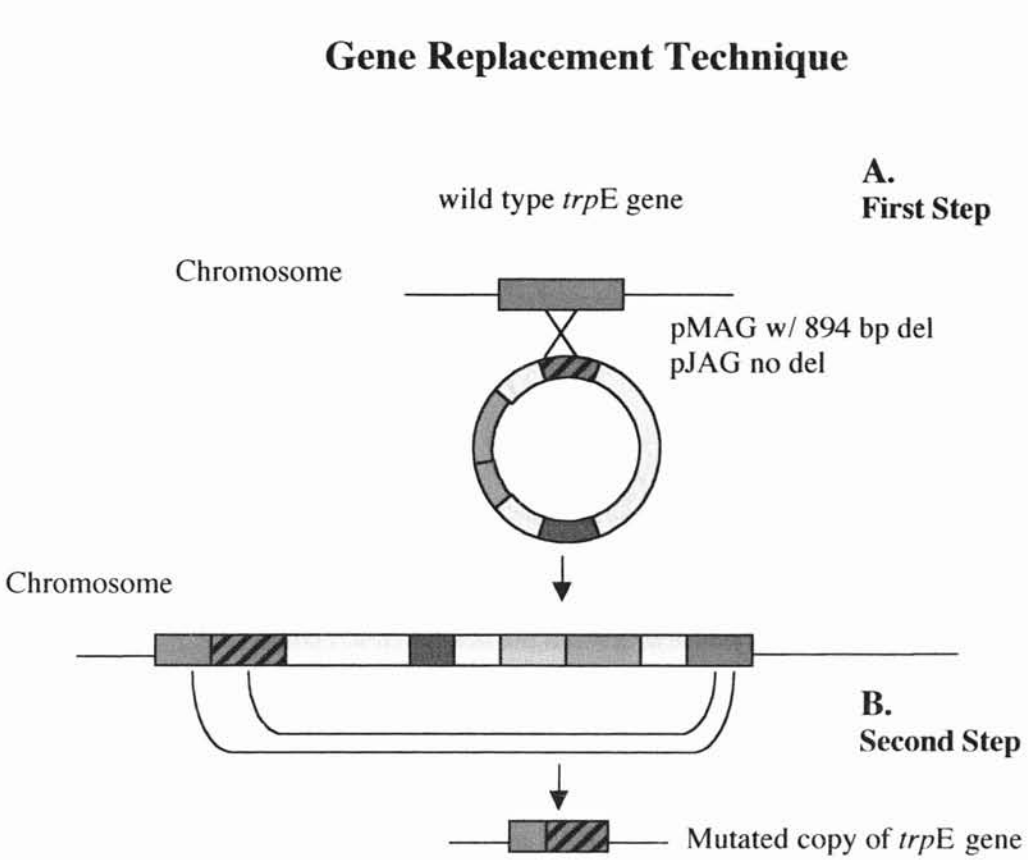

Figure 12. Gene Replacement Technique

A. First step, pMAG and pJAG are electroporated in *Brucella* cells. A single-crossover event occurs between the wild type chromsomal gene and the disrupted plasmid-borne gene. This results in the integration of the plasmid into the chromsome.

B. Second step, there is a second recombination event between the wild type chromsomal gene and the disrupted plasmid-borne gene. During this event the plasmid vector is lost leaving behind the mutated copy of the gene in the chromsome.

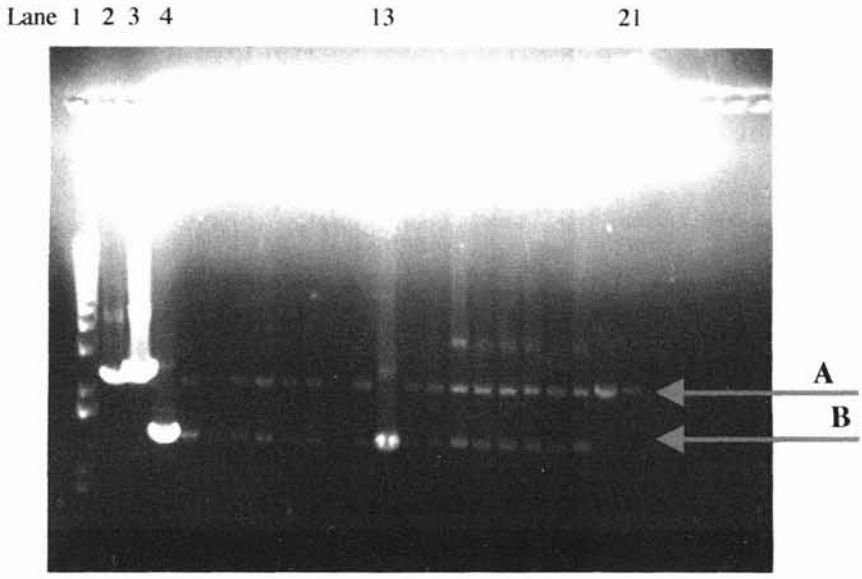

Figure 13. Genomic DNA analyzed by PCR, 33/161 colonies displayed both the wild type and deletion band

Lane (1) $1Kb^{+}$
(2) wild type *B. abortus* strain 2308
(3) pRE271
(4) $pMAG_{12\ min}$
(13) $pMAG_{13\ min}$
(5-23) genomic DNA.

A. Lanes 5-12 and 14-21 have both the wild type and deletion PCR *trp*E band
B. Lanes 22 and 23 have just the wild type *trp*E PCR band

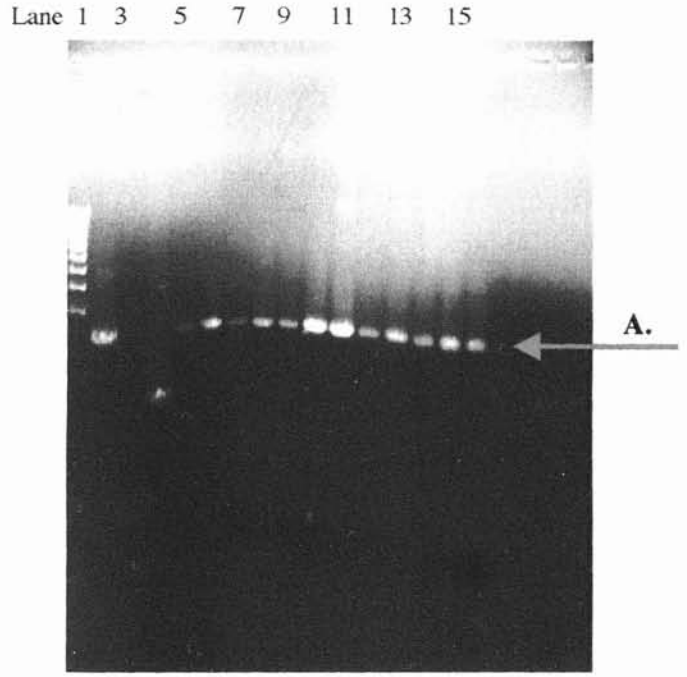

Figure 14. Genomic DNA analyzed by PCR, 117/161 displayed the wild type PCR band only

Lane (1)) Kb^{+}

(2) wild type *B. abortus* strain 2308

(3) empty

(4) $pMAG_{12\ min}$

(5-16) genomic DNA with the wild type *trp*E PCR band

A. Wild type PCR band

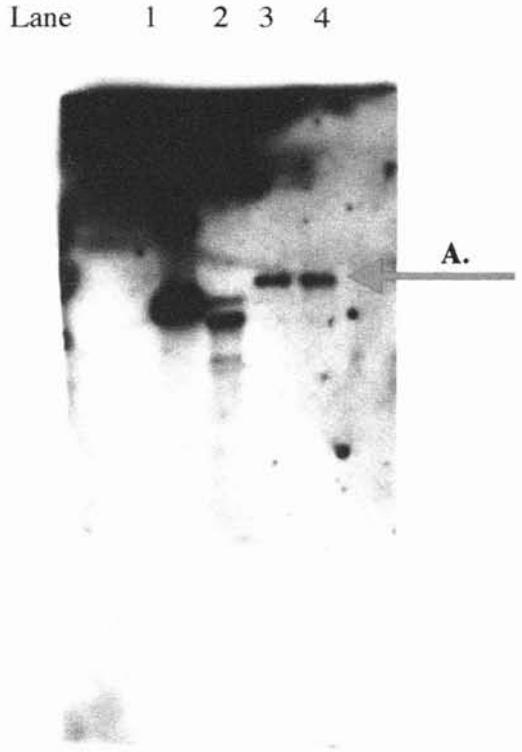

Figure 15. Southern blot analysis
The blot was exposed for 30 min. The probe pRE271 was digested with *Bgl*I. DNA in Lanes 1-4 was digested with *Pst*I.
Lane (1) control- pRE271
(2) control-pUM24
(3) $12_{A \rightarrow C}$
(4) $12_{A \rightarrow I}$
A. The band that represents the *trp*E sequence

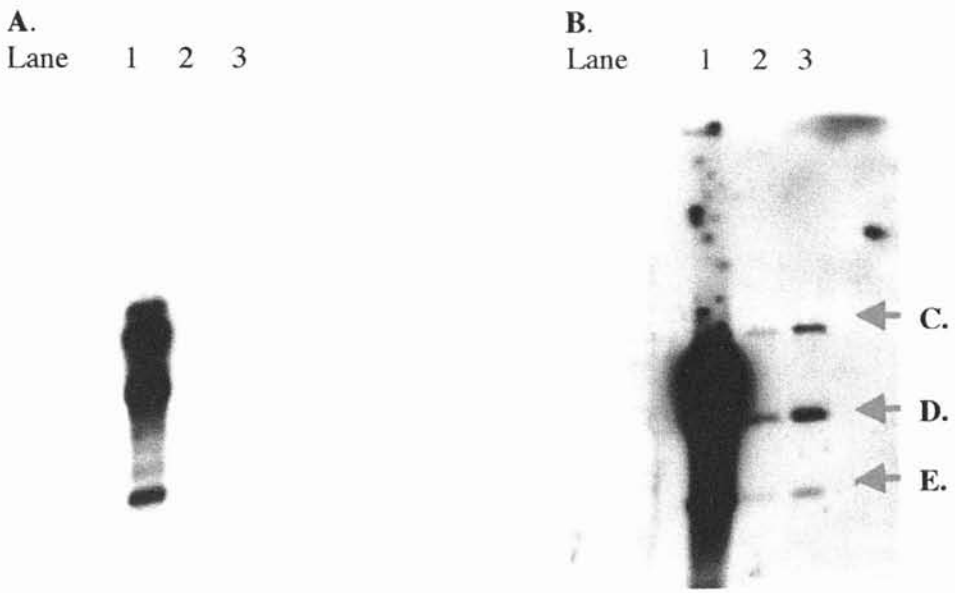

Figure 16. Southern blot analysis.
The probe pUM24 is digested with *Pst*I.
The DNA is digested with *Pst*I.
A. The blot was exposed for 5 min.
Lane (1) control-pUM24
(2) $12_{A \rightarrow C}$
(3) $12_{A \rightarrow I}$

B. The blot was exposed for 30 min.
Lane (1) control-pUM24
(2) $12_{A \rightarrow C}$
(3) $12_{A \rightarrow I}$

(**C**) 3.8 Kb band from the vector
(**D**) 2.6 Kb band from the *sac*B
(**E**) 1.6 Kb band from the kan
D and **E** represent the 3.8 Kb *sac*B-kanamycin cassette from pUM24

Lane 1 2 3 4 5 6 7 8

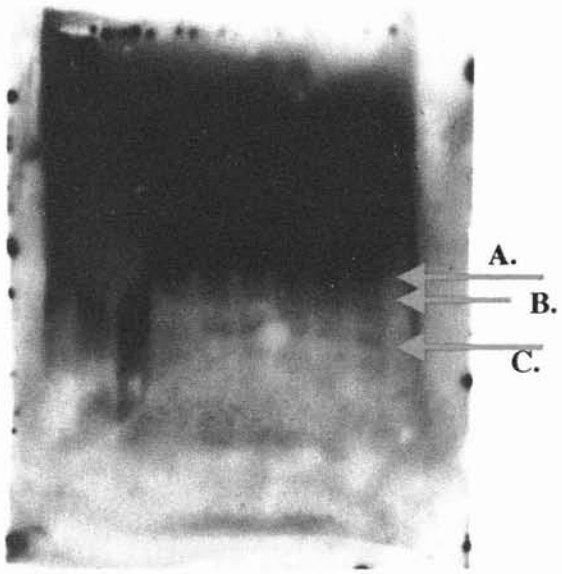

Figure 17. Southern blot analysis
The probe pRE271 is digested with *Bgl*I.
The DNA digested with *Pst*I.
The blot was exposed for 15 min.
Lane (1) control-pRE271
(2) control-pUM24
(3-8) genomic DNA that contained the wild type and PCR deletion band (amp^r-kan^r-suc^r colonies)

(**A**) 3.8 Kb band from the vector
(**B**) 2.6 Kb band from the *sac*B
(**C**) 1.6 Kb band from the kan
B and **C** represent the 3.8 Kb *sac*B-kanamycin cassette from pUM24

Figure 18. Southern blot analysis
The probe pUM24 was digested with *Pst*I.
The DNA was digested with *Pst*I.
The blot was exposed for 30 min.
Lane (1) control - pRE271
(2) control - pUM24
(3-8) genomic DNA that contained the wild type and PCR deletion band (amp^r-kan^r-suc^r colonies)

The probe did not hybridize with any sequences from the digested genomic DNA in lanes 3-8.

Chapter IV

Discussion

In this study *B. abortus trp*E gene, which encodes anthranilate synthase, the first enzyme involved in the biosynthesis pathway of tryptophan, was identified and characterized. This enzyme is responsible for the conversion of chorismate and glutamine to anthranilate, glutamate, and pyruvate (Neidhart et al., 1999). The predicted protein encoded by this gene shows high similarity to other proteins within the *Brucella* species as well with closely related intracellular symbionts and pathogens such as *B. melitensis*, *B. suis*, *Rhizobium*, and *Rhodobacter*.

A mutation was constructed in the *trp*E gene by deleting 894 bp of the coding region and the goal was to replace the deleted region with a *sac*B-kanamycin cassette but after several attempts by different methods, the goal was not reached. Therefore, it became necessary for me to construct a suicide plasmid with the mutated gene and *sac*B-kanamycin cassette and incorporate the mutated gene into the genome of the wild-type *B. abortus* strain 2308 by allelic exchange. The *trp*E gene, which was cloned and mutated in a plasmid, then transformed into a *trp*E$^-$ strain of *E. coli* and plated on minimal media with and without tryptophan. The cells were not able to grow on plates without tryptophan because the deletion in the gene inactivated *trp*E.

The approach used to create a *trp*E mutant of *B. abortus* included the use of pBluescript as the vector bearing the mutated *trp*E gene, ampicillin resistance *bla* gene, and the *sac*B-kanamycin cassette. Following electroporation the primary recombinants are selected on amp-kan plates to allow the single crossover event to take place, which is the incorporation of the plasmid into the wild-type *B. abortus's* chromosome. Since the

plasmid is integrated into the chromosome, the cells should be sensitive to sucrose because of the *sac*B gene. The cells are then plated on tryptose medium to allow the second crossover event to take place between the duplicated regions in which the plasmid vector is lost, leaving behind the mutated copy of the gene. These suc^r clones are checked for the mutant phenotype by plating on *Brucella* Minimal Media (BMM) with and without tryptophan. During the first set of electroporations this approach had low efficiency, out of 189 clones that met the criteria for undergoing both crossover events, 2 (1%) out of 189 had no growth on plates without tryptophan, which is the characteristic I want. 10 (5%) out of 189 clones exhibited slow growth on plates without tryptophan, meaning when the clones were checked after 4 days of incubation on the BMM plate at 37°, 5% CO_2 there was no growth on the plates but several days later the clones started to grow. 4 (2%) clones displayed growth on plates without tryptophan but no growth on plates with tryprophan. This is the opposite of what is expected.

For the second set of electroporations the colonies were amp^r and kan^r which meant that a single crossover event occurred but they were suc^r in the presence of 5% sucrose. The colonies should have been suc^s because the plasmid vector is only lost during the second crossover event. To double check the phenotype of the colonies they were restreaked onto plates containing amp and kan and the single colonies were streaked onto plates containing 5% sucrose and once again the colonies were suc^r. I randomly selected 151 amp^r -kan^r -suc^r colonies and tested them on BMM plates with and without trp. None of the colonies were trp^-.

A total of 161 colonies had their genomic DNA subjected to PCR and Southern blot analysis. 20% (33/161) of the colonies had the wild type and the deletion band

present, 73% (117/161) displayed just the wild-type band, and 6.8% showed no band at all when analyzed by PCR. None contained just the deletion band.

For the first Southern blot the genomic DNA from the two clones $12_{A\rightarrow C}$ and $12_{A\rightarrow I}$ that failed to grow on BMM plates without tryptophane but had both the wild type and deletion PCR band present and the controls pRE271 & pUM24 was digested with *Pst*I. The probe pRE271, which is carrying the wild type *trp*E gene, was digested with *Bgl*I. $12_{A\rightarrow C}$ and $12_{A\rightarrow I}$ both had one band that hybridized with the probe (Fig. 15), which is expected because the genomic DNA has incorporated both copies of the gene into its sequence and there are no *Pst*I sites in the *trp*E sequence. During the second Southern blot $12_{A\rightarrow C}$ and $12_{A\rightarrow I}$, and pUM24 were digested with *Pst*I. The probe, this time pUM24, was also digested with *Pst*I. There were three bands that hybridized with the probe (Fig 16): the 2.6 kb band from the *sac*B, the 1.6 kb band from kan that make up the 3.8 kb *sac*B-kanamycin cassette from pUM24 and the last band which is around 3.8 kb, possibly the plasmid vector. These two clones met the phenotypic criteria of undergoing both crossover events so they should have lost the *sac*B-kanamycin cassette and the vector but the results of the Southern blot did not indicate this. For some strange reason even though $12_{A\rightarrow C}$ and $12_{A\rightarrow I}$ appeared to undergo both recombination events, it is possible that only the first event took place. During the second cross over event the plasmid vector, *sac*B, ampicillin (*bla*), and kanamycin (*aph*) genes are suppose to be lost but this is not the case here. So the whole plasmid is getting incorporated into the chromosome without a second event taking place. This means there could be a problem with the *sac*B gene but I transformed this gene into *E. coli* cells and they were sensitive to 5% sucrose.

Six colonies that were amp^r-kan^r -suc^r, which contained the wild type and PCR deletion band, were digested with *Pst*I and probed with pRE271 digested with *Bgl*I. In figure17 the seven colonies have multiple bands, which is expected because the strain is still amp^r and kan^r. This means the plasmid did not get removed during the second recombination event. In the last Southern blot the seven clones and the probe pUM24 were digested with *Pst*I and no bands were present. The clones are suc^r and the only way for that to happen is if the *sac*B gene was not present or not active. The results of the Southern blot confirmed this (Fig. 18) but the colonies should have the *sac*B-kanamycin cassette present because they had undergone the first cross over event. There should have been sufficient sequences to allow the probe to hybridize. It is possible the allelic exchange occurred in two events, but did not lose the entire plasmid vector during the second event.

In the first set of electroporations there was a high electroporation frequency and a high number of colonies that underwent both recombination event. However, only 1% (2/189) were a tryptophan auxotroph of *B. abortus*. This could be credited to the second crossover event, depending on where this event takes place it is possible the recombination and integration will reconstitute a functional gene. One would think there was not enough sequence on each side of the deletion but the *trp*E sequence starts at 1,327 bp and ends at 3,519 bp. The deletion occurs at 1,857 bp and 2,751 bp (an 894 bp deletion), so there is ample sequence on each side of the deletion, 530 bp and 768 bp respectively on each side.

During the last set of electroporations there was a high electroporation frequency but there were also a high number of single cross over events that were probably due to

nonhomologous, illegitimate recombinations. The plasmid was incorporated but none of it was lost. There were no colonies with just the deletion band, which tells me that the deletion in *B. abortus's trp*E gene is lethal. Tryptophan must be necessary for *B. abortus's* intracellular survival because it is not able to synthesize the amino acid with the deletion. It seems that *B. abortus* is not able to obtain tryptophan from other sources and has some type of warning system when the tryptophan supply is low. Therefore, it would not accept just the deleted *trp*E gene.

Microbes living in an intracellular environment must develop mechanisms to access available nutrients because it is crucial for their survival. One approach that was used in an attempt to better understand the environment in which intracellular bacteria inhabit is to analyze which bacterial genes are induced when the bacteria are inside the cell. A gene fusion system based on plasmid pBBR1MCS and the promoterless gene *gfp* encoding green fluorescent protein was developed for *B. suis* allowing isolation of constitutive and inducible genes (Kohler et al., 1999). Bacteria containing transcriptional fusions of random chromosomal DNA inserts to *gfp* were visualized by fluorescence microscopy and examined by flow cytometry. Twelve clones containing promoters induced inside J744 murine macrophages were isolated and further characterized. Sequence analysis of the transcriptional fusions to *gfp* and alignments of the putatively encoded proteins displayed that five of the fragments exhibited no significant similarity to sequences in the Swissprot database. Six others showed similarity to nucleotide-binding proteins and proteins of various transport systems with amino acid similarities ranging from 59% to 83% (Kohler et al., 1999). In *B. melitensis* signature-tagged mutagenesis (STM) was used to identify genes required for the in vivo pathogenesis of

Brucella. The attenuation of eighteen mutants was confirmed and the mutants were further characterized by their ability to replicate in murine macrophages and in HeLa cells. The sequences disrupted by the transposon in the mutants have similarity to genes coding for proteins of different functional classes such as: transport, amino acid metabolism, transcriptional regulation, peptidoglycan synthesis, a chaperone like protein, and proteins of unknown functions (Lestrate et al., 2002). In *B. suis* STM was used to identify new virulence factors in an in vitro human macrophage infection model. Eighteen mutants were identified and their attenuation was confirmed in THP1 macrophages and HeLa cells. Transposon integration had occurred in fourteen different genes. Some of the genes were known virulence factors involved in intracellular survival or biosynthesis of smooth lipopolysaccharide (*vir*B operon and *man*B), this helped to validate the in vitro human macrophage infection model because STM is usually done in animal models. The second group involved genes in the regulation of gene expression and the third group of genes encodes enzymes involved in the metabolic pathways like glucose metabolism, amino acid biosynthesis, and purine nucleotide biosynthesis (Foulongne et al., 2000). The STM screens all have one thing in common: mutants were attenuated in both macrophages and HeLa cells by metabolic defects rather than lesions in classical virulence factors. These results indicate that nutrients are not freely available to the pathogen inside the phagosome.

My goal was to construct a tryptophan auxotroph of *B.abortus*, and see what effects this mutation has on *Brucella's* ability to survive and replicate inside bovine macrophages. Previous work in *Brucella* indicated that nutrient and amino acid biosynthesis pathways are critical for virulence, because auxotrophic mutants for leucine,

arginine, and aromatic amino acids are attenuated (Hondalus et al., 2000). For example, deletions in the *pur*E gene of *B. melitensis* led the mutant to have attenuated replication in human monocyte derived macrophages (Drazek et al., 1995). In *B. suis* there has been success in constructing an *aro*C mutant, which encodes chorismate synthase, the final enzyme in the synthesis of chorismate. This mutant was highly attenuated in tissue culture (THP1 macrophages and HeLa cells) and murine virulence models (Foulongne et al., 2001). Since chorismate is the precursor to tryptophan and there was success in disrupting the synthesis of chorsimate one could assume the same results for tryptophan. I was able to make a tryptophan auxotroph *B. abortus* but when the genomic DNA was analyzed by PCR there were two bands present: the wild type and the deletion *trp*E bands. Since the colonies that appeared to undergo both recombination events required tryptophan for growth and contained both the wild type and the deletion *trp*E bands, it is possible that only the deleted gene is being expressed. During the cross over events the promoter from the wild type chromosomal copy of the gene could be integrated into the mutated plasmid-borne copy.

Future directions include: trying to get the *sac*B-kanamycin cassette inserted into the sequence of the *trp*E gene; finding another system for integrating the mutated gene into the genome of wild type *B. abortus* strain 2308; finding out how *B. abortus* is able to keep both the wild type and mutated PCR band in its genome; once the genomic DNA is analyzed by PCR and the results show that only the deletion *trp*E band is present and a Southern blot analysis confirms this, then bovine macrophage cells will be used to test the virulence of the mutant and try to understand the nature of the intracellular environment in which *Brucella* resides.

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VITA

Bobby Alfonso Gramling, Jr.

Candidate for the Degree of

Master of Science

Thesis: A STUDY ON *BRUCELLA ABORTUS'S TRP*E GENE

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data: Born in Augusta Georgia, on April 19, 1976, the son of Bobby and Xoan Gramling.

Education: Graduated from Bad Krueznach American High School in Bad Krueznach, Germany in May 1994. Attended Langston University in Langston, Oklahoma and received a Bachelor of Science in Chemistry July 1998. Completed the requirements for the Master of Science degree with a major in Biochemistry and Molecular Biology at Oklahoma State University in Stillwater, Oklahoma in December 2002.

Experience: Employed by the Department of Biochemistry and Molecular Biology at Oklahoma State University as a Research Assistant from July 1998 to December 2002.