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Matthew David Edmonds

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CREATION AND CHARACTERIZATION OF 25 KDA OUTER MEMBRANE PROTEIN (OMP25) DELETION MUTANTS IN BRUCELLA SPECIES

A Dissertation
Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Interdepartmental Program in Veterinary Medical Sciences through the Department of Veterinary Microbiology and Parasitology

by
Matthew David Edmonds
B.S., Saint Mary College, 1996
August 2000
For my father, A. John Edmonds
In loving memory
I want to especially thank Dr. Philip Elzer, my graduate advisor, for his support and guidance during my studies. In his laboratory, I have had the pleasure of working with both basic molecular biology and large animal in vivo trials. This has resulted in a truly unique educational opportunity.

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A2.4. Western immunoblot analysis of cell lysates from Δomp25 mutants with MAb A19/12B10/FO4 specific for the Omp25 protein. ..................................................183
The genus *Brucella* is composed of Gram-negative, facultative intracellular pathogens that impact man and animals. *Brucella* species express a 25 kDa major outer membrane protein termed Omp25. To determine the role of Omp25 in virulence, mutants were created in *Brucella abortus* (BA25), *Brucella melitensis* (BM25), and *Brucella ovis* (BO25) that contain disruptions in the *omp25* gene (Δomp25 mutants).

In the murine brucellosis model, each Δomp25 mutant was attenuated when compared to the virulent parental strain. To assess the ability of the *B. abortus* and *B. melitensis* mutants to colonize and induce abortions in the ruminant host, cattle and goats in late-gestation were inoculated with BA25 or BM25, respectively. Significant decreases in the abortion and colonization profiles of BA25-infected cattle and BM25-infected goats were observed when compared to animals inoculated with the parental strains (*P*<0.05, *n* = 10). While virulent *B. abortus* colonized 10/10 calves and induced abortions in 5/10 dams, BA25 colonized only 3/10 calves and caused one abortion. BM25 did not cause abortions and failed to colonize 5/9 caprine dams compared to 6/6 colonized dams and abortions in goats inoculated with the parental strain. The capacity of brucellae to replicate inside macrophages and chorionic trophoblasts plays an important role in fetal pathogenesis. The *in vitro* replication rate of the *B. abortus* Δomp25 mutant in these cells is significantly less than the replication rate of the parental strain (*P*<0.05, *n* = 5). Such differences may explain the attenuation of both BA25 and BM25 *in vivo*.
Because they appeared attenuated, the effectiveness of the mutants as vaccine candidates was subsequently determined. Vaccination of mice with either BM25 or BO25 provided significant protection against challenge with the homologous virulent parental strain ($P<0.01$, n = 5). Vaccination of goats with BM25 provided protection against abortion and colonization at levels equal to the current caprine vaccine, $B.\ melitensis$ strain Rev. 1. Unlike strain Rev. 1 which causes abortions when given to pregnant goats, BM25 is safe for pregnant goats and may be a possible vaccine alternative to strain Rev. 1. In summary, the described $Brucella\Deltaomp25$ mutants are attenuated and efficacious against virulent challenge.
CHAPTER 1
RESEARCH OBJECTIVES

Introduction

*Brucella* species (spp.) are facultative, intracellular Gram-negative bacteria that infect a wide variety of hosts (Corbel, 1998). *Brucella melitensis* and *Brucella abortus* are recognized causes of abortion during the third trimester of pregnancy in goats and cattle, respectively (Enright, 1990). Infection of humans with either strain causes the disease undulant fever, which is characterized by severe fever, arthritis, depression, and spondylitis (Young, 1995). Although non-pathogenic to humans, *Brucella ovis* causes epididymitis, orchitis, and infertility in rams (Blasco, 1990).

The cell envelope of *Brucella* spp. is composed of an inner cell membrane, a periplasmic space, a peptidoglycan layer, and an outer cell membrane containing lipopolysaccharide (LPS) (Corbel, 1998). Located on the distal end of the LPS is the hydrophilic O-polysaccharide (O-side chain) (Corbel, 1998). The outer membrane contains outer membrane proteins (Omps), including a 94 kDa structural protein (group 1), a 41-43 kDa porin protein (group 2), and two proteins of 25 and 31 kDa (group 3) (Verstreate et al., 1982; Cloeckaert et al., 1996a). The function of the 25 kDa Omp (Omp25) is unknown while the 31 kDa Omp (Omp31) may be a second porin (Cloeckaert et al., 1992; Zecaino et al., 1996). Omp25 is a transmembrane protein that is expressed on the surface of the outer cell membrane and is covalently bound to the peptidoglycan layer of the cell (Dubray and Charriaut, 1983; Cloeckaert et al., 1990; Cloeckaert et al., 1992). Omp25 may be a structural outer membrane protein. 

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protein that stabilizes the outer membrane by binding the underlying peptidoglycan layer and outer cell membrane.

Omp25 may also be an important component of the protective immune response against *Brucella* spp. Immunization of mice with whole-cell sonicates of *Escherichia coli* expressing recombinant Omp25 provides protection against challenge with rough strains of *B. melitensis* which lack the LPS O-side chain (Bowden et al., 1998). Murine vaccination with the sodium dodecyl sulfate insoluble cell wall fraction of *B. abortus*, which contains approximately 50% outer membrane protein, provides protection equal to that of killed-whole cells (Dubray and Bezard, 1980; Cloeckaert et al., 1996c). Cattle and mice vaccinated with native cell envelope which was enriched for Omp25 in an oil base adjuvant developed lymphocyte blastogenesis responses and delayed hypersensitivity reactions to Omp25 one to three months following vaccination (Winter and Rowe, 1988). A hot saline extract (HS) of the *B. ovis* outer membrane, which is rich in Omp25, protected mice against challenge with virulent *B. ovis*, and passive transfer of both T lymphocytes and antibodies from similarly vaccinated mice provided protection (Jimenez de Bagues, 1994a). Based on these findings, Omp25 might be a good candidate for inclusion in a subcellular vaccine for brucellosis.

Alterations in the cell envelope of Gram-negative bacteria can produce mutants with diminished pathogenicity for the host. In *E. coli* K-1 the lack of the structural outer membrane protein A (OmpA) results in mutants that are attenuated in both neonatal rats and embryonated chick eggs (Weiser and Gotschlich, 1991). A *Campylobacter jejuni* mutant lacking the CadF outer membrane protein was unable to
colonize the cecum of newly hatched chicks (Ziprin et al., 1999). Both OmpA and CadF are outer membrane proteins with structures similar to that of Omp25 (Baldermann et al., 1998).

**Hypotheses**

The lack of Omp25 in *Brucella* spp. may affect the ability of these organisms to cause disease in the ruminant host. As a transmembrane protein spanning the outer cell membrane and peptidoglycan, the lack of Omp25 may affect the integrity of the outer membrane. One hypothesis for this research is that the lack of Omp25 will render *Brucella* spp. attenuated in ruminants.

Alternatively, the absence of Omp25 in *Brucella* spp. may alter the host’s immune response to this pathogen. Brucellae lacking Omp25 may fail to generate a typical cell-mediated or humoral immune response in infected animals, resulting in an exacerbation of disease. The alternative hypothesis for this research is that the lack of Omp25 may render *Brucella* spp. more virulent in ruminants due to an altered immune response.

**Statement of Research Objectives**

The goal of this work is to investigate the importance of Omp25 in *Brucella* spp. by analyzing the pathogenicity of Δomp25 mutants of *B. melitensis*, *B. abortus*, and *B. ovis* in the murine and ruminant models. The research objectives for these studies are:

1. To create and verify Δomp25 mutants of *B. melitensis*, *B. abortus*, and *B. ovis*. 

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2. To initially characterize Omp25 mutants of *B. melitensis*, *B. abortus*, and *B. ovis* in the murine brucellosis model.

3. To evaluate the pathogenic potential of the *B. abortus* Omp25 mutant in the ruminant host utilizing *in vitro* assays with bovine phagocytes and trophoblasts and an *in vivo* trial with pregnant cattle.

4. To analyze the colonization and pathogenic potential of the *B. melitensis* Omp25 mutant in the ruminant host with non-pregnant and pregnant goats.

5. To determine if the *B. melitensis* and *B. ovis* Omp25 mutants are efficacious vaccine candidates in the murine brucellosis model.

6. To evaluate the vaccine efficacy of the *B. melitensis* Omp25 mutant in pregnant goats.

In this dissertation, the research results are presented as four separate chapters. A review of the biology and pathology of *Brucella* infections, focusing on key aspects for virulence, precedes these sections. The chapters are as follows:

Chapter 2. Literature Review.

Chapter 3. Generation and Initial Description of *Brucella melitensis*, *Brucella abortus*, and *Brucella ovis* Mutants Lacking a 25 kDa Outer Membrane Protein (Omp25).

Chapter 4. A *Brucella abortus* Δomp25 Mutant in Cattle.

Chapter 5. A *Brucella melitensis* Δomp25 Mutant in Goats.
Chapter 6. Vaccine Trials with *Brucella melitensis* and *Brucella ovis* Mutants Lacking a 25 kDa Outer Membrane Protein (Omp25).

Chapter 7. Assessments and Perspectives.
CHAPTER 2
LITERATURE REVIEW

In this review of *Brucella* species (spp.), emphasis will be placed on pathogenesis and those aspects of brucellae important for virulence. The general cell envelope structure of brucellae and specific disease caused by each species will be described. The ability of these organisms to survive inside the professional phagocyte and to cause abortions will be discussed. Aspects of the immune system important for protective immunity will be explained along with available vaccines. Based on this information, the pathogenic importance of a *Brucella* structural outer membrane protein will be hypothesized.

**Genus Brucella**

*Brucella* spp. are Gram-negative, non-motile, non-encapsulated, non-spore forming, aerobic, facultative intracellular pathogens (Corbel, 1998). The bacteria are small coccobacilli which are 0.6-1.5 μm in length and 0.5-0.8 μm in width (Corbel, 1998). There are currently six recognized species in the genus *Brucella*: *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella neotomae*, *Brucella ovis*, and *Brucella canis*. Brucellae are important zoonotic and agricultural pathogens, infecting a variety of hosts with varying degrees of pathology. *Brucella* spp. contain two chromosomes and a G + C DNA content of 58-59 mol% (Corbel, 1998). Due to a sequence homogeneity of >90%, the genus may contain only a single species, which is separated into different biovars. It has been proposed that all *Brucella* spp. be considered biovars of *B. melitensis* (Meyer, 1990). However, this nomenclature has not been accepted, and the traditional terminology is currently used.
The genus is a member of the $\alpha_2$ subdivision of the Proteobacteria (Corbel, 1998). Other members of this group include the animal pathogens of the *Bartonella* genus, the plant symbionts of the *Rhizobium* genus, and the plant pathogens of the *Agrobacterium* genus (Zinder, 1998). The phylogenetic relationship of this group has been further supported by the finding of common antigenic determinants between *Brucella* spp. and other members of the $\alpha_2$ Proteobacteria (Cloeckaert et al., 1999).

**Cell Envelope**

The cell envelope of *Brucella* spp. is comprised of an inner cell membrane, the periplasmic space, a peptidoglycan layer, and the outer cell membrane containing lipopolysaccharide (LPS). The outer cell membrane and associated LPS is approximately 9 nm thick and the muramic acid-containing peptidoglycan layer is 3-5 nm thick (Corbel, 1998). The LPS of Gram-negative bacteria is composed of three parts: the proximal, hydrophobic lipid A region; the distal, hydrophilic O-polysaccharide region (O-side chain) that protrudes from the outer cell membrane; and the core polysaccharide region (Nikaido and Vaara, 1987). The core polysaccharide is attached to the lipid A by 2-keto-3-deoxyoctulosonic acid (KDO) (Nikaido and Vaara, 1987). In *Brucella* spp. the glycoside backbone of lipid A is made of 2,3-diamino-2,3-dideoxyglucose and 2-amino-2-dideoxyglucose (Corbel, 1998). The core polysaccharide is comprised of glucose, mannose, and 6-amino-6-deoxyglucose (quinovosamine).

Both *B. abortus* and *B. melitensis* O-side chain consist of 4,6 dideoxy-4-formamido-D-mannose ($N$-formyl-D-perosamine). However, while the O-side chain of *B. abortus* biovar 1 comprises approximately 100 glycoside residues linked $\alpha$-1,2, the O-side chain of *B. melitensis* biovar 1 consist of a repeating pattern of 4 residues linked $\alpha$-1,2.
followed by one residue linked α-1,3 (Meikle et al., 1989; Corbel, 1998). The difference in O-side chain structure between *B. abortus* biovar 1 and *B. melitensis* biovar 1 causes antigenic differences that can be identified by serological analysis. The O-side chain structure of *B. melitensis* biovar 1 is termed the M antigen while that of *B. abortus* biovar 1 is termed the A antigen (Meikle et al., 1989). *Brucella suis* biovar 1 generates both M and A antigen-specific antibodies in the infected host due to the presence of both α-1,2 and α-1,3 linkages in the O-side chain (Meikle et al., 1989).

The cell envelopes of *Brucella* spp. are more resistant to lysozyme digestion than are the cell envelopes of enterobacterial organisms, such as *Escherichia coli*, which suggest that brucellae have unusual properties to the peptidoglycan structure (Corbel, 1998). The *Brucella* peptidoglycan consist of a glycan skeleton of D-glucosamine and muramic acid linked by short chains of alanine, glutamic acid, and α,ε-diaminopimelic acid. The phospholipid composition of *Brucella* spp. is unusual when compared to other Gram-negative organisms. The primary phospholipid of *Brucella* spp. is phosphatidylcholine, which is uncommon in both *E. coli* and *Salmonella typhimurium* (Cornan and Rock, 1987; Corbel, 1998). The phospholipids phosphatidylethanolamine and cardiolipin, while being major lipid components of *E. coli* and *S. typhimurium*, are only a small percentage of the total phospholipids in brucellae (Cornan and Rock, 1987; Corbel, 1998).

Contained within the cell envelope are three groups of outer membrane proteins (Omps) that are classified as group 1 (94 kDa), group 2 (41-43 kDa), and group 3 (25-31 kDa) (Dubray and Bezard, 1980; Verstreate et al., 1982). The 94 kDa
group 1 Omps are considered minor components of the cell envelop (Verstreete et al., 1982). The group 2 Omps are porins that form a trimer in the native state (Douglas et al., 1984). Ficht et al. (1988, 1989) have shown that the Brucella group 2 porin is encoded and potentially expressed by two closely related genes, \textit{omp2a} and \textit{omp2b}. The two genes share 85% DNA homology at the nucleotide level, are separated by 900 bp, and are oriented in opposite directions on the chromosome. The predicted amino acid sequence of both genes shares 30-40% identity with the RopA protein of \textit{Rhizobium leguminosarum}, an \(\alpha_2\) Proteobacteria (Cloeckaert et al., 1996a).

The group 3 Omps comprise two separate proteins of 25 kDa (Omp25) and 31 kDa (Omp31) (Cloeckaert et al., 1996a). Based on the predicted amino acid sequence, Omp31 shares 34% identity with Omp25 and 35% identity with the structural outer membrane protein RopB of \textit{R. leguminosarum} (Vizcaino et al., 1996). Omp31 is present on the outer cell membrane, associates with the peptidoglycan, and appears to form oligomers resistant to sodium dodecyl sulfate (SDS) denaturation at low temperatures, which is typical of bacterial porins (Cloeckaert et al., 1990; Cloeckaert et al., 1992; Vizcaino et al., 1996). Based on these findings, Omp31 is proposed to be a porin (Vizcaino et al., 1996). The protein is expressed by all brucellae except for \textit{B. abortus} due to a 17,119 bp genomic deletion (Vizcaino et al., 1996; Vizcaino et al., 1999). This leaves Omp25 as the only group 3 Omp in \textit{B. abortus}.

The Omp25 protein is expressed on the outer cell membrane of each \textit{Brucella} spp. (Cloeckaert et al., 1996c). The protein is tightly associated with the peptidoglycan layer by a possible covalent bond, and association of Omp25 with LPS is important for the reconstitution of conformational epitopes on purified Omp25.
Analysis of the Omp25 gene (omp25) with PCR-RFLP and nucleotide sequencing indicates that the gene is highly conserved among the *Brucella* spp. (Cloeckaert et al., 1995; Cloeckaert et al., 1996b). Only 12 variations in nucleotide sequence were observed in the 639 bp length *omp25*. The greatest variation was a short 36 bp deletion detected at the 3' end of *omp25* from *B. ovis* (Cloeckaert et al., 1996b).

The Omp25 protein is a highly conserved structural outer membrane protein of *Brucella* spp. that is associated with both the LPS and peptidoglycan. The protein was originally considered a homologue of the *E. coli* structural outer membrane protein, OmpA, based on amino acid composition (Verstreate et al., 1982). However, sequencing of *omp25* found the predicted amino acid sequence to have no homology with the OmpA proteins of *E. coli*, *S. typhimurium*, *Serratia marcescens*, *Enterobacter aerogenes*, or *Shigella dysenteriae* (de Wergifosse et al., 1995). Baldermann et al. (1998) have shown that while Omp25 and OmpA do not share amino acid sequence homology, they do contain similar secondary structural properties.

The *Brucella* Omp25 does have 43% identity with the structural outer membrane protein RopB of *R. leguminosarum* (Cloeckaert et al., 1996a). Analogous to Omp25, RopB is covalently associated with the peptidoglycan and shares structural similarities with Omps from a variety of Gram-negative bacteria (Roest et al., 1995; Baldermann et al., 1998). Research indicates that RopB may play a role in the formation of the bacteroid (Roest et al., 1995). Under low nitrogen conditions, *Rhizobium* spp. are able to interact with the roots of leguminous plants to form nitrogen-fixing nodules. During the establishment of a root nodule, *Rhizobium*
differentiates into a bacteroid form that undergoes distinct morphological and biochemical changes, including a dramatic reduction in the expression of RopB (Roest et al., 1995). A more complete understanding of the role of RopB in the formation of the bacteroid may lend additional information on the function of Omp25 in *Brucella* spp.

*Brucella melitensis*

In 1883 a small micrococci was isolated by Bruce from the spleen of a British soldier that had died of a febrile illness common in soldiers stationed on the island of Malta (cited in Madkour, 1989; cited in Hall, 1989). The bacterium was termed *Micrococcus melitensis*, with *melitensis* being derived from the Roman name for Malta, “Melita”. The first accurate description of the human disease was published in 1897 by Hughes, and the illness named “undulant fever” due to the recurring severe pyrexia (cited in Madkour, 1989). Zammit identified goat’s milk as the source of *Micrococcus melitensis* infection of humans on the island of Malta in 1905 (cited in Madkour, 1989; cited in Hall, 1989). Based on cell morphology and agglutination profile, *Micrococcus melitensis* was renamed *Brucella melitensis* by Meyer and Shaw in 1920 (cited in Madkour, 1989).

Besides being a human pathogen, *B. melitensis* is also an agricultural problem of economic impact. Pregnant goats and sheep infected with *B. melitensis* will often abort during the last trimester of pregnancy. In crowded facilities the pathogen can spread quickly following an abortion, posing a serious health threat to the flock and animal handlers. Many scientists consider the coastal areas of the Mediterranean to be the origin of *B. melitensis* (Alton, 1990a; Garin-Bastuji et al., 1998). However, the
organism is found in goats and sheep throughout the Middle East, Mongolia, Russia, Mexico, and Latin America (Alton, 1990a; Garin-Bastuji et al., 1998; Kahler, 2000). A recent serological survey indicates that $B. \text{melitensis}$ may also be common in goats in Uganda (Kabagambe et al., 2000). Except for a few sporadic cases of $B. \text{melitensis}$, of which the last report was in 1970, the U.S. was thought to be free of this pathogen. However, in October 1999, four cows and a large flock of goats and sheep from Southern Texas were found to be infected with $B. \text{melitensis}$ (Kahler, 2000). This is the first report of a large population of animals infected with $B. \text{melitensis}$ in U.S. history. The probable source of the infection is goats from Northern Mexico, which may have been introduced into this naive population.

$\text{Brucella suis}$

In 1914, Traum isolated what appeared to be $B. \text{abortus}$ from an aborted pig obtained from an Indiana farm (cited in Madkour, 1989; cited in Hall, 1989). However, subsequent work by Huddleson found the organism to be a new species of $\text{Brucella}$ that was termed $B. \text{suis}$ (cited in Madkour, 1989; cited in Hall, 1989). Like $B. \text{melitensis}$, $B. \text{suis}$ is also a cause of abortion, in this case, in pregnant sows. Feral and domestic pigs in the U. S., South America, China, Polynesia, Australia, Germany, and Poland are infected with $B. \text{suis}$ biovar 1 (Alton, 1990b; Robson et al., 1993; Lord et al., 1997; Heinritzi et al., 1999).

Infection of sows with $B. \text{suis}$ is characterized by abortions in late-gestation. Often an infected sow will deliver some piglets live and healthy, while others will be born dead or die soon after birth (Alton, 1990b). Unlike $B. \text{abortus}$ and $B. \text{melitensis}$ where the male rarely develops clinical disease, boars colonized by $B. \text{suis}$ present
with epididymitis, orchitis, and subsequent infertility. *Brucella suis* is essentially a venereal disease that is passed from the infected boar to the naive sow (Alton, 1990b). Besides the reproductive complications of *B. suis*, swine can also develop partial paralysis due to bursitis and spondylitis. Like *B. melitensis*, *B. suis* is a major zoonotic pathogen that will become increasingly important in developing countries with the introduction of large-scale swine operations (Alton, 1990b; Gibbs, 1997)

**Brucella abortus**

The organism *Bacillus abortus* was isolated as a cause of infectious bovine abortion by Bang in 1897 and later renamed *Brucella abortus* in 1920 (cited in Madkour, 1989). Infection of naive cows with *B. abortus* often causes abortions during the last trimester of pregnancy (Enright, 1990). Like both *B. melitensis* and *B. suis*, the organism is also a zoonotic agent which causes undulant fever in man (Young, 1995). Due to the agricultural and human health risk associated with bovine brucellosis, the U.S. government led a sixty-year campaign to eradicate this pathogen from domestic cattle in the U.S. The success of this program is attested to by the fact that only six states were classified “infected” as of February 1, 2000 (Stevens et al., 1997; Kahler, 2000). *Brucella abortus* is found in cattle throughout the world and has been reported in numerous locations including: South America, Mexico, South Africa, and India (Erasmus, 1995; Brown and Hernandez de Anda, 1998; Isloor et al., 1998; Samartino et al., 1999).

**Brucella canis**

In 1968 a new *Brucella* spp. was isolated that was responsible for an epizootic of canine abortion, epididymitis, and sterility in several large commercial breeding
kennels (Carmichael and Kenney, 1968). *Brucella canis* has been confirmed to cause abortions in pregnant bitches between 45 and 59 days of gestation (Carmichael, 1990; Johnson and Walker, 1992). Besides abortion, infected bitches often present with no clinical signs. In contrast, the stud develops epididymitis, infertility, abnormal semen quality, and testicular atrophy (Carmichael, 1990; Johnson and Walker, 1992). The bacteria are spread both venereally and by contamination of kennel floors with infected maternal uterine tissues and fluids (Carmichael, 1990; Johnson and Walker, 1992). Long-term antibiotic treatment can control the infection; however, recurrence is common and the damage to the epididymis from the pathogen is often permanent (Carmichael, 1990; Nicoletti, 1991). Due to the difficulty in treating infected animals, whole kennel depopulation is considered the only method to ensure elimination of the infection (Johnson and Walker, 1992). *Brucella canis* has been diagnosed in the U. S., Japan, Germany, Spain, Czechoslovakia, Tunisia, China, Mexico, Central America, and South America (Carmichael and Shin, 1996).

*Bruceella ovis*

*Bruceella ovis* is a causative agent of epididymitis, orchitis, and infertility in sexually mature rams (Blasco, 1990). The organism was first characterized in sheep from New Zealand and termed *B. ovis* (Buddle and Boyes, 1953). *Bruceella ovis* has since been found in Australia, the U. S., South America, Russia, Europe, and South Africa (Blasco, 1990; Robles et al., 1998). Infections can occur in both juvenile and adult males; however infection rates increase with age (Blasco, 1990; Cerri et al., 1999). Venereal transmission via the ewe and direct contact between young rams are the main methods of transmission (Blasco, 1990; Grillo et al., 1999). Ewes rarely
develop clinical signs; however a small percentage do abort in the last trimester of pregnancy (Libal and Kirkbride, 1983; Blasco, 1990; Grillo et al., 1999).

*Brucella ovis* colonizes the epididymides, testicles, vesicular glands, bulbourethral glands, and ampullae of the infected ram (Blasco, 1990). Infected males eventually develop severe bilateral epididymitis, resulting in permanent sterility.

While bacteriological analysis of samples from both the semen and epididymis will usually yield brucellae organisms, the primary method of detection is physical examination for epididymitis (Blasco, 1990; Blasco, 1997). Identification and removal of infected rams, along with vaccination of sexually immature males, are the main control strategies in an infected herd (Blasco, 1990; Blasco, 1997).

**Other Brucella Species**

*Brucella neotoma* was isolated by Stoenner and Lackman in 1957 from a desert wood rat; the organism has not been isolated since and no disease has been associated with this specie (cited in Madkour, 1989). A recent development has been the isolation of brucellae from whales, sea otters, porpoises, seals, and dolphins stranded on either the North American or English coasts (Foster et al., 1998; Ewalt et al., 1998). Analysis of the marine mammal isolates with the standard biochemical tests used to differentiate *Brucella* spp. indicates that these organisms are distinct from the six recognized species (Foster et al., 1998; Ewalt et al., 1998). It has been suggested that the marine mammal isolates should be considered biovars of a new *Brucella* spp. (Ewalt et al., 1998; Jensen et al., 1999). Further research is needed to clarify the taxonomic organization of these new isolates.
Wildlife Brucellosis

While the U.S. government nears completion of the eradication of *B. abortus* from bovine herds, a growing concern is brucellae infected wildlife reservoirs. Bison and elk in the Greater Yellowstone Area (GYA), infected with *B. abortus*, represent the largest reservoir in the U.S. (Davis, 1990). Data indicates that 37% of the elk and 30-40% of the bison from GYA have agglutinating antibodies to *B. abortus* O-polysaccharide (Cheville et al., 1998). The presence of these antibodies is indicative of field strain exposure and/or infection. These free-ranging animals may contact domestic cattle on drive trails or on surrounding pasturelands. Under controlled conditions, non-infected cattle developed brucellosis when held in the same padlock with experimentally infected bison (Davis et al., 1990); and similar studies were preformed in elk (Thorne et al., 1979). More importantly, co-mingling of bison and cattle on a ranch in North Dakota resulted in bison infected with a field strain of *B. abortus* passing the infection to naive cattle (Flagg, 1983). It is clear from this work that bison and elk infected with *B. abortus* do pose a threat to the elimination of bovine brucellosis from the U.S. (Davis, 1990; Cheville et al., 1998). In an effort to address this problem, research is currently being performed with potential new vaccine candidates for brucellosis in bison and elk (Cheville et al., 1998; Elzer et al., 1998a).

Feral swine in the Southern U. S., Australia, and Germany are known to be infected with *B. suis* biovar 1 (Davis, 1990; Robson et al., 1993; Heinritzi et al., 1999). Wild hogs colonized by *B. suis* present with the same clinical signs as domestic pigs, including abortion, orchitis, arthritis, osteomyelitis, and partial paralysis (Davis, 1990). In both the U.S. and Australia, hunters and abattoir employees have been...
infected with \textit{B. suis} while handling hunter-killed carcasses (Robson et al., 1993; Gibbs, 1997). Besides posing a threat to human health, feral pigs can also infect domestic bovine and swine herds (Davis, 1990). Based on the risk to both domestic animals and humans, new methods of control for \textit{B. suis} are needed. Since wild hogs in the Southern U.S. are often found in inaccessible locals, including dense forests and swamps, removal or direct vaccine inoculation is an unrealistic option. An oral vaccine candidate for wild hogs that could be mixed with attractive baits such as corn and pecans is currently being investigated (Edmonds et al., 2000b).

Caribou, reindeer, and moose from Alaska, Canada, and Siberia are infected with \textit{B. suis} biovar 4 based on culture and serological analysis (Dieterich, 1981; Davis, 1990; Ferguson, 1997; Edmonds et al., 1999b). The clinical signs of rangiferine brucellosis include abortion, stillbirth, weak fawns, orchitis, epididymitis, arthritis, lameness, and nephritis (Dieterich, 1981; Forbes and Tessaro, 1993). The percentage of animals with antibodies to the O-polysaccharide varies widely between herds and within the same herd over time with values of 0% to 57% reported (Dieterich, 1981; Forbes and Tessaro, 1993; Ferguson, 1997). Based on prevalence rates in caribou, Ferguson (1997) proposes that most Inuit caribou hunters on Baffin Island, Northwest Territories, Canada, are eventually exposed to \textit{B. suis} biovar 4 (Dieterich, 1981; Forbes and Tessaro, 1993). Based on the data presented on caribou, reindeer, moose, feral swine, elk, and bison, it is clear that wildlife reservoirs of \textit{Brucella} spp. are a health threat to humans and domestic livestock.
Human Brucellosis

*Brucella* *melitensis*, *B. suis*, *B. abortus*, and *B. canis* are all potential human pathogens in decreasing order of virulence (Young, 1995). *Brucella neotomae* and *B. ovis* have not been reported to cause disease in humans (Madkour, 1989). *Brucella melitensis* is a serious public health threat in many areas of the world where goats and sheep are an important source of food and fiber stock (Alton, 1990a; Young, 1995). The main method of infection with this species and *B. abortus* is via ingestion of unpasteurized milk and cheese, and other modes of transmission include direct handling of infected tissues and fluids by animal handlers and abattoir employees (Alton, 1990a; Young, 1995). *Brucella suis* is a growing problem in many areas of the world due to the development of large scale swine operations and the hunting of feral hogs, which brings humans into closer contact with infected swine (Robson et al., 1993; Gibbs, 1997).

Human brucellosis (undulant fever) is a febrile illness that can present with a variety of clinical symptoms (Young, 1995). Patients often complain of cyclic fever, fatigue, anorexia, weight loss, depression, and back pain. Bone and joint complications are common and can include sacroiliitis, peripheral joint arthritis, spondylitis, osteomyelitis, and bursitis. Respiratory problems including cough and dyspnea are reported in approximately 15% of cases. Men infected with *B. suis* and *B. abortus* can develop acute orchitis and epididymitis (Gibbs, 1997). Endocarditis caused by *Brucella* spp. is a serious complication that occurs in less than 2% of cases but is responsible for the majority of *Brucella*-associated deaths (Young, 1995). Rare cases of undulant fever can include meningitis, liver cirrhosis, ileitis, and colitis. The
recommended treatment for human brucellosis is doxycycline (200 mg/day) plus rifampin (600 – 900 mg/day) once daily for 6 weeks. A common problem with brucellosis is relapse after having taken the antibiotics for the prescribed period of time (Young, 1995).

**Pathogenesis**

**Overview**

Infection of domestic ungulates with *Brucella* species can result in abortion during the third trimester of pregnancy (Corbel, 1998). Following entry into the host by the oral mucosa, nasopharynx, conjunctival sac, or genital tract, the bacteria are phagocytosed by macrophages and neutrophils in an effort to eliminate the pathogen (Enright, 1990; Thoen et al., 1993). Once inside these phagocytes *Brucella* spp. inhibit oxidative burst and phagosome-lysosome fusion, enabling the organisms to replicate inside these cells (Kreutzer et al., 1979; Frenchick et al., 1985; Baldwin and Winter, 1994; Liautard et al., 1996). Macrophages containing replicating brucellae migrate to the draining lymph node via the lymphatics (Enright, 1990; Thoen et al., 1993). Within 2-3 weeks following infection, the lymph node becomes hemorrhagic due to the break down of the vasculature within the node. During this process some macrophages are lysed, and the brucellae enter the bloodstream and a subsequent bacteremia develops (Payne, 1959; Enright, 1990; Thoen et al., 1993). During the bacteremic phase the organism is disseminated throughout the host, localizing in the reticuloendothelial system.

During pregnancy, *Brucella* spp. will colonize and replicate to high numbers inside the trophoblastic cells of the placenta (Anderson et al., 1986; Enright, 1990;
The resulting tissue necrosis causes damage to the fetal membranes, allowing transmission of the bacteria to the fetus. For reasons that are not completely understood, infection of the fetus results in an abortion during the third trimester of pregnancy (Anderson et al., 1986; Enright, 1990; Thoen et al., 1993). Following an abortion, the offspring, placenta, uterine exudate, and maternal milk have high numbers of bacteria that contaminate the pasture and handling facilities with infectious organisms which poses a threat to naive animals in the same area (Enright, 1990; Bercovich, 1998).

Phagocyte Evasion

During the process of phagocytosis of a pathogen, the organism is engulfed inside a vacuole termed the phagosome in which the microbe is exposed to a variety of bactericidal agents (Rook, 1998; Kaufmann, 1999). Upon entry into the cell, the phagosome is exposed to reactive oxygen intermediates (ROIs), including superoxide anions, hydroxyl radicals, hydrogen peroxide, and singlet oxygens, which are all highly reactive forms of oxygen that damage bacterial DNA, lipids, and proteins (Kaufmann, 1999). In the macrophage, fusion of the phagosome and lysosome results in formation of the phagolysosome. In this environment the organisms are exposed to low pH and bactericidal products including defensins and lysozyme. Also present in these vacuoles are nitric oxide and other reactive nitrogen intermediates (RNIs) and the myeloperoxidase system which halogenates water to form hypochlorous acid and chloramines which both destroy biologically important microbial proteins (Rook, 1998; Kaufmann, 1999).
Brucella spp. are able to resist or inhibit many of the bactericidal mechanisms of macrophages and neutrophils. Kreutzer et al. (1979) showed that incubation of both smooth and rough strains of *B. abortus* with either guinea pig or human neutrophils resulted in less than 1% of the bacterial cells dying by 120 minutes post-infection. Based on electron microscopy studies, degranulation and oxidative burst were hindered after ingestion of *Brucella* spp. due to either the lack of a proper stimulus or active inhibition by the brucellae. Since no difference was observed in percent survival between the rough and smooth *Brucella* strains, the authors hypothesized that the O-side chain of the LPS was not responsible for the observed resistance to intracellular killing (Kreutzer et al., 1979).

Soluble water extracts of *B. abortus* strain 2308 but not *B. abortus* strain 19 have been found to inhibit phagosome-lysosome fusion in murine peritoneal macrophages (Frenchick et al., 1985). *Brucella abortus* strain 2308 is a virulent challenge strain that has been documented by numerous laboratories to cause abortions in experimentally infected cattle while strain 19 is an attenuated-live cattle vaccine (Enright, 1990; Nicoletti, 1990a; Cheville et al., 1996; Elzer et al., 1998b). Preincubation of murine macrophages with low (90 ng) or high (300 μg) concentrations of a water soluble cell envelope extract from strain 2308 caused a significant decrease in the rate of phagosome-lysosome fusion when compared to untreated controls (Frenchick et al., 1985). Use of strain 19 cell extract at concentrations five times those of strain 2308 failed to demonstrate a similar decrease in percent fusion. Chemical analysis of the cell extract found it to consist of cell
surface material including carbohydrates, glycopeptides, lipids, and LPS (Frenchick et al., 1985).

Research by Porte et al. (1999) indicates early acidification of the macrophage phagosome may be essential for the survival of *B. suis* in murine macrophages. In murine J774 cells, the pH of phagosomes containing *B. suis* was found to drop from 6.0 to 4.0–4.5 by 60 min post-infection. The addition of a vacuolar proton-ATPase (bafilomycin A₁) prior to infection blocked the acidification of the phagosome and resulted in a 34-fold decrease in bacterial numbers by 6 hours post-infection (Porte et al., 1999). The low pH of the phagosome may act as an intracellular signal on the regulation of *Brucella* genes involved in survival and multiplication within the macrophage. The authors hypothesize that the typical decrease in viable brucellae during initial infection of macrophages *in vitro* may result from this period of specific adaptation and gene regulation induced by low pH (Porte et al., 1999).

**Trophoblasts**

Besides being able to grow in the professional phagocytes, *Brucella* species are also known to colonize and replicate inside non-professional phagocytes. An important aspect of *Brucella* virulence is the capacity of these organisms to undergo extensive replication in the trophoblast cells of the placenta (Enright, 1990; Thoen et al., 1993). The placenta develops between the fetus and dam as a means of nutrient and waste exchange. In ruminants there are six cellular layers separating the maternal and fetal blood streams which are the maternal endothelium, maternal connective tissue, uterine epithelium, the trophoblasts (the outermost fetal cell layer), the fetal connective tissue, and the fetal endothelium (Flood, 1991; Frandson and Spurgeon, 22
The fetal trophoblast, connective tissue, and endothelium layers are referred to collectively as the chorion. Inside the chorion are two fluid-filled sacs that separate the fetus from the placenta. Immediately inside the chorion is the allantoic sac that surrounds both the fetus and inner amniotic sac. The amnion membrane forms a barrier between the outer allantoic sac and the inner amniotic sac, in which the fetus is suspended (Flood, 1991; Frandson and Spurgeon, 1992).

The blood from the dam and fetus do not mix, but the chorion and maternal endothelium must be close enough to allow the exchange of oxygen, nutrients, and waste. Variations exist between animal species as to the structure and exact nature of this exchange. The bovine, caprine, ovine, and porcine placentas are termed epitheliochorial since the trophoblast cells lie against the uterine epithelium (Flood, 1991; Frandson and Spurgeon, 1992). In contrast, the canine placenta is endotheliochorial because the fetal trophoblast cells interact directly with the maternal endothelium; the maternal connective tissue and uterine epithelium are not present. The placenta of primates and some rodents is termed hemochorial since all three maternal cell layers, including the endothelium, are lost and the fetal trophoblast lies directly against the maternal blood (Flood, 1991; Frandson and Spurgeon, 1992).

The area of placental attachment between the dam and fetus also varies between species. A diffuse placentation occurs in the porcine placenta where almost the entire chorion is directly in contact with the uterine epithelium (Flood, 1991; Frandson and Spurgeon, 1992). The canine placenta is termed zonary since a band of fetal and uterine membrane interchange develops only at the midline of the fetus. The hemochorial placenta of primates and lower rodents is discoidal since a disk-shaped
area of contact occurs. The placental attachment of ruminants is unique since the epitheliochorial contact of the fetus and uterus occurs in localized mushroom-like areas referred to as placentomes. Outside the placentome, the fetal and uterine membranes do not interact. The fetal portion of the placentome is termed the cotyledon while that of the dam is the caruncle. The caruncles project inward from the surface of the uterus and form small villi that interdigitate with the fetal trophoblast cells. The bovine caruncle forms a dome that extents outward into the chorion while that of the ovine forms a concave bowl that the fetal villi grow into. The caprine caruncle also projects outward into the chorion, however the maternal villi do not extend as far into the fetal epithelium as in the bovine placenta (Flood, 1991; Frandson and Spurgeon, 1992).

The ability of *Brucella* spp. to invade and replicate to high numbers in the chorionic trophoblasts is thought to play a major role in the abortion process (Enright, 1990; Thoen et al., 1993). In 1959, Payne documented that experimental infection of naive cows with a virulent strain of *B. abortus* resulted in ulceration of maternal and fetal tissue in the placentome and the multiplication of brucellae inside the chorionic trophoblasts (Payne, 1959). The ability of *B. abortus* to replicate in the bovine trophoblast has also been demonstrated in vitro with trophoblastic cell lines and chorioallantoic membrane explants (Samartino, 1991; Samartino et al., 1994; Samartino and Enright, 1996). Three trophoblastic cell lines obtained from a 13-15 day old bovine embryo, a 5-month placenta, and 8-month placenta were able to be experimentally infected with strain 2308 (Samartino et al., 1994). While extensive bacterial replication occurred in both the 5-month and 8-month trophoblast cell lines.
up to 30 hours post-infection, no statistically significant increase in bacterial numbers were observed in the early-gestational cell line after 4 hours post-infection. Similar results were obtained from chorioallantoic membrane explants obtained from early-gestational (60-120 days) and late-gestational (180-240 days) cattle (Samartino and Enright, 1996). The increased replication rate of *B. abortus* in the late-gestational trophoblasts when compared to early-gestational cell lines is thought to play a role in the characteristic late-term abortion of bovine brucellosis (Samartino, 1991; Samartino et al., 1994; Samartino and Enright, 1996).

The sugar erythritol may play a role in the tropism of *Brucella* to the reproductive tract (Sangari and Aguero, 1996). Smith et al. (1962) proposed that erythritol caused the localization of *B. abortus* in the bovine placenta based on the presence of the sugar in the chorion and the ability of *B. abortus* to metabolize it. However, the erythritol-sensitive-vaccine *B. abortus* strain 19 and virulent *B. ovis*, which does not utilize erythritol, both still cause abortions (Enright, 1990; Nicoletti, 1990a; Sangari and Aguero, 1996; Corbel, 1998). Based on these data, erythritol is not the only factor involved in the tropism of *Brucella* spp. for the chorionic trophoblasts.

Based on experimental infection of pregnant goats with *B. abortus*, Anderson et al. (1986) demonstrated that *Brucella* spp. first gain entry into the placenta via the erythrophagocytic trophoblasts. In the central depression of the placentome, some maternal blood escapes from the capillaries and is actively phagocytosed by the first fetal layer of cells, the erythrophagocytic trophoblasts. During the bacteremia that occurs following *Brucella* infection, some erythrophagocytic trophoblasts may not
only take up maternal erythrocytes but also live brucellae. The erythrophagocytic
trophoblasts then infect the underlying chorioallantoic trophoblasts. In the
chorioallantoic trophoblasts, the bacteria grow to excessive numbers resulting in
necrosis and spread to adjacent cells and the uterine lumen. Via hematogenous
dissemination and ulceration of chorionic membranes, the fetus becomes infected with
brucellae (Anderson et al., 1986). Often, the net result of fetal colonization is abortion
during the third trimester of pregnancy.

The exact mechanism by which fetal infection by brucellae causes abortions is
unknown. The impairment of fetal circulation due to placentitis has been suggested as
a cause of fetal abortion (Payne, 1959). However, considerable variation occurs in the
distribution and severity of placental lesions following an abortion with all the
placentomes rarely being affected (Enright, 1990; Thoen et al., 1993). LPS from E.
coli has been documented as causing abortions in cattle during the first trimester of
pregnancy, however in vitro inoculation of bovine trophoblast cell lines with purified
Brucella LPS did not cause cellular damage or detachment (Giri et al., 1990;
Samartino et al., 1994). Preliminary data indicates that infection of the fetus by
brucellae may alter the normal hormonal levels of the uterus and induce delivery prior
to maturation of the fetus (Enright, 1990). Further research is needed to determine the
critical cause of abortion following fetal infection by Brucella spp. However, it is
clear that bacterial colonization of the chorionic trophoblasts play an integral role in
the abortion process.
Immune Response

As facultative intracellular bacteria, *Brucella* spp. are able to reside inside the host's cells undetected by the humoral immune system. Therefore, as with most intracellular pathogens, the role of T cells and activated macrophages in resistance to brucellosis in paramount (Winter, 1990; Kaufmann, 1999). Immune CD4 T cells produce interferon-γ (IFN-γ) for macrophage activation, and CD8 T cells recognize infected cells via the major histocompatibility class I complex (MHC class I) receptor resulting in cell death. During bacteremia and following lysis of infected cells, antibodies aid in opsonization of extracellular bacteria and bind to the macrophage Fc receptor which results in increased triggering of the respiratory burst in IFN-γ activated macrophages (Winter, 1990; Elzer et al., 1994a).

**Murine Brucellosis Model**

The BALB/c murine model of brucellosis established by Montaraz and Winter (1986) has provided much of the current understanding of immunity to brucellosis. The model is valuable since the murine colonization profile of virulent type strains and vaccine candidates mimics what is often attenuated in the ruminant host (Montaraz and Winter, 1986; Winter, 1990; Schurig et al., 1991; Elzer et al., 1996; Stevens et al., 1997; Edmonds et al., 2000a; Edmonds et al., 2000b). The murine model is also useful due to practical concerns including availability of reagents, cost, and population homogeneity (Montaraz and Winter, 1986; Garcia-Carrillo, 1990; Winter, 1990).

The majority of work published on the immune response in the murine brucellosis model has been with virulent *B. abortus* strain 2308 and attenuated strain 19 (Winter, 1990). Intraperitoneal (i.p.) injection of mice with $5 \times 10^4$ colony forming units...
units (CFU) of strain 2308 results in a chronic infection that is not cleared by six months post-infection (Montaraz and Winter, 1986). *Brucella abortus* strain 19 at 1 week post-infection replicates to the same extent as strain 2308 and by 2 weeks post-infection is 1.5 logs higher than strain 2308. However, by 4 weeks post-infection, strain 19 drops 1 log lower than strain 2308 and by 8 weeks is almost cleared from the spleen (Montaraz and Winter, 1986).

**Murine Immune Response**

By utilizing strain 2308 and strain 19 in mice, Winter and co-workers were able to develop a better understanding of the murine immune response to brucellosis (Montaraz and Winter, 1986; Montaraz et al., 1986; Araya et al., 1989; Winter et al., 1989; Araya and Winter, 1990; Winter, 1990; Elzer et al., 1994a; Elzer et al., 1994b). Injection of mice either i.p. or intravenously (i.v.) with strain 2308 or strain 19 results in localization of *brucellae* in the reticuloendothelial system and specifically in the spleen, as is the case with other facultative intracellular pathogens (Montaraz and Winter, 1986; Kaufmann, 1999). Following splenic colonization, large numbers of monocytes infiltrate the spleen and begin to ingest *brucellae* by means of complement receptors (Winter, 1990). These phagocytes do not attain a sufficient state of activation to kill either strain 2308 or strain 19 due to the lack of activation factors (Araya et al., 1989; Winter 1990; Zhan and Cheers, 1993; Elzer et al., 1994a; Kaufmann, 1999). Zhan and Cheers (1993) have shown that the macrophage activating cytokine, IFN-γ, is important in the controlling and/or killing of intracellular *brucellae* since depletion of this cytokine with anti-IFN-γ monoclonal antibodies

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(MAbs) resulted in a significant increase in splenic colonization compared to
treated controls.

By 3 weeks post-infection antibody specific for *Brucella* begins to appear in
the mouse (Araya et al., 1989). Elzer et al. (1994b) found that at this time, as the
numbers of strain 19 began to decrease, IgG3 antibodies developed that were effective
at opsonizing strain 19 and that likely bind to the macrophage Fc receptor resulting in
phagocytic activation and bacterial killing. IgG3 opsonized strain 19 is unable to
replicate in this hostile environment and is quickly eliminated while opsonized strain
2308 resists the killing processes of the macrophage as previously described (Kreutzer
et al., 1979; Frenchick et al., 1985; Araya et al., 1989; Araya and Winter, 1990; Elzer
et al., 1994a; Liautard et al., 1996).

Serum from mice vaccinated with strain 19 that contained predominately O-
side chain specific antibodies, was also demonstrated to provide between 2.0-2.5 logs
of protection against challenge when passively transferred to naive animals (Araya et
al., 1989; Araya and Winter, 1990). The serum from mice obtained prior to 3 weeks
post-vaccination was not protective, and serum obtained at 6 weeks post-vaccination
afforded the greatest protection, approximately 2.5 logs. Monoclonal antibodies
specific for the LPS O-side chain have also been shown by different laboratories to
provide significant protection against virulent challenge in mice (Montaraz et al.,
1986; Winter et al., 1989; Cloeckaert et al., 1991; Jacques et al., 1992). Antibodies
specific for the O-side chain opsonize extracellular *Brucella* for phagocytosis by
professional phagocytes. Based on these data, antibody to the LPS O-side chain is
important for protective immunity against murine brucellosis.
The importance of T cells for immunity to brucellosis was demonstrated by Araya et al. (1989) who found that the passive transfer of T cells from strain 19 vaccinated mice provided approximately 2.0 logs of protection against virulent challenge in naive mice. Both the CD8 and CD4 T cell populations were required for protection since depopulation of either T cell type resulted in an approximate 0.5 log reduction in protection. Combined transfer of both immune serum and T cells enhanced protection over that observed with either T cells or serum alone by approximately 1 log. Passive transfer of T cells from mice vaccinated less than 4 weeks did not protect against virulent challenge (Araya et al., 1989). Zhan and Cheers (1993) found that depletion of endogenous IFN-γ from recipient mice prior to passive transfer of protective T cells blocked the adoptive transfer of resistance indicating the importance of IFN-γ in the cell-mediated immune (CMI) response through macrophage activation.

The long protracted infection of strain 2308 in BALB/c mice indicates that the presence of protective T cells is not enough to eliminate the pathogen (Montaraz and Winter, 1986; Araya et al., 1989). Winter (1990) proposed that recovery from strain 2308 infection might depend on the formation of both immune T cells and the development of antibodies with high affinity and the required isotype. Elzer et al. (1994b) found that mice infected with *Brucella* demonstrated a 38 fold increase in *Brucella*-specific IgG3 and a 12 fold increase in IgG2a from 4 to 8 weeks post-infection. The authors proposed that the observed increase in both IgG3 and IgG2a was driven by IFN-γ which was derived in a T cell-independent manner until approximately 4-5 weeks post-infection when T cells became the primary IFN-γ
producers. In murine brucellosis both the humoral and CMI response are important for protection. O-side chain specific antibodies may opsonize extracellular bacteria and activated macrophages to a bactericidal state via the Fc-receptor. Meanwhile, CD8 T cells lyse infected cells and CD4 T cells activate macrophages through IFN-γ production.

**Ruminant Immune Response**

The bovine, ovine, and caprine immune responses to brucellae have not been as well elucidated as the murine immune response. Activated macrophages and T cells are both required for a protective immune response in ruminants (Nicoletti, 1990b; Nicoletti and Winter, 1990; Smith, 1990). Joubert and Valette (1967) found that macrophages from strain 19 vaccinated cattle reduced the growth of virulent *B. abortus* by 54% *in vitro* when compared to macrophages from naive animals. The importance of bovine macrophages as antigen presenting cells (APC) for T lymphocytes was documented *in vitro* by incubating bovine macrophages expressing *Brucella* antigens with a MAb specific for the MHC class II receptor (Splitter and Everlith, 1986). The ability of T cells to proliferate in response to the APC macrophages was evaluated by measuring the incorporation of [3H]thymidine. T cells incubated with anti-MHC class II treated macrophages showed a 9-fold decrease in lymphocyte proliferation when compared to untreated controls (Splitter and Everlith, 1986).

Numerous authors have shown that cattle vaccinated with either strain 19 or the rough cattle vaccine, *B. abortus* strain RB51, are protected against infection and produce lymphocytes that proliferate in response to *Brucella* antigens (Kaneene et al.,...
The fact strain RB51 lacks the LPS O-side chain and protects cattle against brucellosis indicates that *Brucella* O-side chain specific antibody titer does not correlate with protection in strain 19 vaccinated cattle and that antibodies to the O-side chain are not required for a protective immune response in cattle (Kaneene et al., 1979; Nicoletti, 1990b; Stevens et al., 1995; Stevens et al., 1996). In contrast to cattle, goats vaccinated with strain RB51 or a rough mutant of *B. melitensis* develop a limited protective immune response to brucellosis (Elzer et al., 1998c; P.H. Elzer, personal communication). Currently the only successful vaccine for caprine brucellosis is smooth *B. melitensis* strain Rev. 1 (Alton, 1990a; Blasco, 1997). Based on these findings, antibodies to the O-side chain may be necessary for a protective caprine immune response to *B. melitensis* (Elzer et al., 1998c).

**Vaccines**

The vaccines currently used for ruminant brucellosis are all attenuated-live mutants that were empirically derived (Adams, 1990; Nicoletti, 1990a; Nicoletti, 1990b). These vaccines exhibit limited replication in the host, and they induce a protective immune response. They are characterized by attenuation, induction of a protective CMI response, and stability upon multiple passages *in vivo* (Adams, 1990; Nicoletti, 1990a; Nicoletti, 1990b).

**Bovine Brucellosis**

In 1923, Buck isolated an attenuated-live organism termed *B. abortus* strain 19 from a culture of virulent *B. abortus* that was left at room temperature for over one year (cited in Graves, 1943). Work by numerous scientists has documented that strain
19 when given subcutaneous (s.c.) at $1 \times 10^9$ to $5 \times 10^{10}$ CFU to sexually immature cattle provides 65-75% protection against abortion when challenged with a virulent strain as adults (Cotton et al., 1934; McDiarmid, 1957; Garcia-Carrillo, 1980; Confer et al., 1985). In a seven-year study with approximately 500 cattle kept under strict isolation conditions, McDiarmid (1957) found that vaccination of 6 month old calves with strain 19 protected these animals against experimental challenge through their fifth pregnancy. The attenuation of strain 19 was documented as being stable following seven i.v. passages through pregnant cattle (Taylor and McDiarmid, 1949). In 1940 strain 19 was introduced as the official U.S. bovine brucellosis vaccine (Stevens et al., 1997).

Although strain 19 provides protection against *B. abortus*, the vaccine has several drawbacks. *Brucella abortus* strain 19 and virulent field strains both carry the immunodominant LPS O-side chain (Cotton et al., 1934; McDiarmid, 1957; Garcia-Carrillo, 1980; Confer et al., 1985; Nicoletti, 1990a). This is important because all accepted, current serologic tests for the diagnosis of brucellosis identify infected animals by detecting antibodies against the LPS O-side chain thus making it difficult to readily differentiate between strain 19-vaccinated and field strain-infected cattle (Alton et al., 1988; Stevens et al., 1997). Animals under one year of age vaccinated with strain 19 typically lose anti-O-side chain antibodies by the time of sexual maturity (Nicoletti, 1990a). As a result, full dose strain 19 vaccination ($5 \times 10^{10}$ CFU s.c.) is only recommended for sexually immature females (Nicoletti, 1990a).

A problem associated with strain 19 calfhood vaccination is that some cattle become chronically infected and retain a low-level titer to the LPS O-side chain.
Meyer and Nelson (1969; Duffield et al., 1984). Meyer and Nelson (1969) in a three-year study documented ten separate dairies where 11 sexually mature Holsteins had low level titers to the LPS O-side chain and were found to secrete strain 19 in the milk for the length of the study. Rarely, strain 19 also induces abortions if given to pregnant cattle at $3 \times 10^8$ to $5 \times 10^{10}$ CFU s.c., as is sometimes done in areas of high infectivity where animals are housed together in close proximity (Nicoletti, 1976; Beckett and MacDiarmid, 1985). In two separate studies, strain 19 s.c. vaccination of 10,000 animals with $5 \times 10^{10}$ CFU and 300 animals with $3 \times 10^8$ CFU resulted in abortion rates of $<1\%$ and $<3\%$ respectively (Nicoletti, 1976; Beckett and MacDiarmid, 1985).

Reports exist of three bulls vaccinated at approximately 6 months of age which were colonized by strain 19 in the reproductive tract and developed orchitis, epididymitis, sterility, and high titer to the LPS O-side chain by 6 months post-vaccination (Danks, 1943; Lambert et al., 1964). Due to the orchitis and persistent O-side chain antibody titer that can occur in vaccinated bulls, the use of strain 19 is restricted to female cattle (Nicoletti, 1990a). This vaccine can also cause undulant fever in humans if ingested or injected (Nicoletti, 1990a; Nicoletti, 1990b; Young, 1995). These problems associated with strain 19 have lead scientists to find a new vaccine for bovine brucellosis. This new vaccine would show decreased pathogenicity for the host, provide levels of protection similar to strain 19, and would not induce anti-O-side chain antibodies.

In 1996 the U.S. Department of Agriculture, Animal and Plant Health Inspection Service approved the new calfhood brucellosis vaccine *B. abortus* strain.
RB51 that was developed to overcome the serologic problems associated with strain 19 (Stevens et al., 1997). Schurig et al. (1991) reported the development of strain RB51, a stable, rough, rifampin-resistant, variant of virulent strain 2308 that was isolated by 51 serial passages of smooth strain 2308 on rifampin-containing media plates. This new vaccine is devoid of the LPS O-side chain, and strain RB51 vaccinated animals do not produce antibodies to the O-side chain thus alleviating the serodiagnostic problems associated with strain 19 (Schurig et al., 1991; Stevens et al., 1994; Stevens et al., 1995; Stevens and Olsen, 1996; Palmer et al., 1996; Palmer et al., 1997; Elzer et al., 1998b; Edmonds et al., 1999a). The vaccine provides protection equal to that of strain 19 and does not induce abortions in pregnant cattle (Cheville et al., 1996; Palmer et al., 1996; Palmer et al., 1997; Elzer et al., 1998b; Lord et al., 1998b). Multiple passages in vivo have shown the vaccine to be stable; and after its use by veterinarians and scientist worldwide, strain RB51 does not appear to be pathogenic for humans (Schurig et al, 1991; Stevens et al., 1997). Vaccination of bulls with strain RB51 did not result in orchitis and vaccinated dams did not shed the organism in their secretions (Edmonds et al., 1999a). Based on these studies, strain RB51 provides protection equal to strain 19 but without the undesirable side effects associated with strain 19.

**Caprine and Ovine Brucellosis (Brucella melitensis)**

A suitable vaccine for small ruminants against *B. melitensis* did not exist until the report of attenuated *B. melitensis* strain Rev. 1 by Elberg and Faunce in 1957 (cited in Jones et al., 1964). *Brucella melitensis* strain Rev. 1 is a smooth streptomycin-independent isolate obtained from a mutant streptomycin-dependent population of *B.*
In both controlled experimental studies and in field trials throughout the world, strain Rev. 1 has proven efficacious against virulent *B. melitensis* in either goats or sheep when given at $1 \times 10^9$ CFU s.c. (Jones et al., 1964; Alton, 1966; Alton, 1987; Alton, 1990a; Blasco, 1997; Garin-Bastuji et al., 1998; Scharp et al., 1999). Six serial *in vivo* passages in pregnant goats with strain Rev. 1 found no reversion to a virulent phenotype (Alton et al., 1967). Alton (1966) documented that s.c. vaccination of 3 to 7 month old sexually immature goats with strain Rev. 1 provided protection against virulent challenge 2.5 years following vaccination. Due to its capability to provide protection against abortion and infection, the strain Rev. 1 vaccine has been utilized throughout the world, including South America, Europe, Africa, and Asia (Alton, 1990a).

As in the case of *B. abortus* strain 19 vaccine, problems do exist with the use of strain Rev. 1. Vaccination of pregnant goats or sheep s.c. with the standard dose of $1 \times 10^9$ CFU can cause abortions 40-60 days post-vaccination (Jones et al., 1964; Alton, 1966; Alton et al., 1967; Alton, 1987; Alton, 1990a; Blasco, 1997; Garin-Bastuji et al., 1998). Blasco (1997) reported that during large field trials with over 200 animals, abortion rates >75% occurred when sheep were vaccinated s.c. with $5 \times 10^8$ CFU of strain Rev. 1 during the second and third months of pregnancy. A second problem with the use of smooth strain Rev. 1 is the residual antibody titer to the LPS O-side chain (Alton, 1987; Alton 1990a; Blasco, 1997; Garin-Bastuji et al., 1998). Alton (1987) states that 5-15% of goats vaccinated as adults with the standard Rev. 1 dose are positive for agglutinating antibodies to the O-side chain 1-2 years post-vaccination. As a result of the problems associated with adult vaccination with strain
Rev. 1, full dose vaccination is recommended for only sexually immature animals between 3 and 8 months of age (Alton, 1987; Alton, 1990a; Blasco, 1997). *Brucella melitensis* strain Rev. 1 must also be handled carefully since s.c. injection can cause undulant fever in humans (Blasco and Diaz, 1993).

In developing countries where management practices are limited and infection rates are high, the recommended vaccination of only 3-8 month old sexually immature animals is often impractical (Blasco, 1997). As an alternative, some authors have suggested whole-flock vaccination every two years with a reduced dose of strain Rev. 1 (5 x 10^4 to 1 x 10^6 CFU) in addition to yearly vaccination of sexually immature animals with the full dose (Alton, 1987; Alton, 1990a; Scharp et al., 1999).

Alternative routes of inoculation have also been suggested, including via the conjunctival sac which appears to reduce both the occurrences of abortions and the production of long-term anti-O-side chain antibodies (Alton, 1987; Alton, 1990a). Scharp et al. (1999) reported that in Kuwait the mass vaccination of goats and sheep with the reduced Rev. 1 dose of 1 x 10^7 CFU s.c. resulted in a 65% decrease in the rate *B. melitensis* infection in small ruminants from 1993 to 1997 (5.8% to 2.02%). Serological analysis showed no evidence of persistent anti-O-side chain titers and no increases in abortions (Scharp et al., 1999).

Several authors disagree with the use of low dose Rev. 1 mass vaccination or conjunctival inoculation (Zundel et al., 1992; Blasco, 1997; Garin-Bastuji et al., 1998). Blasco (1997) reported that in Spain mass s.c. vaccination with the reduced dose of strain Rev. 1 (1 x 10^6 CFU) resulted in “thousands of vaccine-induced abortions” that occurred usually 40-60 days post-vaccination. *Brucella melitensis* strain Rev. 1 was
consistently isolated from abortions; and as a result, both low and high dose s.c.
vaccination was suspended in Spain (Blasco, 1997). Zundel et al. (1992) found that
conjunctival vaccination of pregnant ewes and goats with $1 \times 10^8$ CFU of strain Rev. 1
resulted in a 20% abortion rate. As a result, these authors consider mass conjunctival
vaccination with $1 \times 10^8$ CFU of strain Rev. 1 unsafe for pregnant animals (Zundel et
al., 1992). Due to the problems associated with low dose vaccination, Blasco (1997)
recommends standard Rev. 1 vaccination of 3 to 8 month old small ruminants and
conjunctival vaccination of adults at least 1 month prior to breeding and/or after the
completion of lactation (Alton, 1990a; Blasco, 1997; Garin-Bastuji et al., 1998).

In the field, vaccination of adults one month prior to breeding and after
completion of lactation is difficult and often unrealistic in remote or financially
constrained areas (Blasco, 1997; Garin-Bastuji et al., 1998). An efficacious vaccine
that could be given to both pregnant adults and sexually immature animals would be a
major advantage in developing countries. Recent work with a rough mutant of \textit{B.
melitensis} containing a disruption in a O-side chain synthesis gene or rough \textit{B. abortus}
strain RB51 found neither vaccine efficacious against virulent \textit{B. melitensis} (Elzer et
al., 1998c; P.H. Elzer, personal communication).

**Ovine Brucellosis (\textit{Brucella ovis})**

\textit{Brucella melitensis} strain Rev. 1 is considered the best vaccine currently
available for the prevention of ovine brucellosis due to \textit{B. ovis} (Blasco et al., 1987;
Blasco, 1990; Jimenez de Bagues et al., 1995). Under experimental conditions, s.c.
vaccination of 3 to 5 month old rams with $1 \times 10^9$ CFU of strain Rev. 1 provides
between 75-100% protection against colonization by virulent \textit{B. ovis} (Blasco et al.,
strain Rev. 1 induces persistent anti-O-side chain antibodies and is pathogenic for both humans and pregnant sheep (Alton, 1990a; Blasco and Diaz, 1993; Blasco, 1997). In countries such as the U.S. where *B. melitensis* has rarely been isolated, the use of attenuated *B. melitensis* strains such as Rev. 1 is not allowed (Blasco, 1990; Jimenez de Bagues et al., 1995). Therefore in the U.S. and similar countries, no vaccines are available for ovine brucellosis.

**Porcine Brucellosis**

No vaccines are currently available for *B. suis* infections of domestic or feral swine (Alton, 1990b). In Venezuela, Lord et al. (1998a) found that oral vaccination of gilts with *B. abortus* strain RB51 did protect against infection under field conditions where the mating boars were secreting up to $1 \times 10^4$ CFU/ml of *B. suis* in the semen. Under experimental conditions, sows fed $>1 \times 10^{10}$ CFU of strain RB51 mixed with pecans and corn were transiently colonized in the draining lymph node and mounted a detectable humoral immune response (Edmonds et al., 2000b). Based on these two studies, it may be possible to vaccinate infected feral swine with strain RB51 using attractive baits such as corn and pecans (Lord et al., 1998a; Edmonds et al., 2000b).

**Wildlife Brucellosis**

One possible method to control the problem of bison and elk infected with *B. abortus* in the Greater Yellowstone Area (GYA) is vaccination. Davis et al. (1991) showed that vaccination of pregnant bison with *B. abortus* strain 19 resulted in abortions, chronic infections, and prolonged serologic response to the LPS O-side chain. Due to the problems associated with strain 19 in bison, the new cattle vaccine
\textit{B. abortus} strain RB51 is now being evaluated in these animals. Vaccination of ten adult bison bulls, seven calves, and six females in the first trimester of pregnancy with strain RB51 resulted in clearance of the vaccine by 13 weeks post-vaccination and no abortions (Elzer et al., 1998a). Initial trials with bison indicate that strain RB51 may also be efficacious against virulent \textit{B. abortus}, however additional studies are necessary (Cheville et al., 1998; P.H. Elzer, personal communication). The Wyoming Game and Fish Department (WGFD) has vaccinated over 36,000 elk with \textit{B. abortus} strain 19 at WGFD-managed elk feeding grounds since 1985 (Cheville et al., 1998). A 50\% decrease in the prevalence of brucellosis at one WGFD feeding ground was attributed to strain 19 vaccination (Cheville et al., 1998). Elzer et al. (cited in Cheville et al., 1998) found that oral vaccination of female elk with \textit{B. abortus} strain RB51 resulted in a 40\% decrease in abortions and 70\% decrease in colonization following challenge with virulent \textit{B. abortus}. While additional studies are necessary, it is clear that vaccination of bison and elk is a realistic option for the control of brucellosis in the GYA.

\textbf{Outer Membrane Proteins}

\textit{Immune Response}

Besides work with live vaccines, several research groups have shown in mice that extracts of the \textit{Brucella} cell wall contain important vaccine components. Dubray and Bezard (1980) demonstrated that boiling the \textit{B. abortus} cell wall in sodium dodecyl sulfate resulted in an insoluble cell fraction (SDS-I) that provided some protection when administered to mice. Analysis of this SDS-I fraction in a SDS-PAGE gel revealed three major protein bands of 37 kDa, 25 kDa, and 15 kDa (Dubray
and Bezard, 1980). In *B. abortus* these proteins were later identified to be the 41-43 kDa porin protein, the 25 kDa outer membrane protein (Omp25), and a 15 kDa lipoprotein, respectively (Verstreate et al., 1982; Dubray and Charriaut, 1983; Douglas et al., 1984; Verstreate and Winter, 1984; Cloeckaert et al., 1996a; Cloeckaert et al., 1996c). Each major protein from the SDS-I fraction was eluted from the gel and contained <5% of the other proteins (Dubray and Bezard, 1980). Chemical analysis of the SDS-I fraction found it to consist of 30% peptidoglycan, 20% Omp25, 13.5% porin, 4.5% lipoprotein, and an additional 32% of unidentified products including a small amount of LPS (Dubray and Bezard, 1980).

To study the vaccine properties of the SDS-I fraction, mice were vaccinated with either the hydrolyzed SDS-I fraction, Omp25, porin, or lipoprotein combined with Freund’s incomplete adjuvant (FIA) (Dubray and Bezard, 1980). As positive controls, an additional group of mice were vaccinated with killed-whole cells of *B. melitensis* strain H38, which provides protection in mice (Dubray and Bezard, 1980). Both Omp25 and the porin provided approximately 1.7 logs of protection against virulent *B. abortus* 2 weeks following challenge. In comparison, killed-whole cells of strain H38 provided 2.07 logs of protection while the entire SDS-I fraction provided approximately 2.3 logs of protection. Based on the vaccine properties of SDS-I, Dubray and Charriaut (1983) concluded that Omp25, the 36 kDa porin, and LPS were the three most important antigenic surface components in this fraction.

Following the isolation of the major outer membrane proteins (Omps) of *B. abortus* by Verstreate et al. (1982), Winter and coworkers performed a series of experiments testing the immunogenicity of these Omps in both cattle and mice.
(Winter et al., 1983; Baldwin et al., 1985; Montaraz and Winter, 1986; Winter et al., 1986; Winter and Rowe, 1988). Vaccination of mice with the native Omps of *B. abortus* in an oil-based adjuvant was found to provide significant protection against virulent *B. abortus* at 1 and 4 weeks post-challenge (Montaraz and Winter, 1986). Vaccination of cattle with the cell envelope from *B. abortus* containing Omps, peptidoglycan, and some LPS in a oil-based adjuvant resulted in both a positive delayed type hypersensitivity reaction to the purified Omp25 protein and the production of anti-Omp25 antibodies up to 7 months post-vaccination (Winter et al., 1986). Winter and Rowe (1988) showed that vaccination of mice with either the native cell envelope of *B. abortus* or the SDS-I fraction described previously resulted in equivalent levels of protection in mice 1 and 4 weeks post-challenge. Winter and Rowe (1988) also showed that vaccination of cattle with either *B. abortus* cell envelope, purified Omps, or SDS-I fraction in combination with an oil-based adjuvant simulated Omp25-specific lymphocyte proliferation and delayed type hypersensitivity reactions over a 3 month period. Based on this work, Winter and Rowe (1988) concluded that the native cell envelopes of *B. abortus* and the SDS-I fraction were vaccines of comparable quality based on measurements of protective immunity in mice and of immunogenicity in cattle.

Besides work with the SDS-I fraction and native cell envelope Omps, Jimenez de Bagues et al. (1994a) have shown that a hot saline extract (HS-PCP) of the *B. ovis* outer membrane containing <1% LPS is protective against virulent *B. ovis* challenge in mice. The HS-PCP fraction consist of 93% protein, including high levels of the group 3 Omps, Omp25 and Omp31, on an polyacrylamide gel (Gamazo et al., 1989;
Cloeckaert et al., 1996a; Cloeckaert et al., 1996b; Vizcaino et al., 1996). Vaccination of mice with HS-PCP plus an adjuvant was found to provide 2.57 logs of protection against virulent *B. ovis* challenge while the official ovine brucellosis vaccine, *B. melitensis* strain Rev. 1, provided 1.77 logs of protection. The passive transfer of both T cells and antibodies from mice vaccinated with a hot saline extract containing 35% R-LPS and 65% protein also provided significant protection (Jimenez de Bagues et al., 1994a). Based on the protection seen in mice with HS-PCP, the outer membrane proteins may be major components necessary for a protective immune response to *B. ovis*.

The cloning and expression of the *omp25* gene in *E. coli* made it possible to study the immunogenicity of purified Omp25 in *vivo* (de Wergifosse et al., 1995; Cloeckaert et al., 1996b; Bowden et al., 1998). Vaccination of mice with the denatured, purified recombinant Omp25 did not provide significant protection against *B. melitensis* nor did these mice produce Brucella-specific antibodies (Cloeckaert et al., 1996c; Bowden et al., 1998). However, vaccination with whole-cell sonicates of *E. coli* expressing Omp25 provided significant protection against rough *B. melitensis* in the murine model (Bowden et al., 1998). Mice vaccinated with the killed whole-cells produced Omp25-specific antibodies from 15 to 90 days post-vaccination that were able to bind to rough strains of *B. melitensis* more effectively than smooth strains. Vaccination of mice with the recombinant whole-cell lysate did not provide significant protection against smooth strains of *B. melitensis*. Bowden et al. (1998) concluded that antibodies to well-exposed conformational epitopes on Omp25 might be important for protective immunity to *B. melitensis* in mice. Based on work with
cell envelope extracts containing high levels of Omp25 and whole-cell lysates of *E. coli* expressing recombinant Omp25, this protein may play an important role in a protective humoral and cell-mediated immune responses to *Brucella* spp.

**Virulence Factors**

Work in other pathogenic bacterial species has shown that besides being important for a protective immune response, certain outer membrane proteins may also be important virulence factors. Baldermann et al. (1998) have shown that the *Brucella* Omp25 shares structural homology with Omps from over 25 different Gram-negative bacteria, including CadF from *Campylobacter jejuni* and OmpA from *E. coli*.

*Campylobacter jejuni* is a commensal of the intestinal tract of chickens that is passed to humans by eating undercooked poultry (Nachamkin, 1995). The bacteria causes over 2 million cases of acute diarrhea in U.S. citizens each year (Nachamkin, 1995). Indicative of the important role of the Omps in pathogenesis, Ziprin et al. (1999) found that a *C. jejuni* mutant containing a disruption in the *cadF* gene was attenuated *in vivo*. CadF is a 37 kDa fibronectin-binding Omp that may be an important adhesin allowing the bacteria to colonize the cecum of chickens (Konkel et al., 1997). In order to investigate this hypothesis *in vivo*, one-day-old chicks were exposed orally to either the CadF* parental strain or the Δ*cadF* mutant (Ziprin et al., 1999). At 7 days post-challenge, 43/60 chicks exposed to the parental strain were colonized by *C. jejuni* while an equal number of chicks exposed to the Δ*cadF* mutant were free of infection. The lack of CadF rendered the mutant unable to colonize the cecum of newly hatched chicks (Ziprin et al., 1999).
Escherichia coli K-1 is a known etiologic agent of neonatal septicemia and meningitis (Levine, 1984; Selander et al., 1987). The outer membrane protein A (OmpA) of E. coli K-1 is a 35 kDa protein that is known to serve as a mediator of F-dependent conjugation and as receptor for both colicins and phages (Nikaido and Vaara, 1987). In an effort to investigate if OmpA was also a virulence factor for E. coli K-1, Weiser and Gotschlich (1991) tested a ΔompA mutant in two models of E. coli K-1 infection, 10-day-old embryonated chick eggs, and neonatal rats.

Inoculation of chick embryos with 10 to 1 x 10^5 CFU of the virulent parental strain resulted in a 27 to 62% mortality rate, respectively (Weiser and Gotschlich, 1991). The ΔompA mutant demonstrated a three- to fivefold decrease in virulence in the same procedure (10 to 18%) while complementation of the ompA mutation restored a level of virulence equivalent to the parental strain. In a model of bacteremia, neonatal rats were orally inoculated with an equal mixture of the OmpA+ parental strain and the ΔompA mutant. At 72 hours post-inoculation, the incidence of bacteremia due to the mutant decreased sevenfold compared to the parental strain while complementation of the ompA mutation again restored virulence (Weiser and Gotschlich, 1991). The lack of OmpA rendered E. coli K-1 attenuated in both neonatal rats and embryonated chick eggs. Based on these studies, Omps appear to be important in the virulence of both E. coli K-1 and C. jejuni.

**Role of Omp25 in Brucella spp.**

The importance of Omp25 for virulence in Brucella spp. is unknown. This protein may be an important structural Omp that stabilizes the outer membrane by binding the underlying peptidoglycan layer and outer cell membrane. Omp25 is
known to be covalently bound to the peptidoglycan layer, and reconstitution of conformational epitopes on purified Omp25 requires association with LPS (Dubray and Charriaut, 1983; Cloeckaert et al., 1992; Cloeckaert et al., 1996c). As demonstrated with *C. jejuni* CadF and *E. coli* K-1 OmpA mutants, the absence of Omp25 from *Brucella* spp. may render these mutants attenuated in ruminants (Weiser and Gotschlich, 1991; Ziprin et al., 1999).

However vaccination of mice with cell envelope extracts containing high levels of Omp25 or whole-cell lysates of *E. coli* expressing recombinant Omp25 provides some protection against brucellosis (Dubray and Bezard, 1980; Montaraz and Winter, 1986; Winter and Rowe, 1988; Bowden et al., 1998). Inoculation of cattle with these same cell extracts has been shown to induce Omp25-specific humoral and CMI responses (Winter et al., 1986; Winter and Rowe, 1988). Therefore, the absence of Omp25 in *Brucella* spp. may alter the host’s immune response to this pathogen. Ruminants exposed to brucellae mutants lacking Omp25 may fail to generate the proper immune response and display an exacerbation of disease.

In the following chapters the creation and characterization of *B. melitensis*, *B. abortus*, and *B. ovis* Δomp25 mutants will be described. The goal of this work is to determine the role of Omp25 in virulence. The lack of this protein may render these mutants attenuated due to the lack of an important structural protein or inoculation of them into ruminants may induce heightened pathogenesis due to an altered immune response.
CHAPTER 3
GENERATION AND INITIAL DESCRIPTION OF *Brucella melitensis*, *Brucella abortus*, AND *Brucella ovis* MUTANTS LACKING A 25 kDa OUTER MEMBRANE PROTEIN (Omp25)

Introduction

*Brucella* species (spp.) are Gram-negative, facultative, intracellular pathogens that pose threats to both agribusiness and human health throughout the world. The different *Brucella* spp. are classified by the preferred natural host and basic biochemical tests (Meyer, 1990). Bovine and caprine brucellosis are characterized by late-term abortions caused by *Brucella abortus* and *Brucella melitensis*, respectively (Enright, 1990; Thoen et al., 1993). Rams infected with *Brucella ovis* develop orchitis and epididymitis and subsequent infertility (Blasco, 1990). Both *B. melitensis* and *B. abortus* are pathogenic for humans and cause the febrile illness, undulant fever. Infected individuals can present with a variety of clinical symptoms including pyrexia, sacroiliitis, arthritis, spondylitis, osteomyelitis, and bursitis (Young, 1995).

*Brucella* spp. have three major groups of outer membrane proteins (Omps) termed group 1 (94 kDa), group 2 (41-43 kDa) and group 3 Omps (25-31 kDa) (Dubray and Bezard, 1980; Verstreete et al., 1982). The group 1 Omps are considered minor components of the outer membrane while the group 2 Omps have been identified as functional porins (Verstreete et al., 1982; Douglas et al., 1984). The group 3 Omps consist of two separate proteins of 25 and 31 kDa. The function of the 25 kDa Omp (Omp25) is unknown while the 31 kDa Omp (Omp31) is proposed to be a second porin (Cloeckaert et al., 1992; Vizcaino et al., 1996).
Omp25 is a transmembrane protein that is expressed on the outer membrane of brucellae and is covalently bound to the underlying peptidoglycan layer of the cell (Dubray and Charriaut, 1983; Cloeckaert et al., 1990; Cloeckaert et al., 1992). PCR-RFLP analysis of the *omp25* gene with nine restriction enzymes indicates that the gene is conserved in the *Brucella* genus (Cloeckaert et al., 1995). Two deviations in the *omp25* gene are a short 36 nucleotide deletion near the 3' end of the *B. ovis* gene and the lack of an *EcoRV* restriction site in the *B. melitensis* gene (Cloeckaert et al., 1995). Association of Omp25 with lipopolysaccharide (LPS) is required for the reconstitution of conformational epitopes on purified Omp25 (Cloeckaert et al., 1996c). This protein may be a highly conserved structural Omp that stabilizes the outer membrane by binding the underlying peptidoglycan layer and outer cell membrane.

In addition to the structural role of this protein, Omp25 is also antigenic in both mice and cattle. Vaccination of mice with *Brucella* cell envelope extracts containing concentrated levels of Omp25 or normal cell envelope levels of Omp25 in an oil-based adjuvant provides significant protection against virulent challenge (Dubray and Bezard, 1980; Montaraz and Winter, 1986; Winter and Rowe, 1988). Bowden et al. (1998) demonstrated that whole cell lysates of *Escherichia coli* expressing recombinant Omp25 could protect mice against challenge with divergent rough strains of *B. melitensis*. Cattle vaccinated with the native Omp25 from *B. abortus* in an oil-based adjuvant demonstrated Omp25-specific lymphocyte proliferation and delayed type hypersensitivity reactions over a 3 month period (Winter and Rowe, 1988).

As a transmembrane protein spanning the outer cell membrane and peptidoglycan layer, the lack of Omp25 in *Brucella* spp. may affect the ability of these...
organisms to cause disease in the ruminant host. Alternatively, the absence of Omp25 may alter the host's immune response to this pathogen. Brucellae lacking Omp25 may fail to generate a typical cell-mediated or humoral immune response in infected animals resulting in an exacerbation of disease. To determine the role of Omp25 in brucellae, mutants of *B. abortus*, *B. melitensis*, and *B. ovis* lacking the Omp25 protein were created. To determine the pathogenic potential of each mutant *in vivo*, the murine BALB/c brucellosis model was utilized.

**Materials and Methods**

**Bacterial Strains**

Three *Brucella* strains were utilized for these studies. *Brucella abortus* strain 2308 is a virulent challenge strain used by several different laboratories that is documented to cause abortions in cattle (Enright, 1990; Cheville et al., 1996; Elzer et al., 1998b). *Brucella melitensis* strain 16M is also a virulent challenge strain causing caprine abortions (Phillips et al., 1997; Elzer et al., 1998c). *Brucella ovis* strain LSU99 was isolated from the semen of a ram experimentally infected with *B. ovis* strain PA. *Brucella ovis* strain PA was isolated from a naturally infected ram and passaged in mice prior to experimental inoculation of the ram. At the time strain LSU99 was isolated, the ram had developed bilateral epididymitis and subsequent infertility.

**Plasmids**

In order to obtain deletion mutants, suicide plasmids containing a disrupted *omp25* gene from either *B. ovis* strain 63/290 (plasmid pAC2555) or *B. melitensis*
strain 16M (plasmid pAC2553) were provided by Dr. Axel Cloeckaert, Institut National de la Recherche Agronomique, Nouzilly, France (Appendix 1).

Electroporation

Glycerol stocks of *B. abortus* strain 2308, *B. melitensis* strain 16M, and *B. ovis* strain LSU99 were thawed and plated on Schaedler agar (Difco Laboratories, Detroit, MI) supplemented with 5% bovine blood. Whole bovine blood was obtained from a healthy steer with no brucellosis vaccination history. Following incubation for three days at 37°C in a 5% CO₂ atmosphere, fresh brucella broth (Difco) was inoculated with either *B. abortus* strain 2308 or *B. melitensis* strain 16M and incubated overnight in a shaking water bath at 37°C. Following overnight growth, 30 ml of either *B. abortus* strain 2308 or *B. melitensis* strain 16M inoculated broth was divided into micro-centrifuge tubes and spun at 15,000 x g for 5 min (min) to pellet the bacteria. The resulting pellets were combined into a total volume of 1.2 ml of sterile distilled water (dH₂O) and subsequently washed three additional times with 0.5 ml of sterile dH₂O to remove any salts that would interfere with the electroporation. Following the final wash, the resulting bacterial pellet was resuspended in 0.1 ml of sterile dH₂O. Due to its slow growth and inability to grow in broth, *B. ovis* strain LSU99 was grown on Schaedler blood agar plates (SBA) for four days and the plates subsequently harvested with 30 ml of sterile dH₂O. Following harvest, the brucellae were washed as previously described.

To perform the electroporations, an Eppendorf 2510 Electroporator (Eppendorf Scientific Inc., Madison, WI) was used with Eppendorf cuvettes containing a 2-mm gap width and total potential volume of 0.4 ml. To 40 µl of washed bacteria...
suspended in sterile dH₂O, 2 μl of either pAC2553 (pUC19 containing the disrupted 
omp25 of B. melitensis strain 16M) or pAC2555 (disrupted omp25 of B. ovis strain 
63/290) was added. Both plasmids were provided purified and resuspended in sterile 
dH₂O. The resulting mixture was electroporated at 2500 V for approximately 5 to 6 
milliseconds. Immediately following electroporation, the bacteria were resuspended 
in 0.5 ml of sterile SOC-B broth and placed in a 37°C shaking water bath overnight. 
The SOC-B broth was filter sterilized prior to use and consisted of 6% Tryptic Soy Broth (TSB) (Difco), 10 mM NaCl, 2.5 mM MgCl₂, 10 mM MgSO₄, and 20 mM 
glucose. Following electroporation the inoculated broth was plated on SBA plates 
containing 45 μg/ml kanamycin (Sigma Chemical Company, St. Louis, MO). The 
plates were incubated at 37°C in a 5% CO₂ atmosphere for 2 weeks. Any colonies 
that appeared were subsequently patched to kanamycin plates and transferred to SBA 
plates containing 100 μg/ml of ampicillin (Sigma).

The presence of the 5’ and 3’ ends of native omp25 in the suicide vectors 
allows for homologous recombination to occur between the native gene and the 
disrupted omp25 on pAC2553 or pAC2555. Insertion of the omp25 gene from the 
suicide vector would prevent Omp25 production due to the replacement of the internal 
159 bp Styl fragment of omp25 with the 1.3 kb insertion containing the kanamycin 
resistance gene from pUC4K. Single-crossover mutants will be kanamycin resistant 
(Kan’) and ampicillin resistant (Amp’) due to the presence of both the kanamycin 
resistance determinant and the penicillin beta-lactamase gene bla. However, a double­
crossover mutant will remain Kan’ due to the insertion and will be ampicillin sensitive 
(Amp *) since the bla gene of pUC19 is found outside the 5’ and 3’ ends of the
disrupted *omp25* gene. Isolates that were both Amp' and Kan' or only Kan' were frozen in 25% sterile glycerol and 75% brucella broth and held at -80°C. Isolates were never passaged more than three times in the laboratory before returning to the original glycerol stock.

Double-crossover mutants were complemented via electroporation with plasmids pAC2580 and pAC2581 containing the complete *omp25* gene from *B. melitensis* strain 16M and *B. ovis* strain 63/290 inserted into the multiple cloning site of the broad-host-range plasmid pBBR1MCS4 (Kovach et al., 1994). The complementation plasmids were provided by Dr. Axel Cloeckaert. Transformants were selected on SBA plates containing 100 μg/ml of ampicillin (Sigma). Previous studies have found this plasmid to be highly stable in all six *Brucella* species both *in vivo* and *in vitro* (Elzer et al., 1995).

**Western Immunoblot**

To verify that Kan' isolates were *Brucella* spp., western immunoblot was performed with polyclonal serum from animals experimentally infected with either virulent *B. abortus* expressing the LPS O-polysaccharide side chain (O-side chain) or divergent, rough spp. lacking the O-side chain. To confirm that Kan' isolates were not expressing Omp25, immunoblot was performed with a series of monoclonal antibodies (MAbs) specific for Omp25.

Cell lysates of suspected isolates were obtained by thawing and plating original glycerol stocks onto SBA and incubating for three to four days as described previously. All isolates were streaked for isolation and observed daily prior to harvest to ensure no contamination. Plates were harvested with 2ml of sterile dH₂O and the
resulting suspension sonicated with a Heat Systems-Ultrasonics W-38S Sonicator (Farmingdale, NY) using a tapered microtip with a 1 second (sec) pulse for 10 min. After sonication, the resulting cell lysate was then diluted 1:2 with Laemmli sample buffer (Tris-HCl 62.5mM, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue) (BioRad, Hercules, CA) and boiled for 10 min (Laemmli, 1970).

Electrophoresis was performed with a BioRad Mini-Protean II unit utilizing the Laemmli discontinuous SDS-PAGE method with 0.5 µl to 5 µl of cell lysate per 20 µl well depending on cell lysate concentration (Laemmli, 1970). For electrophoresis Tris-HCl 10 – 20% acrylamide linear gradient gels with a 4% stacking gel were used (BioRad). Electrophoresis was terminated when the dye front was approximately 1 cm from the bottom of the gel. BioRad Kaleidoscope prestained standards were used as molecular weight markers with 5 µl per lane. Cell lysates were analyzed for LPS O-side chain utilizing western immunoblot.

Nitrocellulose transfer was performed for 1 hour (h) with 100 V in a Mini-Trans-Blot Electrophoretic Transfer Cell (BioRad). After transfer, the blots were blocked with 0.25% cold water fish skin gelatin (Sigma) for 30 min followed by washing with Tris-buffered saline (TBS) solution containing 0.5% Tween-20. Following blocking, the nitrocellulose blot was incubated with polyclonal serum from a cow experimentally infected with smooth *B. abortus* strain 2308. The LPS O-side chain of *Brucella* spp. produces a nondiscrete, ladder-like smear from 29 to 68 kDa based on protein markers which migrate concurrently on the same gel (Edmonds et al., 1999b). Cell lysates were also analyzed for internal non-O-side chain associated proteins using polyclonal serum from a cow vaccinated with the rough vaccine *B.*
abortus strain RB51. Cell lysates from both smooth and rough *Brucella* spp. produce numerous discrete bands when incubated with anti-RB51 polyclonal serum (Edmonds et al., 1999a). Each blot was incubated overnight at room temperature (RT) with a 1:40 dilution of the polyclonal serum in TBS. The blots were then washed and incubated with rabbit anti-bovine IgG horseradish-peroxidase-conjugate (Sigma) at a dilution of 1:800 for 45 min in TBS. After washing, the substrate 4-chloro-1-naphthol (Sigma) was added for color development.

A set of murine MAb s specific for Omp25 were described and provided by Dr. Axel Cloeckaert (Cloeckaert et al., 1990). While the specific epitope for each MAb is unknown, competitive inhibition assays have shown that they do not bind to the same epitope (Cloeckaert et al., 1996c). Monoclonal antibodies were diluted 1:800 and rabbit anti-mouse IgG horseradish-peroxidase-conjugate was used as the secondary antibody and the blots were then prepared as described previously. As a positive control, purified Omp25 was included on each gel. Purified Omp25 was provided by Dr. Alex Winter, Department of Veterinary Microbiology, Immunology and Parasitology, New York State College of Veterinary Medicine, Cornell University, Ithaca, NY.

**Genomic DNA Preparation**

Large-scale genomic DNA was extracted from Kan' isolates and from the parental strains for analysis by polymerase chain reaction (PCR) and Southern blotting. A 100 ml brucella broth culture of each isolate was grown overnight in a shaking water bath at 37°C. Following incubation an equal volume of acetone was added to each culture and stirred overnight under a fume hood. Cells were harvested
by centrifugation at 6,000 x g for 30 min and washed 3 times with 0.2 x saline EDTA buffer (SEDTA) (0.15 M NaCl, 0.01 M EDTA, pH 8.0). The cells were resuspended in 25 ml of 0.2 x SEDTA and mixed with 75 ml of chloroform and stirred for 1 h at RT. The aqueous phase containing the bacteria was removed, and the cells harvested by centrifugation at 2,000 x g for 15 min and washed three times with 2.5 ml of 0.2 x SEDTA. The cells were resuspended in 2.5 ml of 0.2 x SEDTA and incubated for 1 h at 37°C with 30 μl of a 50 mg/ml concentration of lysozyme (Sigma). Following the lysozyme treatment, 30 μl of a 20 mg/ml concentration of proteinase K (Sigma) was added for 1 h at 37°C. The cells were then lysed by the addition of SDS to a final concentration of 1%.

The lysed suspension was extracted twice using an equal volume of phenol-chloroform. Following a 5 min incubation the aqueous phase was removed from the chloroform by centrifugation at 17,000 x g for 10 min. Following the second extraction, the DNA was resuspended in SEDTA and precipitated by the addition 0.7 v/v isopropanol followed by centrifugation at 17,000 x g for 30 min. The resulting precipitate was resuspended in 0.5 ml of 1 x TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5) and mixed with 0.1 ml of Dnase-free Rnase (1 mg/ml stock) (Sigma) and incubated for 1 h at 37°C. The phenol-chloroform extraction was repeated as previously described. For the final precipitation, 10 M ammonium acetate was added until a final concentration of 2 M was achieved along with 0.7% isopropanol. After centrifugation at 17,000 x g for 30 min, the pellet was resuspended in 1-4 ml of 1 x TE depending on pellet size.

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**Plasmid Preparation**

Small-scale isolation of plasmid DNA was performed as previously described (Elzer et al., 1995). A 1.5 ml overnight broth culture of each isolate carrying the desired plasmid was harvested by centrifugation in a micro-centrifuge at 15,000 x g for 10 min and resuspended in 100 μl of Tris buffered solution (50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl, pH 8.0). The bacteria were killed by the addition of an equal volume of chloroform for 5 min. The cells were centrifuged at 15,000 x g, and the aqueous phase removed and plasmid DNA isolated by the alkaline lysis “mini-prep” procedure of Birboim and Doly (1979). To do this, the bacteria were first resuspended in 100 μl of ice-cold Tris buffered solution containing 5 mg/ml lysozyme (Sigma). Following incubation at RT for 5 min, 200 μl of fresh 0.2 N NaOH and 1% SDS w/v were added for lysis; and the solution incubated for 5 min on ice. Cellular debris was then precipitated with 150 μl of 5 M potassium-acetate and 11% w/v glacial acetic acid. The aqueous phase containing plasmid DNA was then extracted with phenol-chloroform as previously described and precipitated with 0.7 v/v isopropanol. The resulting pellet was resuspended 1 x TE and stored at -20°C for later use.

**Polymerase Chain Reaction**

Polymerase chain reaction was utilized to determine if the *omp25* gene was disrupted with the 1.3 kb Kan’ insertion. The use of PCR primers to the 5’ and 3’ ends of *omp25* from *B. ovis* strain LSU99 should amplify an approximately 600 bp fragment. In contrast, use of the same primers on a mutant containing the disrupted *omp25* gene should generate a product of approximately 1.75 kb. To verify insertion...
of the Kan$^\text{r}$ gene, primers were devised to the 5$'$ and 3$'$ ends of the published nucleotide sequence for \textit{omp25} from \textit{B. ovis} (Cloeckaert et al., 1996b). The sequence chosen is the same in all six \textit{Brucella} species (Cloeckaert et al., 1996b). The two 24-mer primers chosen were OMP25F (5$'$-ATGCGC\textit{ACTC}\textit{TTAAGTCTCTCGTA}-3$'$) and OMP25R (5$'$-CGCGTC\textit{GCATCGGCTACAAGTTC}-3$'$).

To further confirm the insertion of the disrupted \textit{omp25} gene, primers were also devised for the kanamycin resistance gene from pUC4K (Amersham Pharmacia Biotech). Using the published sequence in GenBank (accession number X06404) the devised primers should amplify an internal 700 bp sequence (Taylor and Rose, 1988). The two 21-mer primers chosen were KANF (5$'$-GCTGATTTATATGGGTATAA-3$'$) and KANR (5$'$-CAATACCATATTTTGGAAAAA-3$'$).

To verify the Kan$^\text{r}$ and Amp$^\text{r}$ isolates were double-crossovers mutants, primers were also contrived for the \textit{bla} gene from pUC19. Such primers would yield a gene product in the case of single-crossover mutants. As mentioned before, the Amp$^\text{r}$ cassette of pUC19 is found outside the 5$'$ and 3$'$ ends of the disrupted \textit{omp25} gene and hence Amp$^\text{r}$ is lost in double-crossover mutants. As a positive control, genomic DNA was isolated from a single-crossover mutant of \textit{B. abortus} that was Kan$^\text{r}$ and Amp$^\text{r}$. The two primers were chosen based on the published nucleotide sequence of the beta-lactamase from pBR322 (GenBank Accession Number VO1119) from which the Amp$^\text{r}$ in pUC19 is derived (Sutcliffe, 1979). The two primers were AMP1 (5$'$-GGGATTTTTGCTCATGAGATT-3$'$) and AMP2 (5$'$-GGAACCCCTATTTGTTTA-3$'$). Primers for \textit{omp25} and \textit{bla} were produced by Integrated DNA Technologies, Inc (Coralville, IA) while primers for the kanamycin determinant were made by GeneLab.
in the Department of Veterinary Microbiology and Parasitology at the Louisiana State University School of Veterinary Medicine (Baton Rouge, LA).

Amplification reactions were prepared in 100 μl volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl (10 x PCR buffer), 1% DMSO, 200 μM (each) dNTPs, 0.2 μM of each primer, approximately 200 ng genomic DNA, 2.5 U AmpliTaq DNA Polymerase, and 1.5 mM MgCl₂ (Perkin-Elmer, Branchburg, NJ) (Mullis and Fallen, 1987). Most reactions were performed on a PTC-100 thermocycler (MJ Research Inc., Watertown, MA) while amplification for the kanamycin determinant was performed on a BioRad Gene Cycler. Amplification of the omp25 gene was performed with a 95°C denature for 4 min followed by 34 repetitions of a 95°C denature for 30 sec, 30 sec annealing cycle at 65°C, and a 72°C extension for 2 min. A final extension for 10 min at 72°C was also performed. Amplification of the kanamycin and ampicillin resistance genes was performed with the same protocol but at an annealing temperature of 45°C and 50°C, respectively.

Agarose Gel Electrophoresis

Following PCR, amplification products were visualized with agarose gel electrophoresis. For each gel, 20 μl of reaction mixture was added to 3 μl of glycerol-dye loading buffer to facilitate loading. The results of both the omp25 and bla reactions were visualized on 0.7% agarose / TAE (40 mM Tris-acetate, 2mM EDTA, pH 8.5) gels while the small size of the kanamycin product necessitated a 0.9% agarose / TAE gel. A 90 V charge was utilized for the agarose gel in a BioRad DNA Sub Cell and stopped when the dye neared the far edge of the gel. As molecular
weight standards, 10-15 μl of λ DNA digested with HindIII were visualized on each gel (BioRad). Upon completion, gels were incubated with 10 μg/ml ethidium bromide for 3 min followed by destaining overnight in dH2O. Bands were detected with an UV transilluminator and documented with a digital camera.

**Southern Blot Analysis**

As a method to validate the results from PCR, Southern blot analysis was also performed with the suspected *B. abortus* mutant. Genomic DNA from the mutant, parental strain, and control plasmids was digested with the restriction enzyme EcoRI (Amersham-Pharmacia) using a reaction of 20 μl of genomic DNA, 120 U of enzyme, and 10 μl of EcoRI buffer in a total volume of 100 μl. Digestions were incubated overnight in a shaking 37°C water bath. Digested DNA was then visualized on a 1.2% agarose gel, and the genomic DNA analyzed by the Southern blot technique (Southern, 1975).

In preparation for transfer, the genomic DNA was partially depurinated with 0.25 M HCl for 15 min and subsequently denatured with 0.4 M NaOH for 25 min. The gel was then transferred to a nylon membrane utilizing the alkaline buffer technique with a Stratagene vacuum blotter (La Jolla, CA) utilizing 0.4M NaOH. The transfer was performed for 2 h with the addition of new solution approximately every 5 min. Following completion of a successful transfer, the nylon was washed with 2 x SSC (3.75 M NaCl and sodium citrate dihydrate, pH 7.0) and dried at 60°C for 20 min. The blot was then prehybridized with 40 ml of hybridization buffer (0.25 M disodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA) at 65°C for 1 h using a rotisserie hybridizer.
To use as a probe, *omp25* from the complementation vector pAC2581 carrying the complete *omp25* gene was amplified via PCR as described previously. The *omp25* probe was denatured in boiling H$_2$O for 5 min and immediately transferred to ice for 5 min. Approximately 25 ng of denatured probe was then biotinylated with 10 µl of biotinylated random octamers, 5 µl of dNTP containing Bio-dUTP, and 1 µl of DNA polymerase 1 – large (Klenow) fragment as via the manufacture's instructions (Tropix, Bedford, MA). The reaction was incubated overnight at 37°C and stopped the next day with 30 µl of stop solution (22 µl of 10 M sodium acetate, 5 µl tRNA, 3 µl 0.25 M EDTA) and 150 µl of ethanol (EtOH). The mixture was incubated at 4°C for 30 min and precipitated with 70% EtOH.

Following prehybridization of the blot, 10ml of hybridization buffer was added containing the biotinylated probe and incubated overnight at 65°C in rotisserie hybridizer. The next day the blot was washed three times with 2 x SSC and 1% SDS at RT for 5 min followed by one wash at 65°C with 0.1 x SSC and 1% SDS for 15 min. As a final step prior to chemiluminescent detection, the blot was washed two times with 1 x SSC at RT for 5 min. For chemiluminescent detection the Tropix Southern-Light system containing the CSPD Ultra-Pure alkaline phosphatase substrate was utilized. The blot was first washed two times with blocking buffer (0.2% casein, 1 x PBS, 0.5% SDS) for 5 min and then incubated with the same buffer for 10 min at RT. Next, alkaline phosphatase conjugated to streptavidin was diluted 1:5,000 and incubated with the blot for 20 min at RT with constant rotation. The blot was again washed one time for 5 min with blocking buffer followed by three washes with rinse buffer (1 x PBS and 0.5% SDS). Next, assay buffer (0.1 M diethanolamine, 1 mM
MgCl₂) was added twice for 2 min. Finally, the chemiluminescent substrate, CSPD, was added at a concentration of 0.25 mM and incubated for 5 min. All excess solution was drained off the blot, and the blot wrapped in Saran Wrap. Bands were immediately visualized by exposure to autoradiographic film (Hyperfilm-ECL, Amersham Pharmacia Biotech) and developed. Exposure times of 30 sec were found to provide optimal results.

**Standard Identification Tests**

Suspected mutants were also checked with several common techniques used to differentiate *Brucella* spp. from other Gram-negative organisms. The production of indophenol oxidase was recorded using the oxidase test (Becton Dickinson and Company, Cockeysville, MD). In this reaction, indophenol oxidase in the presence of atmospheric oxygen oxidizes the redox dye (N,N,N’,N’-tetramethyl-p-phenylenediamine dihydrochloride) resulting in a color change from yellow to purple (Alton et al., 1988). The ability to hydrolyze urea was determined with a modified Christensen’s agar medium (Becton Dickinson and Company) (Alton et al., 1988). Smooth and rough isolates were differentiated by the acriflavine test (Alton et al., 1988). A 1:1000 dilution of neutral acriflavine (Sigma) is mixed with a loop full of brucellae; rough colonies quickly clump while smooth colonies remain in solution. Isolates were also characterized by Gram-stain, colony morphology, and growth rate. *Brucella melitensis* strain 16M and *B. abortus* strain 2308 are both urease positive, oxidase positive, Gram-strain negative, smooth, and grow to isolated colonies in approximately three days on SBA plates at 37°C with a 5% CO₂ atmosphere (Alton et al., 1988). Unlike *B. abortus* and *B. melitensis*, *B. ovis* strain LSU99 is rough, urease...
negative, oxidase negative, and grows to isolated colonies in four days under the same conditions.

A common method used to differentiate *Brucella* spp. from each other is sensitivity to various dyes (Alton et al., 1988). All isolates and the respective parental strains were tested for sensitivity to azure A, basic fuchsin, crystal violet, pyronin, safranin, and thionin using commercial reagents (Key Scientific Products, Round Rock, TX). Approximately $5 \times 10^5$ colony forming units (CFU) of bacteria were plated onto a Trypticase Soy Agar (Difco) supplemented with 5% equine serum. The six dye tablets were placed at equal distances throughout the plate. Unlike *B. melitensis*, *B. abortus* is inhibited by both azure A and thionin (Alton et al., 1988).

**Inoculation Doses**

Inoculation doses were made of all three parental strains, complemented mutants, and potential *omp25* deletion mutants as described previously (Elzer et al., 1996). From original glycerol stocks, fresh three-day cultures were obtained by methods previously described. Bacteria were harvested into sterile brucella broth and the resulting suspension adjusted with a spectrometer (Baush and Lomb, Rochester, NY) to an O.D. reading of 0.15 at 600nm or approximately $1 \times 10^9$ CFU/ml. The inoculum was divided into smaller aliquots and snap frozen in liquid nitrogen prior to being stored at -80°C. Exact viability counts were obtained by serial dilution and plating on SBA.

**Mice**

Female BALB/c mice at approximately 6 to 8 weeks of age were obtained from the Division of Laboratory Animal Medicine in the Louisiana State University...
School of Veterinary Medicine (Baton Rouge, LA) and held at least one week prior to use. All animals were housed in a restricted access facility. All mice were used in accordance with the Animal Care and Use Committee at Louisiana State University and approved protocols were followed.

**Murine Neutrophil Killing Assay**

To investigate if the *B. abortus* mutant exhibited increased sensitivity to killing by neutrophils, a previously described murine neutrophil killing assay was utilized (Elzer et al., 1996). Mice were injected with 1 ml of warm thioglycolate broth (3 gm/100 ml) intraperitoneally to elicit neutrophils into the peritoneal cavity. Four h post-injection the mice were euthanized by halothane inhalation, and the peritoneal cavity injected with 8 ml of cold media (RPMI 1640, 10% fetal calf-serum (FCS), 200 U/ml heparin) (Sigma). The recovered cells were centrifuged at 250 x g for 10 min and subsequently washed and centrifuged twice more with cold media. The recovered pellet was resuspended in 3 ml of cold media, and viability counts obtained via trypan blue exclusion (Sigma).

The enriched neutrophil population was immediately transferred to 96-well plates with 2 x 10^5 cells/well. The resistance of both the suspected mutant and parental strain to killing by murine neutrophils was determined. Prior to infection, both bacterial strains were opsonized with 10% normal murine serum (complement preserved) for 10 min at 37°C. Following opsonization, 2 x 10^7 CFU/well of either strain were added to the neutrophils and incubated at 37°C with 5% CO₂. At 10, 30, 60, and 120 min post-infection, the neutrophils were lysed by the addition of 0.1% deoxycholate (Sigma) for 4 min at RT. Subsequently, viable counts of bacteria were
performed by serial dilution and plating on SBA. Four replicate wells for each strain were evaluated per time point. The entire experiment was repeated four times. The percent survival for each strain was determined by dividing the number of bacteria present at a particular sampling time by the number of brucellae added to the neutrophils at time zero and multiplying by 100.

**Murine Macrophage Killing Assay**

The murine macrophage killing assay was carried out to evaluate the ability of both the *B. abortus* mutant and parental strain to survive and replicate inside murine macrophages. As described previously (Elzer et al., 1996), mice were euthanized by halothane inhalation and 8 ml of cold media injected into the peritoneal cavity. Media used contained RPMI 1640, 10% FCS, and Pen-Strep (100 U penicillin, and 0.1 mg streptomycin) (Gibco, Grand Isle, NY). The recovered cells were centrifuged 250 x g for 10 min and subsequently washed and centrifuged twice more with cold media. The recovered pellet was resuspended in 3 ml of cold media and viability counts obtained via trypan blue exclusion. Ninety-six well plates were inoculated with 5 x 10^5 cells/well, and the cells incubated overnight at 37°C in a humidified atmosphere containing 5% CO₂.

After overnight culture, the macrophages were washed three times with 200 µl/well of warm PBS containing 1% FCS to remove nonadherent cells and any residual antibiotic. Immediately prior to performing the assay, inoculation doses of both the *B. abortus* mutant and the parental strain were opsonized for 30 min at 37°C with murine hyperimmune serum specific for *B. abortus*. The washed cells were then inoculated with 5 x 10^7 CFU/well of the opsonized mutant or parental strain.
Following a 2-h period of phagocytosis at 37°C, all extracellular bacteria were killed by the addition of 50 μg/ml of gentamicin (Sigma) for 1 h at 37°C. Following this 1-h treatment, the high-concentration gentamicin was removed and replaced with media containing a low concentration of gentamicin (12.5 μg/ml). At 0, 4, 24, and 48 h following high-concentration gentamicin treatment, the macrophages were washed three times with warm PBS containing 1% FCS. Cells were then lysed with 0.1% deoxycholate and the number of viable intracellular brucellae determined by serial dilution and plating on SBA. Percent survival was calculated by dividing the number of bacteria present at a particular sampling time by the number of brucellae added to the macrophages at time zero and multiplying by 100. Statistics were performed on four replicate wells per time point and the entire experiment repeated four times.

Pathogen Growth Curves in Mice

The murine BALB/c brucellosis model was utilized to assess the pathogenic potential of all three mutants in vivo. Frozen inoculums of the mutant, parental strain, and complemented mutant were thawed and diluted to $5 \times 10^5$ CFU/ml. Mice were then injected intravenously (i.v.) with 100 μl of this inoculum or $5 \times 10^4$ CFU. Inoculation doses were verified by serial dilution and plating on SBA. At various time points post-infection, mice in groups of five were euthanized by halothane overdose. At necropsy, the whole spleen was removed by aseptic technique for bacteriological analysis while blood was obtained for serological analysis. The spleen was homogenized with a Sorvall Omni-Mixer (Newton, CT) in sterile PBS and subsequently serially diluted and plated on SBA. Mutants were also plated on media containing the appropriate antibiotic to ensure that problems did not occur with
stability \textit{in vivo}. All plates were incubated at 37°C in a 5\% CO$_2$ atmosphere for 3 to 4 days until isolated colonies appeared.

Murine serum was analyzed with both the Card test and western immunoblot (Alton et al., 1988). The Card test (or Rose Bengal Plate Test) is a standard agglutination test used by the United States Department of Agriculture – Animal and Plant Health Inspection Service (USDA-APHIS) for field screening. Thirty microliters of plasma is added to 30 μl of killed \textit{B. abortus} cells stained with Rose Bengal. Infection with smooth \textit{Brucella} strains produces anti-O-side chain antibodies that agglutinate the stained cells. The sample is mixed, allowed to incubate for 4 min, and read as positive if agglutination occurs (Alton et al., 1988). The western immunoblot was performed as previously described with the following modifications. Electrophoresis was performed with a Tris-HCl 15\% acrylamide resolving gel with a 4\% stacking gel containing one 450 μl well and an additional well for the molecular weight standard (BioRad). For each gel, 50 μl of cell lysate was added evenly across the well and electrophoresis performed at 80 V. Serum from mice infected with \textit{B. abortus} was screened with cell lysates from smooth \textit{B. abortus} strain 2308 and rough \textit{B. abortus} strain RB51. Serum from mice infected with \textit{B. melitensis} was analyzed with cell lysate from smooth \textit{B. melitensis} strain 16M and rough \textit{B. abortus} strain RB51. Since \textit{B. ovis} lacks the LPS O-side chain, serum from mice infected with this strain was only analyzed with \textit{B. ovis} strain LSU99 cell lysate. To visualize the complete area of cell lysate transmission on the nitrocellulose blot, pyronin-Y (Sigma) was added at 1 μl (0.1 \% stock) per 50 μl of cell lysate. Following transfer, the nitrocellulose blot was cut in small strips, and each ribbon incubated with a different
serum sample as previously described. For the five mice infected with the same strain at each time point, serum samples were combined.

**Statistics**

For neutrophil and macrophage killing assays, statistical comparisons between experimental groups were performed with the Student $t$-test with $P<0.05$ being considered significant (Snedecor and Cochran, 1989). Data from the murine splenic colonization studies was compared using a one-way analysis of variance (ANOVA) with a Student-Newman-Keuls pairwise multiple comparison procedure when three strains were present at the same time point. If two strains were present at a time point, the Student $t$-test was utilized. For the ANOVA and $t$-test, $P<0.05$ were considered significant (Snedecor and Cochran, 1989). All statistics were performed with the Sigma Stat program (Sigma Stat for Windows, Version 1.0, Jandel Scientific, 1992-1994).

**Results**

**Mutant Strains**

Electroporation of *B. abortus* strain 2308 with pAC2555 containing the disrupted *omp25* gene from *B. ovis* strain 63/290 resulted in isolation of a Kan$^\text{r}$ and Amp$^\text{s}$ colony that was named strain BA25 for *B. abortus omp25* mutant. Subsequently, additional experiments using the plasmid pAC2553 carrying the disrupted *omp25* gene from *B. melitensis* strain 16M resulted in the isolation of strain BM25 from strain 16M. Also, utilizing pAC2555, a mutant of *B. ovis* strain LSU99 was also obtained and termed BO25. All three strains were verified as Amp$^\text{s}$ and Kan$^\text{r}$. 

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The three isolated mutants were also electroporated with the complementation vectors pAC2581 and pAC2580 carrying the complete \textit{omp25} gene from \textit{B. ovis} strain 63/290 and \textit{B. melitensis} strain 16M, respectively. Ampicillin resistant isolates were obtained from BM25 and BA25 with pAC2580 and termed BM25p80 and BA25p80, respectively. An Amp' isolate was also obtained from BO25 utilizing pAC2581 and termed BO25p81.

\textbf{Western Immunoblot}

Analysis of the mutants, complemented mutants and parental strains for LPS O-side chain was performed via western immunoblot utilizing bovine anti-O-side serum (Figure 3.1). \textit{Brucella abortus} strain 2308 along with BA25 and BA25p80 produced the typical O-side chain ladder-like smear (Figure 3.1). \textit{Brucella melitensis} strains 16M, BM25, and BM25p80 also all produced similar O-side chain smears. \textit{Brucella ovis} strains LSU99, BO25, and BO25p81 did not demonstrate a ladder-like smear due to the lack of O-side chain (Figure 3.1). Analysis of all the cell lysates with bovine serum specific for internal non-O-side chain associated antigens produced typical banding patterns for \textit{Brucella} spp. (Figure 3.2). While the banding pattern for BA25p80 is light due to the cell lysate concentration, some similar bands are visible. To verify that the Omp25 protein was not expressed by the three mutants, western immunoblot was performed with five MAbs specific for Omp25. Analysis of BA25, BM25, and BO25 cell lysates with MAb A59/05F01/C09 failed to detect the Omp25 protein (Figure 3.3; Lanes 3, 6, 9). Omp25 was recognized in BA25p80, BM25p80, and BO25p81 indicating that complementation for Omp25 was successful (Figure 3.3; Lanes 4, 7, 10). As positive controls, purified Omp25 was recognized and
Figure 3.1. Western immunoblot analysis of cell lysates from Δomp25 mutants with anti-O-side chain polyclonal serum.
Lane 1: *B. abortus* strain 2308
Lane 2: *B. abortus* strain BA25
Lane 3: *B. abortus* strain BA25p80
Lane 4: *B. melitensis* strain 16M
Lane 5: *B. melitensis* strain BM25
Lane 6: *B. melitensis* strain BM25p80
Lane 7: *B. ovis* strain LSU99
Lane 8: *B. ovis* strain BO25
Lane 9: *B. ovis* strain BO25p81

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Figure 3.2. Western immunoblot analysis of cell lysates from \( \Deltaomp25 \) mutants with anti-RBS1 polyclonal serum.
Lane 1: \textit{B. abortus} strain 2308
Lane 2: \textit{B. abortus} strain BA25
Lane 3: \textit{B. abortus} strain BA25p80
Lane 4: \textit{B. melitensis} strain 16M
Lane 5: \textit{B. melitensis} strain BM25
Lane 6: \textit{B. melitensis} strain BM25p80
Lane 7: \textit{B. ovis} strain LSU99
Lane 8: \textit{B. ovis} strain BO25
Lane 9: \textit{B. ovis} strain BO25p81

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Figure 3.3. Western immunoblot analysis of cell lysates from Δomp25 mutants with MAb A59/05F01/C09 specific for the Omp25 protein.
Lane 1: Molecular weight standards
Lane 2: *B. abortus* strain 2308
Lane 3: *B. abortus* strain BA25
Lane 4: *B. abortus* strain BA25p80
Lane 5: *B. melitensis* strain 16M
Lane 6: *B. melitensis* strain BM25
Lane 7: *B. melitensis* strain BM25p80
Lane 8: *B. ovis* strain LSU99
Lane 9: *B. ovis* strain BO25
Lane 10: *B. ovis* strain BO25p81
Lane 11: Purified native Omp25

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the protein was also detected in cell lysates from all three parental strains (Figure 3.3; Lanes 2, 5, 8, 11). While the molecular weight of Omp25 in the B. melitensis strains 16M and BM25p80 appears shifted, this apparent increase is due to insufficient removal of peptidoglycan during cell lysate preparation (Figure 3.3; Lanes 5, 7). Analysis of these cell lysates with an additional four MAbs specific for Omp25 verified the absence of the protein from each mutant (Appendix 2).

**Polymerase Chain Reaction**

To verify that the *omp25* gene was disrupted with the Kan' insertion, PCR primers specific for the 5' and 3' ends of *omp25* were employed. As shown in Figure 3.4, this resulted in generation of a roughly 1.8 kb product from the genomic DNA of BA25, BM25, and BO25 (Lanes 3, 5, 7). Also, an approximate 0.6 kb fragment was amplified from all three parental strains (Figure 3.4, Lanes 2, 4, 6). These results agree with the anticipated amplification of a 1.75 kb product from the mutants and a 600 bp fragment from the paternal strains. As positive controls, a 0.6 kb gene product was amplified from the complementation vector pAC2581 and an approximate 1.75 kb product was produced from the suicide vector pAC2555 (Figure 3.4, Lanes 8, 9). The size shift of the amplified product is due to replacement of the 159 bp *StyI* internal fragment with the 1.3 kb insertion containing the Kan' gene.

To provide additional data that the *omp25* gene had been disrupted, PCR was also performed with primers specific for an internal 700 bp sequence from the pUC4K kanamycin determinant. As shown in Figure 3.5, an approximate 700 bp fragment was amplified from BA25, BM25, and BO25 (Lanes 3, 5, 7). A 700 bp sequence was
Figure 3.4. Amplified PCR products utilizing primers specific for the 5' and 3' ends of the \textit{omp25} gene.
Lane 1: Molecular weight standards
Lane 2: \textit{B. abortus} strain 2308
Lane 3: \textit{B. abortus} strain BA25
Lane 4: \textit{B. melitensis} strain 16M
Lane 5: \textit{B. melitensis} strain BM25
Lane 6: \textit{B. ovis} strain LSU99
Lane 7: \textit{B. ovis} strain BO25
Lane 8: pAC2581
Lane 9: pAC2555

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Figure 3.5. Amplified PCR products utilizing internal primers for the kanamycin resistance gene from pUC4K.
Lane 1: Molecular weight standards
Lane 2: *B. abortus* strain 2308
Lane 3: *B. abortus* strain BA25
Lane 4: *B. melitensis* strain 16M
Lane 5: *B. melitensis* strain BM25
Lane 6: *B. ovis* strain LSU99
Lane 7: *B. ovis* strain BO25
Lane 8: pAC2555

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also generated from the suicide vector pAC2555 (Figure 3.5, Lane 8). A similar product was not amplified from the parental strains (Figure 3.5, Lanes 2, 4, 6).

Polymerase chain reaction was also performed with primers for the pUC19 bla gene. Amplification was attempted on genomic DNA from *B. abortus* strain 2308, BA25, and a *B. abortus* Kan′ Amp′ isolate. As shown in Figure 3.6, an approximate 900 bp sequence was generated from both the *B. abortus* Amp′ isolate and from the suicide vector pAC2555 (Lanes 3, 5). A gene product was not amplified from either strain 2308 or BA25 (Figure 3.6; Lanes 2, 4).

**Southern Blot Analysis**

Southern blot analysis was also performed on genomic DNA from both BA25 and *B. abortus* strain 2308 to verify that the *omp25* gene was disrupted. Purified DNA from both BA25 and strain 2308 digested with *Eco*RI was probed with the complete *omp25* gene. This resulted in annealing of the probe to an approximate 4 kb fragment in strain 2308 (Figure 3.7, Lane 1) and the detection of two fragments from BA25 migrating at approximately 1.5 kb and 2 kb (Figure 3.7, Lane 2). The identification of Omp25 in a 4 kb fragment from genomic DNA digested with *Eco*RI is in accord with previous findings in other laboratories (A. Cloeckaert, personal communication). The annealing of the *omp25* probe to two smaller fragments in genomic DNA from BA25 occurred due to the presence of both the 5′ and 3′ ends of *omp25* and the inclusion of an *Eco*KL site in the Kan′ insertion. As a positive control, the PCR generated probe did anneal to itself (Figure 3.7, Lane 4). In this experiment, genomic DNA from a second *B. abortus* single-crossover mutant (BA25-2) was also probed and produced results equal to BA25 (Figure 3.7, Lane 3). The *omp25* probe also annealed to a 700 base pair fragment.
Figure 3.6. Amplified PCR products utilizing internal primers for the ampicillin resistance gene (bla) from pUC19.
Lane 1: Molecular weight standards
Lane 2: *B. abortus* strain 2308
Lane 3: *B. abortus* single-crossover mutant (Kan, Amp, OmpZS*)
Lane 4: *B. abortus* strain BA25
Lane 5: pAC2555

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Figure 3.7. Southern blot analysis of genomic DNA from *B. abortus* strains 2308 and BA25 with an *omp25* probe.
Lane 1: *B. abortus* strain 2308
Lane 2: *B. abortus* strain BA25
Lane 3: *B. abortus* strain BA25-2
Lane 4: *omp25* probe
Lane 5: pAC2581
bp sequence in the complementation vector pAC2581 along with several non-specific fragments (Figure 3.7, Lane 5). Southern blot analysis verified that the *omp25* gene was disrupted in BA25. The disruption of the *omp25* gene in each mutant is hereafter referred to as a Δ*omp25* mutation.

**Standard Identification Tests**

Following verification that BA25, BM25, and BO25 did not express the Omp25 protein and that the *omp25* gene was disrupted, each strain was analyzed with standard biochemical tests used to discriminate *Brucella* spp. from other Gram-negative organisms. Analysis of all three strains for urease and oxidase reactions found no difference between each Δ*omp25* mutant and parental strain. All of the *B. melitensis* and *B. abortus* strains were urease and oxidase positive while the *B. ovis* strains were negative. Acriflavine testing supported the previous findings by western immunoblot that BO25 is rough and that BA25 and BM25 contain LPS O-side chain. Furthermore, examination of colony morphology, growth rate, or Gram-stain reaction failed to find any discrepancies between each mutant and parental strain. All three Δ*omp25* mutants were also tested for inhibition against six different dyes used to differentiate the *Brucella* spp. Again no discernable variance was observed between each Δ*omp25* mutant and the parental strain. The *B. abortus* strains were inhibited by azure A and thionin while the *B. melitensis* strains were able to grow in the presence of all six dyes. *Brucella ovis* strain LSU99 and BO25 were both inhibited by all the dyes tested expected for pyronin.
**Murine Neutrophil Killing Assay**

To investigate if the lack of Omp25 would render BA25 more sensitive to the bactericidal mechanisms of the neutrophil, a series of murine neutrophil killing assays was performed. The results of one representative experiment are shown in Figure 3.8. By 10 min post-infection both *B. abortus* strain 2308 and the Δomp25 mutant, BA25, remained near 100% percent survival (Figure 3.8). However, by 30 min post-infection both strains decreased in viable numbers to less than 50% of the inoculum. After 30 min post-infection, the rate of killing for both strains decreased, reaching a mean of 35% survival at 60 min. At 120 min post-infection, strain 2308 recovered to 40% survival while the Δomp25 mutant decreased to 27% of the inoculum. Statistical analysis of individual time points with the Student *t*-test failed to find any statistical difference between BA25 and strain 2308. At each time point in Figure 3.8, the data presented is the mean of five individual wells containing bacteria and cultured phagocytes. The entire experiment was repeated four times with no significant deviations being observed between trials.

**Murine Macrophage Killing Assay**

To determine the ability of the *B. abortus Δomp25* mutant, BA25, to survive and replicate in murine macrophages, a macrophage killing assay was performed. One representative experiment is shown in Figure 3.9. By 4 h post-infection, both *B. abortus* strain 2308 and the Δomp25 mutant decreased in viable numbers to less than 9% of the inoculum (Figure 3.9). At 24 h following infection, both BA25 and strain 2308 exhibited less than 3% survival. However, by 48 h while strain 2308 had recovered to 101% of the initial infection, BA25 had recovered to only 60% survival.
Figure 3.8. Killing of *B. abortus* strains 2308 and BA25 opsonized with normal murine serum by cultured murine neutrophils. The data presented are the results of a representative experiment with five repetitions per strain at each time point. Vertical bars indicate standard error, closed circles strain 2308, and open circles BA25.
Figure 3.9. Killing of *B. abortus* strains 2308 and BA25 opsonized with hyperimmune murine serum by cultured, resident, peritoneal murine macrophages. The data presented are the results of a representative experiment with five repetitions per strain at each time point. Vertical bars indicate standard error, closed circles strain 2308, and open circles BA25. **p<0.01, based on a t-test, n = 5.
Analysis with the Student $t$-test found the difference in percent survival at 48 h to be significant with a $P<0.01$ (n = 5 per treatment group). A statistically significant difference in percent survival between the two strains was not observed at 4 or 24 h post-infection. The data presented in Figure 3.9 is the mean of five individual wells. The entire experiment was repeated four times with no significant variations being observed between trials.

**Pathogen Growth Curves in Mice**

To assess the pathogenic potential of the *B. abortus* ∆omp25 mutant, BA25, *in vivo*, BALB/c mice were infected with *B. abortus* strain 2308, BA25, or BA25p80. At 1, 3, 6, and 20 weeks post-infection, the data presented represents five mice per strain at each time point (Figure 3.10). At 2, 4, 8, 12, and 18 weeks, the data presented for BA25 and strain 2308 represents ten mice per strain at each time point and is the combination of two experiments. The two trials were combined since a statistically significant difference was not observed between groups of mice infected with the same strain from either experiment. From 1 to 12 weeks post-infection, no significant difference was observed in mean CFU/spleen between *B. abortus* strain 2308, BA25, or BA25p80 (Figure 3.10). All three strains increased in mean CFU from 1 to 4 weeks and then decline gradually out to 12 weeks post-infection.

At 18 and 20 weeks post-infection, the ∆omp25 mutant BA25 is nearly cleared from a total of 15 mice while the parental strain remains at a mean CFU/spleen of 4.1 logs by 20 weeks following infection (Figure 3.10). At both of these time points, the number CFU of BA25 is approximately 0.15 logs greater than the $2.1072$ log limit of detection for this assay. The difference in mean CFU/spleen of BA25 and strain 2308 is
Figure 3.10. Splenic colonization of BALB/c mice by *B. abortus* strains 2308, BA25, and BA25p80. Ten mice per BA25 and strain 2308 groups at 2, 4, 8, 12, and 18 weeks post-infection. At all other time points mice are in groups of five. Vertical bars indicate standard error, closed circles strain 2308, open circles BA25, and triangles BA25p80. **P<0.01, ***P<0.001, using an ANOVA for three data points and a *t*-test for two data points.
infected mice was statistically significant at 18 ($P<0.001$, $n = 10$) and 20 weeks post-infection ($P<0.01$, $n = 5$). Complementation of Omp25 production in BA25 restored the mean CFU/spleen to that of the parental strain at 20 weeks post-infection (Figure 3.10). Serological analysis found that all mice, regardless of the inoculation strain, produced antibodies against both the LPS O-side chain and internal Brucella proteins.

Mice were also infected with the \textit{B. melitensis} \textit{Δomp25} mutant, BM25, to assess splenic colonization (Figure 3.11). The experiment was carried out to 8 weeks post-infection and included the parental strain, \textit{B. melitensis} 16M, and complemented mutant, BM25p80. As shown in Figure 3.11, by 4 and 6 weeks post-infection a statistically significant difference was observed in mean CFU/spleen between BM25 infected mice and animals inoculated with the other two strains ($P<0.05$, $n = 5$ per treatment group). This difference continued at 8 weeks post-infection for strain 16M infected mice ($P<0.01$, $n = 5$ per treatment group). Complementation of BM25 again restored the mean CFU/spleen to levels equal to the parental strain at 4 and 6 weeks post-infection. The growth of BM25p80 in mice was not ascertained at 8 weeks post-infection. Serological analysis demonstrated that all mice, regardless of the \textit{B. melitensis} strain used, did produce antibodies against both the LPS O-side chain and internal Brucella proteins.

The colonization profiles of \textit{B. ovis} strain LSU99 and the \textit{Δomp25} mutant BO25 were assessed in mice up to 14 weeks following inoculation. At 1 week post-infection, strain LSU99 replicated to an approximate mean log of 7.1 CFU/spleen from an inoculum of 4.5 logs (Figure 3.12). In contrast, at 1 week following exposure, BO25 replicated to a mean of 5.8 log CFU/spleen ($P<0.01$, $n = 5$). By 2 weeks
Figure 3.11. Splenic colonization of BALB/c mice by *B. melitensis* strains 16M, BM25, BM25p80. Five mice per group at each time point. Vertical bars indicate standard error, closed circles strain 16M, open circles BM25, and open triangles BM25p80. *$P<0.05$, **$P<0.01$, using an ANOVA for three data points and a *t*-test for two data points, $n=5$. 

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Figure 3.12. Splenic colonization of BALB/c mice by *B. ovis* strains LSU99, BO025, and BO25p81. Five mice per group at each time point. Vertical bars indicate standard error, closed circles strain LSU99, open circles BO25, and triangles BO25p81. **P<0.01, ***P<0.001, using an ANOVA for three data points and a *t*-test for two data points, n = 5.
post-infection, while strain LSU99 was >7.5 logs per spleen, BO25 had decreased to a
mean CFU/spleen of 5 logs ($P<0.001$, $n = 5$). As shown in Figure 3.12, this decrease
in mean CFU/spleen of BO25 continued until by 8 weeks post-infection no brucellae
could be detected from the spleens of BO25 mice while the parental strain remained at
5.5 logs ($P<0.01$, $n = 5$). Complementation of BO25 for Omp25 production did not
restore the mean CFU/spleen to levels equal to the parental strain. Statistical analysis
of BO25p81 and BO25 infected mice at 2, 4, and 8 weeks post-infection did not find
any difference in mean CFU/spleen between the two groups. Serological analysis did
detect antibodies against $B$. ovis in all infected mice regardless of the inoculation
strain and antibodies to the LPS O-side chain were not found.

Discussion

Mutants of $B$. abortus (BA25), $B$. melitensis (BM25), and $B$. ovis (BO25)
containing a disruption in the $omp25$ gene ($\Deltaomp25$ mutants) were successfully
created. Western immunoblot analysis of cell lysates from all three mutants with five
MAbs specific for the Omp25 protein verified that it was not expressed. Polymerase
chain reaction with primers specific for the $omp25$ gene and for the kanamycin
resistance determinant verified that the $omp25$ gene was disrupted in all three $\Deltaomp25$
mutants. Complementation of each mutant with a broad-host-range plasmid
pBBR1MCS4 carrying the complete $omp25$ gene restored expression of the Omp25
protein. Analysis of all three mutants with the standard biochemical tests used to
differentiate Brucella spp. failed to find any phenotypic difference due to the lack of
the Omp25 protein.
To assess the pathogenic potential of each Δomp25 mutant in vivo, the BALB/c murine brucellosis model was utilized. In this model of splenic colonization, virulent type strains and vaccine candidates often mimic in the mouse what is observed in the ruminant host (Montaraz and Winter, 1986; Winter, 1990; Schurig et al., 1991; Elzer et al., 1996). Inoculation of mice with virulent B. abortus strain 2308, the parental strain of BA25, resulted in a chronic infection that was at a mean CFU/spleen of 5.5 logs by 18 weeks post-infection (Figure 3.10). This is in agreement with previous studies of strain 2308 in BALB/c mice (Montaraz and Winter, 1986; Winter, 1990). At the same time point, mice infected with BA25 had mean CFU/spleen counts that were at the 2.1 log limit of detection for this assay ($P<0.001$, $n = 10$) (Figure 3.10). Mice infected with the B. melitensis mutant BM25 showed a significant decrease in mean CFU/spleen at 4, 6, and 8 weeks post-infection when compared to the virulent parental strain ($P<0.05$, $n = 5$) (Figure 3.11). The colonization profile of the virulent parental strain, B. melitensis 16M, was in agreement with previous findings (Phillips et al., 1995). Complementation of both BA25 and BM25 for Omp25 restored the number of CFU/spleen to levels equal to the respective, virulent parental strain (Figure 3.10; Figure 3.11).

Mice infected with the B. ovis mutant BO25 were consistently colonized with fewer bacteria from 1 to 8 weeks following infection than mice inoculated with the virulent parental strain, LSU99. At 1 week post-infection, while strain LSU99 replicated to $>7$ logs per spleen, the Δomp25 mutant replicated to approximately 5.8 logs ($P<0.01$, $n = 5$) (Figure 3.12). By 8 weeks post-infection, the mutant was below the limit of detection while the virulent parental strain remained at $>5$ logs per spleen.
(P<0.01, n = 5) (Figure 3.12). The growth of virulent \textit{B. ovis} to >7 logs in the spleens of BALB/c mice has been demonstrated previously (Jimenez de Bagues et al., 1994a).

Unlike the \textit{B. abortus} and \textit{B. melitensis} mutants, complementation of BO25 did not restore virulence in the mouse model (Figure 3.12). The \textit{B. ovis} mutant was attenuated from 1 week post-infection while the Δomp25 mutants BM25 and BA25 did not show statistically significant differences from the parental strains in mean CFU/spleen until 4 and 18 weeks post-infection, respectively. The cell envelope of \textit{B. ovis} may be responsible for the increased attenuation of BO25 in mice when compared to the other two \textit{Brucella} mutants. Unlike \textit{B. abortus} and \textit{B. melitensis}, naturally occurring strains of \textit{B. ovis} do not express the LPS O-side chain (Blasco, 1990). Therefore, the loss of an important structural Omp that binds the underlying peptidoglycan and outer cell membrane may have profound effects on the stability of the \textit{B. ovis} cell envelope. Complementation may not have been successful in BO25 due to abnormal expression of the Omp25 protein in the O-side chain deficient LPS of \textit{B. ovis}. To determine if the \textit{B. ovis} Δomp25 mutant is attenuated in ruminants, studies are currently underway in both rams and ewes. Based on this data from the mouse model, the lack of the Omp25 protein may render BO25 attenuated in the ovine host.

An important component of brucellae virulence in both ruminants and mice is the capacity of these pathogens to survive and replicate inside professional phagocytes. Upon natural exposure of a ruminant to brucellae, the pathogen is phagocytosed by neutrophils and macrophages which migrate via the lymphatics to the draining lymph node (Enright, 1990; Thoen et al., 1993). Injection of mice i.v. with virulent brucellae results in localization of the organism in the reticuloendothelial
system and specifically in the spleen, as is the case with other facultative intracellular pathogens (Montaraz and Winter, 1986; Kaufmann, 1999). In the spleen, large numbers of macrophages phagocytize the brucellae in an effort to control the infection (Winter, 1990). Indicative of the important role of macrophages in murine brucellosis, Zhan and Cheers (1993) demonstrated that in the mouse model, depletion of the macrophage activating cytokine interferon-γ (IFN-γ) with anti-IFN-γ MAbs results in a significant increase in splenic colonization compared to inoculated, nontreated controls.

Incubation of the *B. abortus* mutant BA25 or virulent parental strain 2308 with cultured murine neutrophils produced no difference in percent survival up to 2 h post-infection (Figure 3.8). A significant difference was observed between the ability of BA25 and strain 2308 to replicate inside murine macrophages (Figure 3.9). Both strains demonstrated a similar decrease in viable numbers from 4 to 24 h post-infection. However, by 48 h the virulent parental strain had replicated to >100% survival while BA25 had recovered to 60% of the inoculum (*P*<0.01, n = 5). The inability of the *B. abortus* Δomp25 mutant to replicate as efficiently as the parental strain, in murine macrophages *in vitro*, may partially explain the observed attenuation *in vivo*. While not tested in professional phagocytes, the *B. melitensis* mutant may also be unable to replicate inside the macrophage as quickly as the parental strain.

The proposed inability of the both the *B. melitensis* and *B. abortus* mutants to adjust to the intracellular environment of the macrophage may not be the only factor rendering these mutants attenuated *in vivo*. Mice infected with BM25 and BA25 exhibit CFU/spleen counts that are equivalent to the respective, virulent parental strain.
up to 3 and 12 weeks post-infection, respectively. In the mouse model, IgG3 antibodies effective at opsonizing extracellular brucellae are known to develop 3-4 weeks following infection (Elzer et al., 1994b). These antibodies are proposed to bind to the macrophage Fc receptor resulting in phagocyte activation and bacterial killing. Brucellae that are released by lysed cells are opsonized by these antibodies and phagocytosed by activated macrophages (Elzer et al., 1994b). In the case of the B. melitensis Δomp25 mutant, at 4 weeks post-infection and continuing through 8 weeks, a significant decrease in the number of viable brucellae was observed when compared to the virulent parental strain ($P<0.05$, $n = 5$). This decrease in the number of CFU/spleen may represent an inability of IgG3-opsonized BM25 to survive inside the activated macrophage.

A significant difference in the mean CFU/spleen of mice infected with the B. abortus mutant or parental strain 2308 did not occur until 14 weeks following the development of opsonizing IgG3 antibodies. Mice infected with virulent strain 2308 exhibit a long chronic infection that does not clear with the arrival of protective T cells at approximately 6 weeks post-infection (Montaraz and Winter, 1986). Therefore, Winter (1990) proposed that recovery from strain 2308 infection may depend on the formation of both immune T cells and the development of antibodies with high affinity and the required isotype, specifically IgG2a and IgG3. Conceivably, the observed attenuation of BA25 in vivo may be due to an increased sensitivity to this combined humoral and cell-mediated immune response. Following opsonization of BA25 by antibodies of the correct isotype and affinity, an increased killing by activated macrophages may occur.
The attenuation of both the *B. abortus* and *B. melitensis* mutants in mice supports further studies of these mutants in the ruminant hosts. If BA25 is unable to replicate as efficiently as the virulent parental strain in bovine phagocytes, it could impair the ability of this organism to colonize the ruminant host and to induce abortions. Following initial entry into the host, the bacteria could be eliminated in the primary lymph node preventing spread of the pathogen throughout the dam. Likewise, the data generated in mice with the *B. melitensis* mutant BM25 indicates that this mutant might also be attenuated in pregnant goats. The lack of the Omp25 protein may render BM25 unable to colonize or cause abortions in the caprine host. In the subsequent two chapters, the virulence of the *B. abortus* and *B. melitensis Δomp25* mutants will be examined in the ruminant hosts.

Based on these studies in the murine model, the lack of the Omp25 protein did not result in an exacerbation of disease. In contrast, all three Δomp25 mutants were attenuated in the mouse model when compared to the respective, virulent parental strain. The lack of the Omp25 protein did render BA25, BM25, and BO25 attenuated in BALB/c mice and indicates that these strains might also be attenuated in the ruminant hosts.
Brucellosis or Bang’s disease is a serious economic threat to beef and dairy cattle producers throughout much of the world. The etiological agent, *Brucella abortus*, is a Gram-negative, facultative, intracellular pathogen that causes cattle to abort during the last trimester of pregnancy (Anderson et al., 1986; Enright, 1990; Thoen et al., 1993). *Brucella abortus* is also pathogenic for man causing a variety of possible symptoms, including intermittent fever, arthritis, osteomyelitis, and spondylitis (Young, 1995).

This pathogen gains entry into the host via the mucosal surfaces and is phagocytosed by macrophages and neutrophils in an effort by the host to eliminate the organism (Smith and Ficht, 1990). However once inside the professional phagocyte, *B. abortus* is able to survive and replicate (Kreutzer et al., 1979; Frenchick et al., 1985; Baldwin and Winter, 1994; Liautard et al., 1996). The phagocyte migrates, via the lymphatic system, to the draining lymph node where brucellae infection causes cell lysis and eventual lymph node hemorrhage about 2-3 weeks following exposure (Enright, 1990). During this period of vascular damage, some of the bacteria enter the blood stream; and a subsequent bacteremia develops which results in dissemination of the pathogen throughout the host, including localization in the reticuloendothelial system (Enright, 1990; Smith and Ficht, 1990; Thoen et al., 1993).

If the infected animal is pregnant, *B. abortus* will also colonize and replicate to high numbers in the chorionic trophoblasts of the developing fetus (Anderson et al.,...
1986; Enright, 1990; Smith and Ficht, 1990; Thoen et al., 1993). The resulting tissue necrosis to the fetal membranes allows transmission of the bacteria to the fetus (Anderson et al., 1986; Enright, 1990; Thoen et al., 1993). The net effect of chorionic and fetal colonization is the premature delivery of a dead calf during the last trimester of pregnancy. The exact mechanism by which colonization of the chorionic membranes and fetus results in a late-term abortion is unknown. The impairment of fetal circulation, resulting from placentitis has been suggested as a cause of fetal abortion (Payne, 1959). However, considerable variation occurs in the distribution and severity of placental lesions following abortions with all the placentomes rarely being affected (Enright, 1990; Thoen et al., 1993). The exact nature of the induced abortion may be a combination of factors, which produces sufficient alteration of the fetal/maternal relationship. This alteration results in an abortion occurring during the last trimester of pregnancy.

The predilection of *B. abortus* for late-gestational chorionic trophoblasts has also not been fully explained. Evidence does exist that hormones and possibly the sugar erythritol may play an important role in the tropism of brucellae to the reproductive tract (Smith et al., 1962; Enright, 1990; Sangari and Aguero, 1996). Late-gestational chorionic trophoblasts, when compared to trophoblasts obtained from early-gestational placentas, produce higher levels of erythritol and different concentrations of hormones (Smith et al., 1962; Enright, 1990). *Brucella abortus* is also known to invade and replicate poorly in early-gestational trophoblasts when compared to late-gestational cells (Samartino et al., 1994). The penchant of *B. abortus*
to invade and replicate inside bovine trophoblasts is an important step in the abortion process.

Three major groups of outer membrane proteins (Omps) have been identified in *Brucella* species (spp.) (Dubray and Bezard, 1980; Verstreate et al., 1982). The group 1 Omps (94 kDa) are considered minor components of the outer membrane while the group 2 Omps (41-43 kDa) are functional porins (Verstreate et al., 1982; Douglas et al., 1984). The group 3 Omps range from 25-31 kDa and consist of two different proteins of 25 (Omp25) and 31 kDa (Omp31), respectively (Verstreate et al., 1982; Cloeckaert et al., 1996a). Unlike the other *Brucella* spp., *B. abortus* lacks Omp31 due to a large chromosomal deletion (Vizcaino et al., 1996; Vizcaino et al., 1999). While no function has been described for Omp25, the *omp25* gene has been sequenced and cloned from *B. abortus* (de Wergifosse et al., 1995). The Omp25 protein is highly conserved throughout the *Brucella* genus (Cloeckaert et al., 1996a). This protein is also covalently bound to the peptidoglycan layer of the cell envelope and is expressed on the outer membrane in association with lipopolysaccharide (LPS) (Dubray and Charriaut, 1983; Cloeckaert et al., 1990; Cloeckaert et al., 1992; Cloeckaert et al., 1996c).

To determine the role of Omp25 in virulence, a mutant of virulent *B. abortus* strain 2308 containing a disruption in the *omp25* gene was created (Δ*omp25* mutant). The resulting isolate was termed BA25 for *B. abortus Δomp25* mutant. A significant decrease was observed in the ability of BA25 to replicate inside cultured murine macrophages at 48 hours (h) post-infection, when compared to the virulent parental strain (*P*<0.01, *n* = 5). Analysis of this mutant in the murine BALB/c brucellosis...
model indicated that BA25 was attenuated. Inoculation of mice with BA25 resulted in mean colony forming units (CFU)/spleen counts that were at the 2.1 log limit of detection by 18 weeks post-infection. In comparison, mice infected with the parental strain, *B. abortus* 2308, had >2 logs more bacteria at the same time point (*P*<0.001, *n* = 10). Complementation of BA25 with the broad-host-range plasmid pBBR1MCS4, containing the complete *omp25* gene, restored the CFU/spleen counts to that of strain 2308 infected mice.

Based on these studies in mice with the *B. abortus* Δ*omp25* mutant, it was hypothesized that the lack of the Omp25 protein would render BA25 attenuated in the ruminant host. To determine if the *B. abortus* mutant was attenuated, *in vitro* and *in vivo* experiments were instituted with BA25 in cattle. The ability of the mutant to survive and replicate in bovine professional phagocytes and chorionic trophoblasts *in vitro* was assessed. Cattle in late-gestation were also infected with BA25 to ascertain the potential of the mutant to colonize and cause abortions in the ruminant host.

**Materials and Methods**

**Bacterial Strains**

*Brucella abortus* strain 2308 is a virulent challenge strain used by several different laboratories that is documented to cause abortions in pregnant cattle (Enright, 1990; Cheville et al., 1996; Elzer et al., 1998b). The creation and initial characterization of the *B. abortus* Δ*omp25* mutant, BA25, from strain 2308, has been described previously. Infectious doses containing approximately 1 × 10⁹ and 1 × 10¹¹ CFU/ml were prepared as described previously and snap frozen in liquid nitrogen prior to storage at -80°C. Immediately prior to animal inoculation, samples were
thawed and diluted in sterile saline to the appropriate concentration. Doses were verified by serial dilution and plating on Schaedler agar containing 5\% bovine blood (SBA) (Difco Laboratories, Detroit, MI).

**Bovine Neutrophil Enrichment**

Bovine peripheral blood was obtained from a donor with no history of strain 19 or RB51 vaccination and which was negative on the Card test for agglutinating antibodies to *B. abortus* (Alton et al., 1988). Blood was collected in EDTA-treated tubes and centrifuged at 1000 \( \times \) g for 30 minutes (min). Following centrifugation, 1 ml of blood was collected from under the buffy coat and the red blood cells lysed by dilution in 9 ml of sterile distilled water with gentle agitation for 15 seconds (sec). Subsequently, 1 ml of 10 \( \times \) Dulbecco’s Phosphate Buffered Saline, calcium and magnesium free (PBS) (Sigma Chemical Company, St. Louis, MO), was added, and the suspension centrifuged at 200 \( \times \) g for 10 min. The resulting pellet was suspended in water followed by 10 \( \times \) PBS to ensure complete red blood cell lysis. The cells were washed three times with 1 \( \times \) PBS and centrifuged at 200 \( \times \) g for 10 min. The resulting cells were suspended in RPMI media (Sigma) with 5\% fetal calf serum (FCS) (Gemini Bio-Products, Calabasas, CA) and cell viability assessed by Trypan blue exclusion and counting on a hemacytometer.

**Bovine Neutrophil Killing Assay**

The enriched neutrophil population was immediately transferred to 96-well plates with 2 \( \times \) \( 10^5 \) cells/well. The resistance of both *B. abortus* strain 2308 and BA25 to killing by cultured bovine neutrophils was determined by incubating each strain with these cells. Prior to each infection, both bacterial strains were opsonized with
10% normal murine serum (complement preserved) for 30 min at 37°C. Following opsonization, $2 \times 10^7$ CFU/well of either BA25 or strain 2308 were added to the neutrophils and incubated at 37°C with 5% CO$_2$. At 10, 30, 60, and 120 min post-infection, the neutrophils were lysed by the addition of 0.1% deoxycholate (Sigma) for 4 min at room temperature (RT). Subsequently, viable counts of bacteria were acquired by serial dilution and plating on SBA. Four replicate wells for each strain were evaluated at each time point, and the experiment was repeated three times. The percent survival for each strain was determined by dividing the number of bacteria present at a particular sampling time by the number of brucellae added to the neutrophils at time zero and multiplying by 100.

**Bovine Macrophage Enrichment**

The same donor used to obtain neutrophils was also employed to acquire bovine macrophages. Whole blood was collected in EDTA-treated tubes and subsequently diluted 1:2 in PBS. Twenty ml of the diluted blood was layered onto 15 ml of Sigma Histopaque 1077 (Sigma) and centrifuged at 800 x g for 40 min at RT. Theuffy coat was collected and pelleted at 200 x g for 10 min at RT. The resulting cells were washed six times with PBS and resuspended in RPMI media containing 5% FCS, 100 U/ml penicillin (Gibco, Grand Isle, NY), and 0.1 mg/ml streptomycin (Gibco). Cell viability was evaluated by Trypan blue exclusion and counting on a hemacytometer. Ninety-six well plates were seeded with $5 \times 10^5$ cells/well, and the cells incubated for 72 h at 37°C in a humidified atmosphere containing 5% CO$_2$. Following 24 and 48 h of incubation spent media was replaced.
Bovine Macrophage Killing Assay

The capacity of BA25 and *B. abortus* strain 2308 to survive and replicate in cultured bovine macrophages was determined. Immediately prior to performing the assay, inoculation doses of the Δomp25 mutant and virulent parental strain were opsonized for 30 min with subagglutinating murine hyperimmune serum specific for *B. abortus* at 37°C. After 72 h of culture, the macrophages were washed three times with 1 ml/well of warm PBS containing 1% FCS to remove nonadherent cells and any residual antibiotic. The washed cells were then inoculated with $5 \times 10^7$ CFU/well of opsonized BA25 or strain 2308. Following a 2-h period of phagocytosis at 37°C, all extracellular bacteria were killed by the addition of 50 μg/ml of gentamicin (Sigma) for 1 h at 37°C. Following this 1-h treatment, the high-concentration gentamicin was removed and replaced with media containing gentamicin at a low concentration (12.5 μg/ml).

At 0, 24, and 48 h following high-concentration gentamicin treatment, the macrophages were washed three times with warm PBS containing 1% FCS. Cells were then lysed with 0.1% deoxycholate, and the number of viable intracellular brucellae determined by serial dilution and plating on the appropriate media. Percent survival was calculated by dividing the total number viable intracellular bacteria, present at a particular sampling time, by the number of brucellae added to the macrophages at time zero, and multiplying by 100. Time zero was defined as the point when the high-concentration gentamicin was removed from the cells. Statistics were performed on four replicate wells at each time point, and the experiment was repeated three times.

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Three bovine trophoblast cell lines were used in this study. Dr. D. Stringfellow generously furnished an early-gestational trophoblast cell line that was established from a 13-15 day old bovine embryo (Stringfellow et al., 1987). A mid-gestational cell line derived from the placentomal tissue of a bovine placenta in the fifth month of gestation was charitably provided by Dr. L. Munson (Munson et al., 1988). A late-gestation trophoblast cell line was established by Dr. L. Samartino from the placenta of bovine dam in the eighth month of gestation and was supplied by Dr. F. Enright (Samartino, 1991; Samartino et al., 1994).

All three cell lines were maintained in a complete medium consisting of a 1:1 mixture of Ham's F12 nutrient medium (Sigma) and Dulbecco's modified Eagle medium (DMEM) (GIBCO) (pH 7.3). The media was supplemented with 5 ng/ml selenium, 5 μg/ml transferrin, 5 μg/ml insulin, 10 ng/ml epidermal growth factor (Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) (Hyclone, Logan, UT). All cells were grown in 25 cm² flasks at 37°C in a humidified atmosphere containing 5% CO₂. Following formation of confluent monolayers, cells were detached with 0.25% trypsin-EDTA and viable counts determined with Trypan blue exclusion (0.04%) and a hemacytometer. Cells were subcultured 1:2 in 4 ml of media at 5.5 x 10⁵ cells per 25 cm² flask. Following two to three subpassages, the cells usually reached confluence by four days and were deemed ready for experiments. Subsequent subcultures were split 1:3 into 25 cm² flasks.
Bovine Trophoblast Infection Assay

Prior to infection, each cell line was passaged from the 25 cm² flasks into 24-well plates at a concentration of $1 \times 10^5$ cells/well with 1 ml of antibiotic-free media per well. Twenty-four h following cell passage, inoculation doses of both $B.\,abortus$ strain 2308 and the $Aomp25$ mutant BA25 were thawed and diluted in warm, antibiotic-free cell media. Either bacterial strain was added to the trophoblasts at $1 \times 10^7$ cells/well resulting in a 1:100 ratio of cells to bacteria. The bacteria were incubated with the trophoblasts for 10 h at 37°C with 5% CO₂.

At 10 h post-infection, media was removed and all extracellular bacteria killed by incubation at 37°C with high-concentration gentamicin (50 μg/ml) diluted in warm DMEM or RPMI media. One h later, the high-concentration gentamicin media was removed and replaced with the complete culture media containing a low concentration of gentamicin (12.5 μg/ml). At 0, 12, 24, 36 and 48 h following completion of high-concentration gentamicin treatment, the cells were gently washed with 1 ml of warm PBS containing 1% FCS. Following one wash, 800 μl of PBS was added to each well and the entire plate frozen at -80°C for later bacteriological analysis. All three cell lines were inoculated with both bacterial strains with three replicates per time point. The entire experiment was repeated three times.

Following the completion of each experiment, the 24-well plate was thawed at 37°C and 0.1% deoxycholate added to each well to lyse the cells. Following a 5 min incubation at RT, the cells were serially diluted in PBS and plated on SBA to obtain the viable CFU/ml. Percent survival was calculated by dividing the number of bacteria present at a particular sampling time by the number of brucellae added to the
trophoblasts at time zero and multiplying by 100. Time zero was defined as the point when the high-concentration gentamicin was removed from the cells.

**Cattle**

Twenty pregnant, mixed-breed cows were obtained from private herds. All animals were negative for *Brucella*-specific antibodies by standard Card test and had no previous history of strain 19 or RB51 vaccination. Serological analysis with the western immunoblot utilizing both smooth and rough *B. abortus* cell lysate failed to find evidence of previous vaccination with strain 19 or RB51 (Edmonds et al., 1999a). Throughout the course of the study, all cattle were housed in a United States Department of Agriculture/Animal and Plant Health Inspection Service (USDA/APHIS) approved, restricted-access, large animal isolation facility located at Louisiana State University AgCenter, Baton Rouge, Louisiana. At the conclusion of the study, the adult cows were euthanized at a government-inspected abattoir by use of the captive bolt and exsanguination method. Live calves were euthanized at the research facility by the captive bolt and exsanguination method and disposed of by approved methods.

**Bovine Study**

At approximately 220 days gestation, each cow was inoculated with $1 \times 10^7$ CFU of either *B. abortus* strain 2308 or BA25 by introducing 50 μl of the inoculum into the conjunctival sac of each eye. Ten cows were inoculated with the virulent parental strain while an additional ten animals were inoculated with the *omp25* mutant. Following inoculation, animals were monitored daily for parturition.
The birth status of the calf was recorded as either healthy, weak, or an abortion. Within 12 h of parturition a necropsy was performed on the calf. Samples taken from the calf for bacteriological analysis included the abomasal fluid and lung. Tissue samples were frozen at -4°C for later analysis, while the abomasal fluid was swabbed the day of collection on Farrell’s selective medium (Farrell, 1974). Two days following birth, milk samples from all four quarters of the mammary gland and intrauterine fluid samples were obtained from the dam. Both fluid samples were swabbed the day of collection on Farrell’s selective medium. If \textit{B. abortus} was not cultured from either the milk or uterine fluid, a second sample was obtained 2 weeks later for additional bacteriological analysis. At approximately 35 days post-parturition, the dams were necropsied and tissue samples obtained from the supramammary lymph node, spleen, and liver for the culture of brucellae. Serum was also obtained prior to infection, following parturition, and at necropsy for serological analysis.

**Bacteriological and Serological Analysis**

All tissue samples were thawed and homogenized in a sterile saline (0.9% NaCl) solution and plated onto Farrell’s selective medium containing 5% bovine blood; the limit of detection with this system is 13 CFU/gm or ml. The plates were incubated for 14 days at 37°C in a 5% CO$_2$ atmosphere. \textit{Brucella abortus} was identified on the basis of urease and oxidase reactions, colony morphology, growth rate, smooth phenotype by acriflavine, and Gram-stain reaction (Alton et al., 1988). BA25 was differentiated from \textit{B. abortus} strain 2308 by being kanamycin resistant.
(45 µg/ml). Serum was analyzed for *B. abortus* specific antibodies by the Card test and western immunoblot. Both tests were performed as previously described.

**Statistics**

For neutrophil, macrophage, and trophoblast assays, statistical comparisons between experimental groups were performed with the Student *t*-test for parametric data and the Mann-Whitney Ranked Sum Test for nonparametric data (Snedecor and Cochran, 1989). The rate of abortion between cattle inoculated with strain 2308 or BA25 was compared with a Fisher Exact Probability Test, while median CFU/gm of tissue was compared with the Mann-Whitney Ranked Sum Test (Snedecor and Cochran, 1989). A *P*<0.05 was considered significant for all three tests. All statistics were performed with the Sigma Stat program (Sigma Stat for Windows, Version 1.0, Jandel Scientific, 1992-1994).

**Results**

**Bovine Neutrophil Killing Assay**

To determine if the lack of the Omp25 protein would render BA25 more susceptible to killing by professional phagocytes, the Δomp25 mutant and virulent parental strain were exposed to cultured bovine neutrophils. The results of one representative experiment are shown in Figure 4.1. The difference in percent survival between BA25 and strain 2308 was not significant at 10, 30, 60, or 120 min post-infection. At 30 min post-infection, the percent survival for both strains decreased to approximately 30% and remained statistically the same thereafter (Figure 4.1). At each time point in Figure 4.1, the data presented is the mean of four replicates. The
Figure 4.1. Killing of *B. abortus* strains 2308 and BA25 opsonized with normal murine serum by cultured bovine neutrophils. The data presented are the results of a representative experiment with four repetitions at each time point. Vertical bars indicate standard error, closed circles strain 2308, and open circles BA25.
experiment was repeated three times with no significant deviations being observed between trials.

**Bovine Macrophage Killing Assay**

To ascertain if in the bovine host, the Δomp25 mutant would be more susceptible to the bactericidal mechanisms of the macrophage, a series of *in vitro* killing assays was performed with freshly isolated bovine macrophages. One representative experiment is shown in Figure 4.2. By 24 h following infection, the parental strain had decreased in viable numbers by 28% while BA25 had decreased 93% ($P<0.001$, $n = 4$) (Figure 4.2). At 48 h post-infection, strain 2308 replicated to 549% while the mutant recovered to only 33% ($P<0.01$, $n = 4$) (Figure 4.2). The experiment was repeated three times; and while individual variances were observed, BA25 was consistently killed at a higher rate than *B. abortus* strain 2308.

**Bovine Trophoblasts Infection Assay**

The *B. abortus* Δomp25 mutant was cultured in early, mid, and late-gestational bovine trophoblasts to determine if the lack of Omp25 rendered BA25 attenuated in these chorionic cells. In the early-gestational cell line, no distinction was observed between the survival rates of the two strains at 12 or 24 h post-infection (Figure 4.3). While strain 2308 and BA25 decreased to less than 16% survival at 12 h, both recovered to >100% of the inoculum by 36 h. Unlike the previous time points, at 36 h significant differences occurred between the two strains in percent survival ($P<0.05$, $n = 3$). By 48 h, strain 2308 had replicated more than 1.5 logs greater than BA25 ($P<0.001$, $n = 3$) (Figure 4.3). Two additional replicates of this assay produced no significant variations.
Figure 4.2. Killing of *B. abortus* strains 2308 and BA25 opsonized with hyperimmune murine serum by cultured bovine macrophages. The data presented are the results of a representative experiment with four repetitions at each time point. Vertical bars indicate the 25% and 75% quartiles, closed circles strain 2308 median, and open circles BA25 median. **p<0.01, ***p<0.001, based on a Mann-Whitney Rank Sum Test of nonparametric data, n = 4.
Figure 4.3. Replication of *B. abortus* strains 2308 and BA25 in early-gestational bovine chorionic trophoblasts. The data presented are the results of one representative experiment with three replicates at each time point. Vertical bars indicate standard error, closed circles strain 2308, and open circles BA25. *P<0.05, ***P<0.001, based on a *t*-test, n = 3.

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In a mid-gestational trophoblast cell line, the percent survival for BA25 was consistently lower than *B. abortus* strain 2308 from 12 to 48 h post-infection (Figure 4.4). At 12 h following infection, both strains decreased in percent survival with 2308 at 2.69% and BA25 at 0.38% (*P*<0.01, *n* = 3). By 24 h post-infection, while the Δomp25 mutant recovered to 25% of the inoculum, strain 2308 had replicated to 241% survival (*P*<0.05, *n* = 3). While at consistently lower numbers than the parental strain, the mutant was able to replicate, surpassing the inoculum at 36 and 48 h post-infection (Figure 4.4). In two repetitions of this experiment, similar results were obtained.

In the late-gestational cell line, the Δomp25 mutant again replicated at lower numbers than the parental strain at 24, 36, and 48 h following infection (Figure 4.5). By 24 h post-infection, strain 2308 replicate to 469% survival while BA25 recovered to 85% survival (*P*<0.001, *n* = 3). Significant differences in percent survival between the two strains were also observed at 36 h (*P*<0.01, *n* = 3) and 48 h post-infection (*P*<0.001, *n* = 3). While the mutant did not replicate to the same extent as strain 2308, BA25 was able to surpass the inoculum at 36 and 48 h post-infection (Figure 4.5).

**Cattle Study**

To assess the pathogenicity of BA25 in the ruminant host, pregnant cattle in late-gestation were exposed to either the Δomp25 mutant or the virulent parental strain, *B. abortus* 2308. The results of this study are presented in Table 4.1.

Inoculation of ten pregnant cows with virulent strain 2308 resulted in 5/10 (50%) of the dams aborting. In contrast, only 1/10 (10%) cows inoculated with BA25 aborted (*P*<0.05, *n* = 10). The virulent parental strain was cultured from every strain 2308.
Figure 4.4. Replication of *B. abortus* strains 2308 and BA25 in mid-gestational bovine chorionic trophoblasts. The data presented are the results of one representative experiment with three replicates at each time point. Vertical bars indicate standard error, closed circles strain 2308, and open circles BA25. *P<0.05, **P<0.01, ***P<0.001, based on a t-test, n = 3.

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Figure 4.5. Replication of *B. abortus* strains 2308 and BA25 in late-gestational bovine chorionic trophoblasts. The data presented are the results of one representative experiment with three repetitions at each time point. Vertical bars indicate standard error, closed circles strain 2308, and open circles BA25. **P<0.01, ***P<0.001, based on a t-test, n = 3.
Table 4.1. Abortion rate, colonization rate, and serologic response of 20 pregnant cows inoculated with either *Brucella abortus* strain 2308 or BA25.

<table>
<thead>
<tr>
<th></th>
<th>BA25 Group (n = 10)</th>
<th>Strain 2308 Group (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abortion Rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10%*</td>
<td>50%</td>
</tr>
<tr>
<td>Calf: colonization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abomasal Fluid</td>
<td>20%***</td>
<td>100%</td>
</tr>
<tr>
<td>Fetal Lung</td>
<td>30%*</td>
<td>90%</td>
</tr>
<tr>
<td>Cow: colonization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterine Fluid</td>
<td>30%**</td>
<td>100%</td>
</tr>
<tr>
<td>Milk</td>
<td>50%*</td>
<td>100%</td>
</tr>
<tr>
<td>Supramammary LN</td>
<td>40%* (3/7)*</td>
<td>90% (9/10)</td>
</tr>
<tr>
<td>Liver / Spleen</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Serologic Analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Response</td>
<td>60%</td>
<td>100%</td>
</tr>
</tbody>
</table>

All data is presented as the percentage of cows positive out of ten animals per treatment group. Liver and spleen tissue were homogenized and cultured together.

*Only seven tissue samples were cultured.

*Detection of antibodies specific for the O-side chain by western immunoblot, see text for description.

*P<0.05, **P<0.01, ***P<0.001 by the Fischer Exact Probability Test, n = 10.

LN = lymph node.
inoculated dam and calf. The Δomp25 mutant was cultured from 5/10 (50%) dams and 3/10 (30%) calves \( (P<0.05, n = 10) \) (Table 4.1). In this study, 4/10 (40%) of the BA25 exposed dams did not develop antibodies to \textit{B. abortus} nor were they colonized by the mutant. All of the strain 2308 inoculated animals developed antibodies to \textit{B. abortus} and were colonized.

The median CFU/gm of tissue obtained from the two experimental groups was compared with a Mann-Whitney Ranked Sum Test. The three supramammary lymph nodes colonized by BA25 did have a statistically lower median CFU/gm of tissue than the strain 2308 infected animals \( (P<0.05, n = 3) \). The median CFU/gm of tissue for the 2308 dams was 1000 CFU with 25% and 75% quartiles of 192 CFU and 3250 CFU, respectively. In contrast, the three colonized tissue samples from the BA25 infected dams had a median of 60 CFU/gm with 23 CFU and 128 CFU for quartiles.

**Discussion**

Based on the \textit{in vitro} analysis of BA25 in bovine phagocytes and chorionic trophoblasts, this mutant may be unable to adjust to the intracellular environment as quickly as virulent \textit{B. abortus} strain 2308. As in murine macrophages, incubation of BA25 with bovine macrophages resulted in a significant decrease in percent survival at 48 h post-infection \( (P<0.01, n = 4) \). The data from murine and bovine macrophages indicates that the lack of Omp25 may have influenced the capacity of BA25 to adjust to the bactericidal environment of the activated macrophage.

In bovine trophoblasts from early, mid, and late-gestation, the Δomp25 mutant again did not replicate inside the cell to the same extent as the virulent parental strain, \textit{B. abortus} 2308. In all three cell lines, both strains decreased by more than half a log
at 12 h post-infection. This may reflect a period of adjustment where the bacterium modulates the expression of different genes involved in intracellular growth. While the trophoblast does not have the anti-microbial activity of the macrophage, the conditions inside this cell are different from extracellular growth on a media plate.

Inside the trophoblast, *B. abortus* preferentially metabolizes the sugar erythritol, which is implicated in the tropism of brucellae to the uterus (Smith and Ficht, 1990). The Δomp25 mutant can metabolize erythritol *in vitro* (data not shown). The inability of BA25 to adjust to the intracellular environment of the trophoblast is reflected in the lower percent survival of the Δomp25 mutant at 36 and 48 h post-infection.

The growth of BA25 in macrophages and trophoblasts may explain the attenuation of this mutant *in vivo*. If the Δomp25 mutant is unable to survive and replicate inside the bovine macrophage, the organism may be eliminated from the draining lymph node, preventing dissemination and eventual colonization of the developing fetus. Furthermore, the inability of BA25 to replicate inside the late-gestational bovine trophoblast to the same extent as strain 2308 may also have important consequences in the late-term abortion process. If the bacteria cannot replicate efficiently in the chorionic membranes, the chance of fetal infection and subsequent abortion is lowered.

In the ruminant host, the *B. abortus* Δomp25 mutant was attenuated when compared to the virulent parental strain. Of the ten bovine dams exposed to BA25, four did not develop antibodies to *B. abortus* nor did the bacteria colonize them. These animals may have eliminated the mutant in the draining lymph node thus preventing spread throughout the host. One animal did develop antibodies to *B. abortus*. 

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*abortus*, but both the dam and calf were negative for the culture of brucellae organisms. This animal was colonized for sufficient time to develop antibodies but was able to eliminate the mutant prior to parturition. The inability of BA25 to replicate to the same extent as strain 2308 in bovine macrophages may have resulted in 5/10 pregnant cattle eliminating the mutant prior to parturition.

The remaining five pregnant dams inoculated with the Δ*omp25* mutant were colonized by BA25 but had only three calves that were positive for brucellae organisms. The fact that only 3/5 colonized dams had calves that were likewise positive for *B. abortus* may reflect the inability of BA25 to replicate efficiently inside the late-gestational trophoblast. Following enhanced killing of the mutant by activated macrophages in the draining lymph node, lower numbers of the Δ*omp25* mutant were disseminated throughout the host. Those bacteria that did succeed in colonizing the chorionic membrane were unable to replicate as efficiently as the virulent parental strain. As a result, the necrosis to fetal membranes was decreased and only 3/5 calves were colonized *in utero*. Only one abortion occurred possibly due to alteration of the fetal/maternal relationship, relating to the lower numbers of bacteria present in the placenta.

The lack of the Omp25 protein did render BA25 attenuated in the ruminant host. In the future, the role of Omp25 in the replication of *B. abortus* in bovine macrophages and trophoblasts needs to be furthered explored. A detailed understanding of the physiological role of this protein would provide additional information on the pathogenesis of *Brucella* spp. By understanding the role of Omp25
and other proteins in *Brucella*, the development of new vaccine candidates lacking key virulence factors is possible.
CHAPTER 5
A *Brucella melitensis* Δomp25 Mutant in Goats

Introduction

*Brucella melitensis* is a pathogenic, zoonotic agent found primarily in small ruminants throughout the world (Alton, 1990a; Blasco, 1997; Garin-Bastuji et al., 1998). The primary hosts for *B. melitensis* are goats and sheep, in which the organism causes abortions during the third trimester of pregnancy. The pathogen goes undetected in infected flocks until lambing season when colonized dams abort (Alton, 1990a; Blasco, 1997; Garin-Bastuji et al., 1998). Following an abortion, the infected female excretes copious amounts of the bacteria in both uterine exudate and milk. This state results in spread of the organism throughout the flock and to human caretakers. *Brucella melitensis* is the most virulent *Brucella* spp. for man and is also the least species-specific, infecting goats, sheep, cows, camels, and dogs (Young, 1995). In man, *B. melitensis* causes the disease undulant fever which is characterized by a variety of possible symptoms including pyrexia, arthritis, osteomyelitis, spondylitis, dementia, and in rare cases endocarditis or meningitis (Young, 1995).

*Brucella melitensis* contains three groups of major outer membrane proteins (Omps) (Dubray and Bezard, 1980; Verstreate et al., 1982; Cloeckaert et al., 1996a). The group 1 Omps (94 kDa) are considered minor components of the outer membrane while the group 2 Omps (41-43 kDa) are functional porins (Verstreate et al., 1982; Douglas et al., 1984; Cloeckaert et al., 1996a). The group 3 Omps range from 25-31 kDa and consist of two different proteins of 25 (Omp25) and 31 kDa (Omp31), respectively (Verstreate et al., 1982; Cloeckaert et al., 1996a). The Omp31 protein is

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proposed to be a porin while no function has yet been described for Omp25 (Vizcaino et al., 1996).

The Omp25 protein is expressed on the outer cell membrane of each *Brucella* spp. (Cloeckaert et al., 1996c). The protein is bound to the peptidoglycan layer by a covalent bond, and association of Omp25 with lipopolysaccharide (LPS) is important for the maintenance of conformational epitopes on the protein (Dubray and Charriaut, 1983; Cloeckaert et al., 1992; Cloeckaert et al., 1996c). Analysis of the *omp25* gene with PCR-RFLP and nucleotide sequencing indicates that the gene is highly conserved among the *Brucella* spp. (Cloeckaert et al., 1995; Cloeckaert et al. 1996b).

To investigate if the lack of the Omp25 protein would render *Brucella* spp. attenuated in the ruminant host, a *B. melitensis* mutant containing a disruption in the *omp25* gene was created (*Δomp25* mutant) as described previously. In the BALB/c mouse model, the *B. melitensis* *Δomp25* mutant, BM25, displayed significant attenuation at 4, 6 and 8 weeks post-infection (*P*<0.05, *n* = 5). At 6 and 8 weeks post-infection, mice inoculated with the virulent parental strain, *B. melitensis* 16M, had mean colony forming units (CFU)/spleen counts that were approximately 1 log greater than mice receiving the *Δomp25* mutant (*P*<0.01, *n* = 5). Complementation of BM25 with the broad-host-range plasmid pBBR1MCS4, carrying the complete *omp25* gene, restored protein expression and virulence in the mouse model.

The attenuation of BM25 in mice indicated that the *Δomp25* mutant might also be attenuated in the ruminant host. Previous studies have found that strains of *brucellae* that are attenuated in the ruminant host are also often attenuated in the mouse (Montaraz and Winter, 1986; Winter, 1990; Schurig et al., 1991; Elzer et al., 1993).
1996). Therefore, it was hypothesized that the lack of the Omp25 protein would render BM25 attenuated in pregnant goats. The ability of the *B. melitensis* mutant to colonize and cause abortions in the ruminant host was determined in two experiments.

**Materials and Methods**

**Bacterial Strains**

*Brucella melitensis* strain 16M has been documented to cause abortions in pregnant goats (Phillips et al., 1997; Elzer et al., 1998c). The creation and initial characterization of the *B. melitensis* Δomp25 mutant, BM25 from strain 16M has been described previously. Infectious doses containing approximately $1 \times 10^9$ and $1 \times 10^{11}$ CFU/ml were prepared as described (Elzer et al., 1994c) and snap frozen in liquid nitrogen prior to storage at -80°C. Immediately prior to animal inoculation, samples were thawed and diluted in sterile saline to the appropriate concentration. Doses were verified by serial dilution and plating on Schaedler agar containing 5% bovine blood (SBA) (Difco Laboratories, Detroit, MI).

**Animals**

Thirty sexually mature female Angora goats were obtained from a commercial herd. All goats were negative for *Brucella*-specific antibodies based on western immunoblot (Edmonds et al., 1999a) and the Card test (Alton et al., 1988). Throughout the study, the animals were housed in a restricted access, large-animal isolation facility operated under guidelines approved by the United States Department of Agriculture/Animal and Plant Health Inspection Service (USDA/APHIS). At the conclusion of each study the adult animals were euthanized by the captive bolt and
exsanguination method. Any kids born alive at parturition were euthanized and exsanguinated. These animals were used in the following two studies.

Colonization Study

Six non-pregnant Angora goats were inoculated with approximately $1 \times 10^9$ CFU of *B. melitensis* strain 16M by placing 50 µl of the inoculum into each conjunctival sac. Nine other non-pregnant goats were inoculated with a one log higher dose ($1 \times 10^{10}$ CFU) of the Δomp25 mutant, BM25, via the same method. At 4, 7, and 14 days post-infection, two strain 16M and three BM25 goats were euthanized; and various samples obtained for bacteriological and serological analysis. Tissue samples were obtained from preferred sites of brucellae localization, including the parotid lymph node which drains the site of inoculation, the supramammary lymph node, and the spleen. Following collection, samples were frozen at -4°C and prepared as described below.

Abortion Study

Goats in late-gestation, approximately 124 days, were divided into two groups. The first group of six females was inoculated with $1 \times 10^7$ CFU of the virulent parental strain 16M, by placing 50 µl of the inoculum in each conjunctival sac. At this dose and route of infection, strain 16M has been documented to cause abortions (Phillips et al., 1997; Elzer et al., 1998c). An additional group of nine females were inoculated with an equal dose of BM25, via the same method. Following inoculation, animals were monitored daily for delivery. At parturition, the birth status of the kid was recorded as either an abortion or live-healthy kid. Within 12 h following parturition, the dam was euthanized, as was the kid if born alive. During necropsy samples were
obtained from the dam of the parotid lymph node, prescapular lymph node, prefemoral lymph node, supramammary lymph node, internal iliac lymph node, spleen, liver, and milk for bacteriological analysis. Samples obtained from the kid included the prehepatic lymph node, lung, liver, spleen, thymus, and abomasal fluid. All tissue samples were frozen at -4°C prior to analysis, while the milk and abomasal fluid were plated the day of collection on Farrell’s selective medium (Farrell, 1974). Prior to infection and at necropsy, serum samples were obtained from the adult females for serological analysis.

**Bacteriological and Serological Analysis**

All tissue samples were thawed and homogenized in a sterile saline (0.9% NaCl) solution and plated onto Farrell’s selective medium containing 5% bovine blood; the limit of detection with this system is 13 CFU/gm or ml. The plates were incubated for 14 days at 37°C in a 5% CO₂ atmosphere. *Brucella melitensis* was identified on the basis of urease and oxidase reactions, colony morphology, growth rate, presence of LPS O-polysaccharide (O-side chain) by acriflavine and Gram-stain reaction (Alton et al., 1988). BM25 was differentiated from *B. melitensis* strain 16M by being kanamycin resistant (45 μg/ml). Serum was analyzed for *B. melitensis* specific antibodies by the Card test and western immunoblot. Both tests were performed as previously described (Alton et al., 1988; Edmonds et al., 1999a).

**Statistics**

The number of abortions, colonized dams and colonized kids were compared between the two groups in each study with a Fisher Exact Probability Test, with a $P<0.05$ being considered significant (Snedecor and Cochran, 1989). All statistics
were performed with the Sigma Stat program (Sigma Stat for Windows, Version 1.0, Jandel Scientific, 1992-1994).

Results

Colonization Study

To establish if the *B. melitensis Δomp25* mutant could colonize non-pregnant goats, a short-term colonization study was carried out. Inoculation of nine non-pregnant goats with 1 x 10^{10} CFU of BM25 resulted in colonization of the parotid lymph node at days 4, 7, and 14 post-infection (Table S.1). By day 7 post-infection, BM25 was also obtained from the spleens of 2/3 goats inoculated with the *B. melitensis Δomp25* mutant. The supramammary lymph node was colonized by BM25 at 14 days post-infection. The virulent parental strain, *B. melitensis* 16M, administered at a 1 log lower dose (1 x 10^{9} CFU) also colonized the goats (Table S.1).

Abortion Study

To assess the pathogenicity of BM25 in the ruminant host, pregnant goats in late-gestation were exposed to either the *Δomp25* mutant or the virulent parental strain. The results of this study are presented in Tables 5.2 and 5.3. Inoculation of six pregnant goats with strain 16M resulted in 6/6 (100%) abortions (Table 5.2). In contrast, none (0/9) of the dams inoculated with BM25 aborted (*P*<0.001, *n* = 6). The virulent parental strain was cultured from every strain 16M inoculated dam and kid. The *Δomp25* mutant was cultured from 4/9 (44%) dams and 2/9 (22%) of the kids (*P*<0.05, *n* = 6) (Table 5.2). Of the four dams and two kids colonized by BM25, one dam-kid pair were positive for the culture of brucellae organisms from most of the tissue samples taken (Table 5.3). The two kids colonized by BM25 were from dams...
Table 5.1. Colonization study: bacteriological and serological analysis of non-pregnant Angora goats inoculated with either *Brucella melitensis* strain 16M or BM25.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Days</th>
<th>Parotid LN</th>
<th>Spleen</th>
<th>Supramammary LN</th>
<th>Serologic Response&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4</td>
<td>3/3</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4892 +/- 2372&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>16M&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>BM25</td>
<td>7</td>
<td>3/3</td>
<td>2/3</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7947 +/- 4248</td>
</tr>
<tr>
<td>16M</td>
<td>7</td>
<td>2/2</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1066 +/- 331</td>
</tr>
<tr>
<td>BM25</td>
<td>14</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9704 +/- 2657</td>
</tr>
<tr>
<td>16M</td>
<td>14</td>
<td>2/2</td>
<td>1/2</td>
<td>Neg</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3367 +/- 709</td>
</tr>
</tbody>
</table>

<sup>a</sup>Positive serologic response to *B. melitensis* strain 16M cell lysate via western immunoblot, see text for description.

<sup>b</sup>Inoculum = 1 x 10<sup>10</sup> CFU into the conjunctival sac.

<sup>c</sup>Mean CFU/gm +/- the standard error.

<sup>d</sup>Inoculum = 1 x 10<sup>9</sup> CFU into the conjunctival sac.

LN = Lymph node, Neg = negative.
<table>
<thead>
<tr>
<th>Challenge</th>
<th>Abortion Rate</th>
<th>Adult Colonization Rate</th>
<th>Kid Colonization Rate</th>
<th>Serologic Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM25</td>
<td>0/9***</td>
<td>4/9*</td>
<td>2/9**</td>
<td>9/9</td>
</tr>
<tr>
<td></td>
<td>0%</td>
<td>44%</td>
<td>22%</td>
<td>100%</td>
</tr>
<tr>
<td>16M</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

*aChallenge inoculum = 1 x 10^7 CFU into the conjunctival sac.

*bPositive serologic response to B. melitensis strain 16M cell lysate via western immunoblot, see text for description.

*P<0.05, **P<0.01, ***P<0.001, n = 6, based on a Fischer Exact Probability Test.
Table 5.3. Abortion study: colonization of BM25 inoculated adults and kids.

<table>
<thead>
<tr>
<th>Adult Sample</th>
<th>Number Infected</th>
<th>Kid Sample</th>
<th>Number Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parotid LN</td>
<td>4/9</td>
<td>Abomasal Fluid</td>
<td>2/9</td>
</tr>
<tr>
<td>Supramammary LN</td>
<td>2/9</td>
<td>Lung</td>
<td>1/9*</td>
</tr>
<tr>
<td>Internal Iliac LN</td>
<td>2/9</td>
<td>Thymus</td>
<td>1/9*</td>
</tr>
<tr>
<td>Milk</td>
<td>2/9</td>
<td>Prehepatic LN</td>
<td>0/9</td>
</tr>
<tr>
<td>Spleen</td>
<td>1/9*</td>
<td>Spleen</td>
<td>1/9*</td>
</tr>
<tr>
<td>Liver</td>
<td>1/9*</td>
<td>Liver</td>
<td>1/9*</td>
</tr>
<tr>
<td>Lung</td>
<td>1/9*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prescapular LN</td>
<td>1/9*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LN = lymph node.

*Same dam-kid pair.
that were colonized in the both the supramammary and internal iliac lymph nodes. The remaining two dams infected with BM25 were colonized in only the parotid lymph node and had kids that were negative for the culture of brucellae organisms (Table 5.3). Serological analysis of samples taken prior to infection and during necropsy, found 

\textit{Brucella}-specific antibodies in all the test animals (Table 5.2).

\textbf{Discussion}

A short-term colonization experiment was performed with the \textit{B. melitensis} \textit{\Delta}omp25 mutant to determine if it could survive in the ruminant host. Inoculation of nine non-pregnant goats verified that BM25 was capable of transient \textit{in vivo} colonization. Since the \textit{\Delta}omp25 mutant was capable of short-term colonization in the non-pregnant caprine host, an abortion study was performed. The \textit{B. melitensis} mutant induced no abortions (0/9) while the virulent parental strain, \textit{B. melitensis} 16M, induced a 100\% (6/6) abortion rate ($P<0.001$, $n=6$). The \textit{\Delta}omp25 mutant also colonized fewer dams ($P<0.05$, $n=6$) and kids ($P<0.01$, $n=6$) than strain 16M. Of the four dams colonized by BM25, only two had kids that were colonized upon parturition.

The \textit{Brucella melitensis} \textit{\Delta}omp25 mutant did cause 100\% sero-conversion, indicating that BM25 was not quickly eliminated from the draining lymph node. In contrast, the \textit{Brucella abortus} \textit{\Delta}omp25 mutant, BA25, while causing one abortion \textit{in vivo}, did not induce \textit{Brucella}-specific antibodies or colonize 40\% of the bovine dams (Table 4.1). Also, inoculation of non-pregnant goats with $1 \times 10^{10}$ CFU of the \textit{B. melitensis} mutant (3 logs greater than that used in the abortion study), resulted in the colonization of lymph nodes distant from the site of inoculation including the
supramammary and internal iliac. Based on this data, the attenuation of BM25 *in vivo*
may be due to several factors. As proposed previously for the *B. abortus* mutant, the
*B. melitensis* Δomp25 mutant may be unable to replicate as efficiently as strain 16M in
both caprine macrophages and chorionic trophoblasts. If BM25 is unable to adjust to
the intracellular environment of the caprine trophoblast, then significant alteration of
the fetal/maternal relationship may not occur. Future *in vitro* studies need to be
performed with the *B. melitensis* Δomp25 mutant in caprine trophoblasts and
phagocytes to determine if BM25 is attenuated in these cell types.

The capacity of the *B. melitensis* mutant to transiently colonize the ruminant
host while inducing no abortions makes this mutant an attractive vaccine candidate.
The current vaccine for caprine brucellosis is the live-attenuated *B. melitensis* strain
Rev. 1 (Blasco, 1997; Garin-Bastuji et al., 1998). While it provides protection against
infection, this vaccine can also cause dams to abort if given during pregnancy. (Alton,
1990a; Blasco, 1997; Garin-Bastuji et al., 1998). The fact that BM25 does not induce
abortions raises the possibility that it could be an alternative to strain Rev. 1. In the
following chapter, the potential of BM25 as a vaccine for small ruminants will be
analyzed.
CHAPTER 6
VACCINE TRIALS WITH *Brucella melitensis* AND *Brucella ovis* MUTANTS LACKING A 25 kDa OUTER MEMBRANE PROTEIN (Omp25)

Introduction

*Brucella melitensis* is a serious economic and public health threat in many countries throughout the world (Corbel, 1998). Infection of pregnant goats and sheep with *B. melitensis* results in abortion during the third trimester of pregnancy.

Humans infected with this pathogen develop undulant fever, which is characterized by pyrexia, arthritis, osteomyelitis, and spondylitis (Young, 1995). The attenuated-live vaccine *B. melitensis* strain Rev. 1 has been used successfully in goats and sheep to prevent *B. melitensis* infection (Jones et al., 1964; Alton, 1966; Alton, 1987; Alton, 1990a; Blasco, 1997; Garin-Bastuji et al., 1998; Scharp et al., 1999). Subcutaneous (s.c.) vaccination of three to seven month old sexually immature goats with strain Rev. 1 provides protection against virulent challenge up to 2.5 years following vaccination (Alton, 1966). This vaccine has also been demonstrated to have a stable, attenuated phenotype upon six serial passages *in vivo* (Alton et al., 1967).

However, although attenuated, strain Rev. 1 does cause abortions if given to pregnant goats or sheep s.c. during later stages of gestation (Alton, 1990a; Blasco, 1997; Garin-Bastuji et al., 1998). Based on field trials, some authors have proposed using a reduced dose and/or conjunctival inoculation, which provides immunity while decreasing the risk of abortion (Scharp et al., 1999). However, controlled experimental studies have found that while conjunctival vaccination induces fewer premature deliveries, the abortion rate of approximately 20% is still too high to ignore the pregnancy status of the dam at the time of vaccination (Zundel et al., 1992; Blasco,
As a result, vaccination of mature females is not recommended during the lambing season or less than one month prior to mating (Alton, 1990a; Blasco, 1997; Garin-Bastuji et al., 1998). In areas with an extended breeding season or poor management practices, vaccination is therefore limited to only young animals between three and eight months of age (Blasco, 1997). In endemically infected flocks, vaccination of young lambs does not prevent infected adults from spreading the organism to other mature females and/or animal caretakers (Blasco, 1997). Therefore an efficacious vaccine that could be given to sexually mature and possibly pregnant goats or sheep without fear of abortion would be a major benefit.

The ovine pathogen, *Brucella ovis*, is different from *B. melitensis* in cell envelope structure, host preference, and pathology. Unlike *B. melitensis, B. ovis*, a rough organism, lacks the O-polysaccharide (O-side chain) sugar found on the lipopolysaccharide (LPS) of smooth *Brucella* strains (Blasco, 1990). *Brucella ovis* is also primarily a disease of rams where it induces epididymitis and subsequent infertility (Blasco, 1990; Cerri et al., 1999). Only rarely does this *Brucella* specie induce abortions in pregnant ewes (Libal and Kirkbride, 1983; Grillo et al., 1999). *Brucella ovis* is also non-pathogenic for humans (Blasco, 1990). Ovine brucellosis can have a serious economic impact on the producer since the infection often goes undetected until significant decreases in pregnancy rates are discovered (Blasco, 1990). Subsequent analysis of the breeding rams finds the majority are infected and permanently sterile. This forces the producer not only to lose valuable breeding stock but to also find methods to prevent infection of newly introduced rams.
*Brucella melitensis* strain Rev. 1 is considered the best vaccine for ovine brucellosis (Blasco et al., 1987; Jimenez de Bagues et al., 1995). Recent work with two attenuated-live vaccines, *Brucella abortus* strain RB51 and *Brucella suis* strain 2, have failed to protect rams against virulent challenge with *B. ovis* (Blasco et al., 1993; Jimenez de Bagues et al., 1995). While strain Rev. 1 is efficacious, several problems exist with its use. In countries such as the U.S., the use of attenuated *B. melitensis* strains is prohibited due to the absence of virulent *B. melitensis* in the field.

Therefore, in the U.S. and other countries free of *B. melitensis*, but where ovine brucellosis is a problem, no vaccines are available (Blasco, 1990; Jimenez de Bagues et al., 1995). *Brucella melitensis* strain Rev. 1 can also cause abortions in pregnant ewes and is pathogenic for humans (Alton, 1990a; Blasco and Diaz, 1993; Blasco, 1997; Garin-Bastuji et al., 1998). Strain Rev. 1 is a smooth strain and does induce anti-O-side chain antibodies that are detected on the accepted sero-diagnostic tests (Jimenez de Bagues et al., 1995). In areas attempting to eradicate *B. melitensis* from infected sheep, an animal vaccinated with strain Rev. 1 cannot be serologically distinguished from one infected with virulent, wild-type *B. melitensis*.

The negative aspects of strain Rev. 1 vaccination for the control of both *B. melitensis* and *B. ovis* support the development of new vaccine alternatives. The *B. melitensis Δomp25* mutant, BM25, described previously, is a viable option for the prevention of *B. melitensis* infection. Inoculation of BALB/c mice with BM25 resulted in a significant decrease in the mean colony forming units (CFUs)/spleen from 4 to 8 weeks post-infection, when compared to the virulent parental strain, *B. melitensis* 16M (*P*<0.05, *n* = 5). Furthermore, inoculation of nine pregnant caprine
dams in late-gestation with BM25 resulted in no abortions while the virulent parental strain caused abortions in 6/6 pregnant females ($P<0.001$, $n = 6$). The $\Deltaomp$25 mutant colonized 4/9 dams and 2/9 kids, while strain 16M colonized 6/6 dam-kid pairs ($P<0.05$, $n = 6$). A colonization study of non-pregnant female goats also determined the BM25 can transiently colonize the ruminant host. Since, BM25 does not appear to cause abortions in pregnant caprine dams but is capable of transient colonization, vaccine efficacy trials were performed in both mice and goats.

The $B.\ ovis\ \Deltaomp$25 mutant, BO25, described previously, is also attenuated in the mouse model. Mice infected with BO25 were consistently colonized with fewer bacteria from 1 to 8 weeks following infection than mice inoculated with the virulent parental strain, $B.\ ovis\ LSU99$. At 1 week post-infection, while strain LSU99 replicated to $>7$ logs per spleen, the $\Deltaomp$25 mutant replicated to approximately 5.8 logs ($P<0.01$, $n = 5$). By 8 weeks post-infection, the mutant was below the limit of detection while the virulent parental strain remained at $>5$ logs per spleen ($P<0.01$, $n = 5$).

To ascertain the vaccine efficacy of BM25 and BO25, both mutants were evaluated in the BALB/c mouse model. The potential of the $B.\ melitensis\ \Deltaomp$25 mutant to protect against virulent challenge was also assessed in the ruminant host. The hypothesis for this work is that $Brucella\ \Deltaomp$25 mutants will be efficacious against challenge with the homologous and heterologous virulent parental strains of brucellae.
Materials and Methods

Bacterial Strains

For these studies nine different Brucella strains were utilized. Virulent *B. abortus* strain 2308, *B. melitensis* strain 16M, and *B. ovis* strain LSU99 have been described previously. *Brucella suis* strain 1330 is an established virulent biovar 1 challenge strain in swine and mice (Bosseray and Plommet, 1990). *Brucella canis* strain RM6-66 was generously provided by Dr. Leland E. Carmichael, Department of Veterinary Microbiology, Cornell University, Ithaca, NY. This strain has been documented to cause abortions in experimentally-infected canines (Carmichael, 1990). The official USDA cattle vaccine, rough *B. abortus* strain RB51 (Schurig et al., 1991), was provided by Dr. Gerhardt G. Schurig, Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA. *Brucella melitensis* vaccine strain Rev. 1 was obtained from the manufacturer, Rhone-Merieux, Lyon, France. The creation and initial characterization of the *B. melitensis* (BM25) and *B. ovis* (BO25) Δomp25 mutants has been described previously. All challenge strains were grown on Schaedler blood agar plates (SBA) (Difco Laboratories, Detroit, MI) at 37°C in a 5% CO₂ atmosphere. BM25 and BO25 were grown on SBA supplemented with 45 μg/ml kanamycin (Sigma Chemical Company, St. Louis, MO). Inoculation doses of each strain were prepared as previously described. Immediately prior to infection, frozen inoculums were thawed and diluted to the appropriate concentration with sterile physiological saline. All doses were verified as correct by serial dilution and plating on the appropriate media.
Female BALB/c mice at approximately 6 to 8 weeks of age were obtained from the Division of Laboratory Animal Medicine at the School of Veterinary Medicine at Louisiana State University (Baton Rouge, LA) and held at least one week prior to use. All animals were housed in a restricted access facility. All mice were used in accordance with the Animal Care and Use Committee at Louisiana State University and approved protocols were followed.

Murine BM25 Vaccine Efficacy Trials

Mice were vaccinated intravenously (i.v.) with $5 \times 10^4$ CFU of the *B. melitensis* Δomp25 mutant, BM25. Initially, five mice were infected with BM25 and were found to have cleared the infection by 10 weeks post-infection. For every group of five mice vaccinated with BM25 an additional five non-vaccinated, age-matched mice were held in the same location until challenge. As positive controls, some additional mice were vaccinated with either $5 \times 10^4$ CFU of *B. melitensis* vaccine strain Rev. 1 i.v. or $3 \times 10^8$ CFU of *B. abortus* vaccine strain RB51 intraperitoneal (i.p.). Doses of each strain were based on previously published work in BALB/c mice (Montaraz and Winter, 1986; Jimenez de Bagues et al., 1994a; Jimenez de Bagues et al., 1994b). Prior to vaccination and 8 weeks post-vaccination, mice were bled and the serum was pooled for each group.

Ten weeks following vaccination with either BM25, *B. melitensis* strain Rev. 1, or *B. abortus* strain RB51, both vaccinated and non-vaccinated mice were inoculated with one of the challenge strains. Animals in groups of five were infected with $5 \times 10^4$ CFU i.v. of either *B. melitensis* strain 16M, *B. abortus* strain 2308, *B. suis*
strain 1330, *B. canis* strain RM6-66, or *B. ovis* strain LSU99 (Montaraz and Winter, 1986; Jimenez de Bagues et al., 1994a; Jimenez de Bagues et al., 1994b). Two weeks post-challenge, all mice were euthanized by halothane overdose. At necropsy, the whole spleen was removed by aseptic technique for bacteriological analysis while blood was obtained for serological analysis. Spleens were homogenized with a Sorvall Omni-Mixer (Newton, CT) in sterile phosphate-buffered-saline (PBS) and subsequently serially diluted and plated on SBA. Cell homogenate from BM25 vaccinated mice was also plated on SBA with 45 µg/ml kanamycin to ensure that the mutant had been cleared. All plates were incubated at 37°C in a 5% CO₂ atmosphere for 3 to 4 days until isolated colonies appeared.

**Murine BO25 Vaccine Efficacy Trials**

Mice in groups of five were vaccinated i.v. with 5 x 10⁴ CFU of the *B. ovis* Δomp25 mutant BO25. Age-matched mice were also obtained and held as non-vaccinated controls until challenge. As positive controls, additional mice were vaccinated with either strain Rev. 1 or strain RB51 as previously described. For comparison, mice were also vaccinated with BM25 as described previously. Mice vaccinated with BO25, strain Rev. 1 or strain RB51 were challenged with all five strains 8 weeks post-vaccination. Mice vaccinated with BM25 were challenged 10 weeks post-vaccination as previously described. Two weeks following vaccination, the mice were euthanized and prepared as previously described. Serum samples were obtained prior to vaccination, 6 weeks post-vaccination, and at necropsy for serological analysis.
Goats

Twenty-four sexually mature female Angora goats were obtained from commercial herds. Prior to beginning any experiments, all goats were negative for Brucella-specific antibodies based on western immunoblot (Edmonds et al., 1999a) and the Card test (Alton et al., 1988). Throughout the study the animals were housed in a restricted access, large-animal isolation facility operated under guidelines approved by the United States Department of Agriculture/Animal and Plant Health Inspection Service (USDA/APHIS). At the conclusion of each study, the adult animals were euthanized by the captive bolt and exsanguination method. Any kids born alive were euthanized and exsanguinated.

**Goat BM25 Vaccine Efficacy Trial**

Twenty-four mature, non-pregnant females were divided into three groups. Nine animals were vaccinated s.c. with $1 \times 10^9$ CFU of BM25. A second group of seven animals were vaccinated s.c. with $1 \times 10^6$ CFU of the vaccine *B. melitensis* strain Rev. 1. Previous experiments in goats have shown that this dose of strain Rev. 1 provides protection against challenge with virulent *B. melitensis* strain 16M (Elzer et al., 1998c). A third group of eight animals served as controls and was injected s.c. with 1 ml of the saline diluent. Four weeks following vaccination, a fertile male goat was added to each group. Following confirmation of pregnancy with ultrasound, all animals were challenged at approximately 100 days gestation with $1 \times 10^7$ CFU of virulent *B. melitensis* strain 16M into the conjunctival sac. Following inoculation, animals were monitored daily for delivery. At parturition, the birth status of the kid was recorded as either an abortion or a live-healthy kid. Within 24 h following
parturition, the kid was euthanized if born alive and samples obtained for bacteriologic analysis from the liver, lung, spleen, and abomasal fluid. Two to seven days following parturition, adult females were euthanized; and samples taken from the parotid lymph node, prescapular lymph node, supramammary lymph node, internal iliac lymph node, liver, spleen, milk, and uterine fluid. Tissue samples were frozen at -4°C for later analysis, while milk and uterine fluid were swabbed the day of collection on Farrell’s selective media (Farrell, 1974). Serum samples were obtained prior to vaccination, at challenge, and at necropsy for serological analysis.

**Caprine Bacteriological Analysis**

All tissue samples were thawed and homogenized in a sterile saline (0.9% NaCl) solution and plated onto Farrell’s selective medium containing 5% bovine blood; the limit of detection with this system is 13 CFU/gm or ml. The plates were incubated for 14 days at 37°C in a 5% CO₂ atmosphere. *Brucella melitensis* was identified on the basis of urease and oxidase reactions, colony morphology, growth rate, presence of O-side chain by acriflavine, and Gram-stain reaction (Alton et al., 1988). BM25 was differentiated from *B. melitensis* strain 16M by being kanamycin resistant at 45 µg/ml.

**Serological Analysis**

Serum samples from both mice and goats were analyzed for O-side chain specific antibodies by both the Card test and western immunoblot. The Card test has been described previously (Alton et al., 1988). Pre-vaccination and post-vaccination serum samples from BM25 vaccinated mice were analyzed by western immunoblot utilizing cell lysate for both smooth *B. melitensis* strain 16M or rough *B. abortus* strain...
RB51. Serum from mice vaccinated with BO25 were analyzed prior to challenge with cell lysate from *B. ovis* strain LSU99. Post-challenge serum samples from mice exposed to either *B. abortus* strain 2308 or *B. suis* strain 1330 were analyzed with cell lysate from both *B. abortus* strain 2308 and *B. abortus* strain RB51. Similar serum samples from *B. melitensis*-challenged mice were analyzed with *B. melitensis* strain 16M and strain RB51 cell lysates. Finally, serum samples from all mice challenged with either rough *B. canis* strain RM6-66 or *B. ovis* strain LSU99 were analyzed with cell lysate from rough *B. ovis* strain LSU99. Western immunoblots were performed as previously described.

**Statistics**

For the murine vaccine efficacy trials, median CFU/spleen was obtained for each experimental group following log_{10} conversion. Due to the occurrence of unequal variances between experimental groups, a Kruskal-Wallis Ranked ANOVA utilizing a Dunn’s multiple comparisons versus a control test was used to compare median CFU/spleen between experimental groups (Snedecor and Cochran, 1989). When only two experimental groups were present, the Mann-Whitney Ranked Sum Test was utilized (Snedecor and Cochran, 1989). For both tests, *P*<0.05 was considered significant. Log units of protection were obtained by subtracting the median CFU/spleen count for the control group from the corresponding median CFU/spleen count for each treatment group. For the caprine BM25 vaccine efficacy trial, a Kruskal-Wallis Ranked ANOVA utilizing a Dunn’s multiple comparisons versus a control test was employed with a *P*<0.05 being considered significant.
(Snedecor and Cochran, 1989). All statistics were performed with the Sigma Stat program (Sigma Stat for Windows, Version 1.0, Jandel Scientific, 1992-1994).

**Results**

**Murine BM25 Vaccine Efficacy Trials**

Mice were vaccinated with the *B. melitensis Δomp25* mutant, BM25, to determine if the attenuated-live mutant would be efficacious against virulent challenge. The results of this experiment are shown in Table 6.1. Mice vaccinated with the current caprine vaccine, *B. melitensis* strain Rev. 1, or the *B. melitensis Δomp25* mutant, BM25, had significant levels of protection against challenge with virulent *B. melitensis* strain 16M (*P*<0.01, *n* = 5). Vaccination with either strain resulted in over 2 logs of protection against the challenge strain. In contrast, the current bovine vaccine, rough *B. abortus* strain RB51, did not provide significant protection against strain 16M, with a 0.77 median log reduction in bacterial load (Table 6.1).

The *B. melitensis* mutant was also efficacious against the heterologous virulent challenge strains *B. abortus* 2308 and *B. suis* 1330 (*P*<0.01, *n* = 5) (Table 6.1). Strain BM25 afforded approximately 2 logs of protection against *B. abortus* strain 2308 and 1 log against *B. suis* strain 1330 (Table 6.1). Serological analysis with western immunoblot and the Card test detected antibodies specific for the *Brucella* LPS O-side chain in mice vaccinated with strain Rev. 1 and BM25. Anti-O-side chain antibodies were not detected in serum samples from mice vaccinated with rough *B. abortus* strain RB51.
Table 6.1. Murine BM25 vaccine efficacy trial.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Vaccine</th>
<th>Challenge Strain</th>
<th>Median log_{10} brucellae per spleen</th>
<th>Log units of protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BM25</td>
<td><em>B. melitensis</em></td>
<td>3.38 +/- (3.15 - 3.89)**</td>
<td>2.29</td>
</tr>
<tr>
<td></td>
<td>Rev. 1</td>
<td></td>
<td>3.22 +/- (3.05 - 3.48)**</td>
<td>2.45</td>
</tr>
<tr>
<td></td>
<td>RB51</td>
<td></td>
<td>4.90 +/- (4.53 - 5.58)**†</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>None*</td>
<td></td>
<td>5.67 +/- (5.41 - 5.93)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>BM25</td>
<td><em>B. abortus</em></td>
<td>3.66 +/- (3.57 - 3.74)**</td>
<td>1.99</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td></td>
<td>5.65 +/- (5.18 - 5.79)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>BM25</td>
<td><em>B. suis</em></td>
<td>4.71 +/- (4.12 - 4.75)**</td>
<td>1.04</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td></td>
<td>5.75 +/- (5.69 - 5.75)</td>
<td></td>
</tr>
</tbody>
</table>

*B. melitensis* strains Rev. 1 and BM25 at 5 x 10^4 CFU i.v. *B. abortus* strain RB51 at 3 x 10^6 CFU i.p.

*Brucella melitensis* strain 16M, *B. abortus* strain 2308, *B. suis* strain 1330 at 5 x 10^4 CFU i.v., 10 weeks post-vaccination. Mice were necropsied 2 weeks post-challenge.

Group median CFU/spleen +/- (25% - 75% quartiles), log_{10} conversion.

 Logs protection = median CFU for non-vaccinated controls - median CFU for treatment group.

* n = 10 for this group.

**P<0.01, †No statistical difference, n = 5 unless noted. A Kruskal-Wallis Ranked ANOVA utilizing a Dunn’s multiple comparisons versus a control test was utilized for experimental group 1. A Mann-Whitney Ranked Sum Test was utilized for experimental groups 2 and 3.

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Based on the observed attenuation of the *B. ovis* Δomp25 mutant in mice, a murine vaccine efficacy trial was performed with BO25. Mice vaccinated with the *B. ovis* mutant had 5.65 logs of protection against the virulent parental strain, *B. ovis* LSU99 (Table 6.2). In mice vaccinated with BO25, the median CFU/spleen of strain LSU99 was 2.11 logs, which is the limit of detection for this assay. In two separate murine trials, vaccination with BO25 resulted in complete protection against strain LSU99 (*P*<0.01, *n* = 10). The vaccine strains *B. melitensis* Rev. 1, *B. abortus* RB51, and *B. melitensis* BM25 did not afford the same level of protection as BO25 against virulent *B. ovis* (Table 6.2).

The *B. ovis* Δomp25 mutant also provided significant protection against the other naturally occurring rough *Brucella* species (spp.), *B. canis* strain RM6-66 (*P*<0.05, *n* = 10) (Table 6.2). However, the logs of protection provided by BO25 (1.15 logs) were below that of strain Rev. 1 (1.95 log) or the *B. melitensis* mutant BM25 (1.62 logs). The rough cattle vaccine *B. abortus* strain RB51 was not efficacious against virulent *B. canis* (Table 6.2). The *B. ovis* Δomp25 mutant did not afford significant protection against challenge with the smooth *Brucella* spp., *B. melitensis* strain 16M, *B. abortus* strain 2308, or *B. suis* strain 1330 (data not shown).

Serological analysis detected anti-O-side chain antibodies in mice vaccinated with BM25 and strain Rev. 1. Utilizing *B. ovis* strain LSU99 cell lysate in a western immunoblot, *Brucella*-specific antibodies were detected in strain RB51 and BO25 vaccinates.
<table>
<thead>
<tr>
<th>Exp.</th>
<th>Group</th>
<th>Challenge</th>
<th>Median log_{10} brucellae per spleen</th>
<th>Log units of protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BO25</td>
<td>B. ovis</td>
<td>2.11 +/- (2.11 - 2.11)\textsuperscript{f}**</td>
<td>5.65</td>
</tr>
<tr>
<td></td>
<td>Rev. 1</td>
<td></td>
<td>5.00 +/- (4.53 - 5.49)**</td>
<td>2.76</td>
</tr>
<tr>
<td></td>
<td>RB51</td>
<td></td>
<td>6.49 +/- (5.73 - 7.0)**</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>BM25</td>
<td></td>
<td>5.33 +/- (4.75 - 6.51)**</td>
<td>2.43</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td></td>
<td>7.76 +/- (7.63 - 7.84)\textsuperscript{f}</td>
<td></td>
</tr>
</tbody>
</table>

|      | BO25\textsuperscript{e} | B. canis   | 5.80 +/- (4.90 - 6.52)*             | 1.15                   |
|      | Rev. 1                |           | 5.00 +/- (4.53 - 5.49)*             | 1.95                   |
|      | RB51                  |           | 6.49 +/- (5.73 - 7.0)\textsuperscript{f} | 0.46                   |
|      | BM25                  |           | 5.33 +/- (4.75 - 6.51)*             | 1.62                   |
|      | None\textsuperscript{e} |           | 6.95 +/- (6.62 - 7.24)              |                        |

\textsuperscript{a}Brucella ovis strain BO25, B. melitensis strains Rev. 1 and BM25 at 5 \times 10^6 CFU i.v.

\textsuperscript{b}Brucella abortus strain RB51 at 3 \times 10^6 CFU i.p.

\textsuperscript{c}Group median CFU/spleen +/- (25% - 75% quartiles), log_{10} conversion.

\textsuperscript{d}Logs protection = median CFU for non-vaccinated controls – median CFU for treatment group.

\textsuperscript{e}n = 10 for this group.

\textsuperscript{f}Limit of detection, represents clearance.

\textsuperscript{g}n = 15 for this group.

\textsuperscript{*}P<0.05, \textsuperscript{**}P<0.01, \textsuperscript{†}No statistical difference, n = 5 unless noted.\textsuperscript{a}\textsuperscript{e} A Kruskal-Wallis Ranked ANOVA utilizing a Dunn's multiple comparisons versus a control test was utilized.

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Caprine BM25 Vaccine Efficacy Trial

To determine if the *B. melitensis Δomp25* mutant was efficacious against virulent challenge in the ruminant host, a caprine vaccine efficacy trial was performed. The results of this study are presented in Tables 6.3 and 6.4. The rate of abortions in non-vaccinated dams that were challenged with virulent *B. melitensis* strain 16M was 63% (5/8). In contrast, none of the BM25 or strain Rev. 1 vaccinates aborted (*P* < 0.05, *n* = 7). Bacteriological analysis found 88% (7/8) of the kids from non-vaccinated controls were colonized by the challenge strain, while only 1 kid from each vaccine group was colonized. The one kid colonized by BM25 had ≤ 200 CFU/gm of liver and spleen while the non-vaccinated controls had a median of 44000 CFU/gm (*P* < 0.01, *n* = 7). The challenge strain was also cultured from 100% (8/8) of the non-vaccinated dams. In comparison, only 14% (1/7) of the strain Rev. 1 vaccinates and 44% (4/9) of the BM25 vaccinates were colonized by strain 16M. A significant difference in the median CFU/gm of tissue was demonstrated between each vaccine group and the non-vaccinated controls (*P* < 0.05, *n* = 7) (Table 6.3). No statistical difference was found between the two vaccine groups.

Serological analysis with *B. melitensis* strain 16M cell lysate found all the vaccinated animals to have a positive serologic response post-vaccination with either BM25 or strain Rev. 1 (Table 6.4). The number of animals vaccinated with either BM25 or strain Rev. 1 that had detectable anti-O-side chain antibodies decreased with time. However, at 18 weeks post-vaccination, 58% of the BM25 vaccinates had detectable antibodies to the O-side chain while none of the strain Rev. 1 vaccinated...
### Table 6.3. Caprine BM25 vaccine efficacy trial, abortion and colonization rates.

<table>
<thead>
<tr>
<th>Vaccine Strain</th>
<th>BM25</th>
<th>Rev. 1</th>
<th>Saline Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abortion Rate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0/9</td>
<td>0/7</td>
<td>5/8</td>
</tr>
<tr>
<td></td>
<td>(0%)*</td>
<td>(0%)*</td>
<td>(63%)</td>
</tr>
<tr>
<td>Colonization Kids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abomasal Fluid</td>
<td>0/9c</td>
<td>1/7</td>
<td>7/8 (88%)</td>
</tr>
<tr>
<td>(0%)**</td>
<td>0 (0)**</td>
<td>300 (64 – 850)</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>0/9</td>
<td>0/7</td>
<td>7/8 (88%)</td>
</tr>
<tr>
<td>(0%)**</td>
<td>0 (0)**</td>
<td>&gt;300000 (6400 – 300000)</td>
<td></td>
</tr>
<tr>
<td>Liver/Spleen</td>
<td>1/9</td>
<td>0/7</td>
<td>7/8 (88%)</td>
</tr>
<tr>
<td>(11%)**</td>
<td>0 (0)**</td>
<td>44000 (2095 – 300000)</td>
<td></td>
</tr>
<tr>
<td>Colonization Adults</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1° LN</td>
<td>4/9</td>
<td>1/7</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td>(44%)**</td>
<td>0 (0 – 196)*</td>
<td>745 (390 – 995)</td>
<td></td>
</tr>
<tr>
<td>2° LN</td>
<td>3/9</td>
<td>0/7</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td>(38%)**</td>
<td>0 (0)*</td>
<td>5750 (1650 – 21000)</td>
<td></td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th></th>
<th>Liver/Spleen</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4/9 (44%)</td>
<td>1/7 (14%)</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td></td>
<td>0 (0 – 120)*</td>
<td>0 (0)*±</td>
<td>150 (54 – 2000)</td>
</tr>
</tbody>
</table>

*a Challenge inoculum was *B. melitensis* strain 16M at 1 x 10⁷ CFU in the conjunctival sac.

*b *Brucella melitensis* strains Rev. 1 at 1 x 10⁶ CFU s.c. and BM25 at 1 x 10⁹ CFU s.c.

*c Number colonized / number in treatment group.

*d Median CFU/gm of tissue, (25% - 75% quartiles).

*e One animal had ≤ 25 CFU.

*f Maximum limit of detection.

g One animal had ≤ 200 CFU.

*P < 0.05, **P < 0.01, ***P < 0.001, † No statistical difference, n = 7. Based on a Kruskal-Wallis Ranked ANOVA, with a Dunn’s Test. No statistical difference was found between the vaccine treatment groups.

1°LN = Parotid and prescapular lymph nodes, 2°LN = Supramammary and internal iliac lymph nodes.
<table>
<thead>
<tr>
<th>Vaccine Group</th>
<th>BM25</th>
<th>Rev. 1</th>
<th>Saline Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prior-Vaccination</td>
<td>0/9 (0%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0/7 (0%)</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>2 wks post-vaccination</td>
<td>9/9 (100%)</td>
<td>7/7 (100%)</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>5 wks post-vaccination</td>
<td>9/9 (100%)</td>
<td>5/7 (71%)</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>13 wks post-vaccination</td>
<td>5/9 (56%)</td>
<td>1/7 (14%)</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>18 wks post-vaccination&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5/9 (56%)</td>
<td>0/7 (0%)</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>2 wks post-challenge</td>
<td>4/9 (44%)</td>
<td>2/7 (29%)</td>
<td>1/8 (13%)</td>
</tr>
<tr>
<td>Necropsy</td>
<td>4/9 (44%)</td>
<td>2/7 (29%)</td>
<td>4/8 (50%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Challenge inoculum was *B. melitensis* strain 16M at 1 x 10<sup>7</sup> CFU in the conjunctival sac.

<sup>b</sup>*Brucella melitensis* strains Rev. 1 at 1 x 10<sup>6</sup> s.c. and BM25 at 1 x 10<sup>9</sup> s.c.

<sup>c</sup>Percentage of animals with positive serologic response to *B. melitensis* strain 16M cell lysate via western immunoblot, see text for description.

<sup>d</sup>Day of challenge with *B. melitensis* strain 16M.
animals did. *Brucella*-specific antibodies were not detected in the saline controls until after challenge with virulent strain 16M (Table 6.4).

**Discussion**

In the mouse model, the *B. ovis* Δomp25 mutant, BO25, was found to provide significant protection against both *B. ovis* strain LSU99 and *B. canis* strain RM6-66. BO25 was able to provide 5.64 logs of protection following challenge with virulent *B. ovis*. The mice vaccinated with BO25 had CFU/spleen counts that were below the limit of detection (2.11 logs). The current ovine vaccine, strain Rev. 1, resulted in 2.78 mean logs of protection while BO25 provided twice this level. The reported efficacy of strain Rev. 1 for virulent *B. ovis* is in agreement with previous studies (Jimenez de Bagues et al., 1994a; Winter et al., 1996).

The ability of the *B. ovis* mutant to protect against two naturally occurring rough *Brucella* spp., *B. ovis* and *B. canis*, was demonstrated. However, BO25 did not afford protection against the smooth *Brucella* spp., *B. abortus*, *B. melitensis*, and *B. suis*. The importance of antibodies to the LPS O-side chain for a protective murine immune response to smooth *Brucella* spp. has been demonstrated previously (Araya et al., 1989; Araya and Winter, 1990). Monoclonal antibodies specific for the LPS O-side chain have also been demonstrated by different laboratories to provide significant protection against virulent challenge in mice (Montaraz et al., 1986; Winter et al., 1989; Cloeckaert et al., 1991; Jacques et al., 1992). Therefore, the inability of rough BO25 to protect against the smooth *Brucella* spp. is conceivably due to the absence of the LPS O-side chain on the outer membrane. Mice vaccinated with BO25 do not
produce antibodies to the O-side chain and therefore are not protected against smooth brucellae.

The vaccine efficacy results of BO25 in mice indicate that this mutant might be a promising vaccine candidate for *B. ovis* infection in small ruminants. If the *B. ovis Δomp25* mutant is efficacious for ruminants, it would have several important advantages over the current *B. ovis* vaccine, *B. melitensis* strain Rev. 1. Unlike strain Rev. 1, a *B. ovis* mutant possibly could be approved for field use in the U.S. and other countries free of *B. melitensis* but endemic for *B. ovis*. Being a *B. ovis* derivative the strain should also pose no threat to humans, unlike *B. melitensis* strain Rev. 1 (Blasco and Diaz, 1993). The lack of LPS O-side chain on BO25 means that vaccinated animals will not produce anti-O-side chain antibodies which are detected in the accepted serological assays for *B. melitensis* (Alton, 1990a). Based on the vaccine efficacy data in mice with BO25 and the potential for field use, both safety and vaccine efficacy studies are currently being performed in our laboratory with sexually mature rams and ewes. While not reported in this study, this data will determine if BO25 is a viable vaccine alternative for rams and ewes against *B. ovis* infection.

Vaccination of both mice and goats with the *B. melitensis Δomp25* mutant, BM25, afforded protection against virulent *B. melitensis* strain 16M at levels equal to the current caprine vaccine, *B. melitensis* strain Rev. 1 (Blasco, 1997; Garin-Bastuji et al., 1998). Mice vaccinated with BM25 or strain Rev. 1 had more than a 2 log reduction in bacterial load of the challenge strain (*P*<0.01, *n* = 5). The vaccine strains Rev. 1 and RB51 have been reported previously to provide approximately a log greater protection in mice against virulent *B. melitensis* strain 16M than was observed
in this study (Jimenez de Bagues et al., 1994a; Jimenez de Bagues et al., 1994b; Winter et al., 1996). The lowered efficacy of both vaccine strains may be due to differences in the BALB/c mice. Variances in BALB/c mice with regard to colonization by standard *Brucella* strains has been reported previously (Winter et al., 1989). While the level of protection afforded by strain Rev. 1 was lower than previous studies, both strain Rev. 1 and BM25 significantly lowered the bacterial load of vaccinated mice ($P<0.01$, $n = 5$).

The vaccine efficacy of the *B. melitensis* Δomp25 mutant has also been tested in goats. Vaccination with BM25 or strain Rev. 1 provided 100% protection against abortion following challenge in late-gestation with virulent *B. melitensis* strain 16M ($P<0.05$, $n = 7$). Vaccination with either strain also resulted in a significant decrease in the number of colonized dams and kids ($P<0.05$, $n = 7$). The results of this study indicate that BM25 could be advantageous to strain Rev. 1 in situations where vaccination of pregnant females is warranted. Unlike strain Rev. 1, BM25 does not induce abortions when given to pregnant animals (Alton, 1990a; Blasco, 1997; Garin-Bastuji et al., 1998). Blasco (1997) reported that during large field trials with over 200 animals, abortion rates >75% occurred when sheep were vaccinated s.c. with $5 \times 10^8$ CFU of strain Rev. 1 during the second and third months of pregnancy. Conjunctival inoculation of nine pregnant goats in late-gestation with $1 \times 10^7$ CFU of BM25 resulted in no abortions while virulent strain 16M induced 6/6 inoculated animals to abort (Table 5.2).

In heavily infected flocks, vaccination of only the sexually immature females with strain Rev. 1 will not prevent spread of the infection among adults or infection of
the fetus in utero. Non-vaccinated, infected adults will continue to excrete copious amounts of *B. melitensis* following parturition, which will result in spread of the infection to naive animals and animal caretakers. While test and slaughter could remove these infected adults, lack of financial resources in many countries limits this possibility. Also, without vaccination, test and slaughter often fails to eradicate the infection due to abnormal serological responses from some infected animals (Blasco, 1997). Often the only successful measure to obtain control without vaccination is via complete herd depopulation, which is financially unfeasible in many developing countries. A vaccine such as BM25 that could be given to both sexually mature and immature females, without the risk of abortion, would be a major advantage.

*Brucella melitensis* strain Rev. 1 was empirically derived and the genetic basis for its *in vivo* attenuation is unknown (Alton, 1990a). Through the advent of modern molecular biology, our understanding of the genetics of *Brucella* spp. has increased dramatically. In recent years, numerous genes with a wide variety of functions have been characterized (Sangari and Aguero, 1996). Based on this knowledge, it is now possible to construct potential vaccine candidates with defined genetic disruptions. These new strains can also carry specific genetic and phenotypic markers not found in virulent field strains (Sangari and Aguero, 1996). By targeting one or more genes involved in either virulence or basic cell physiology, attenuated *Brucella* strains can be produced with the proper balance between safety for the host and the capacity to induce long-term immunity. Strains BM25 and BO25 both carry defined disruptions in the *omp25* gene; and unlike the virulent field strains, the attenuated mutants are kanamycin resistant.
Current ongoing studies in our laboratory are evaluating the capacity of BM25 to protect sheep against *B. melitensis*. If BM25 were found to be a safe and efficacious ovine vaccine, the mutant would be useful in areas where sheep and goats commonly intermingle. This would provide a safe and efficacious vaccine for both sheep and goats against *B. melitensis*. Based on this study, the *B. melitensis Δomp25* mutant, BM25, may be efficacious in the ruminant host against *B. melitensis*. 

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Brucella species (spp.) are Gram-negative, facultative intracellular pathogens that impact both man and animals (Corbel, 1998). Both Brucella abortus and Brucella melitensis cause major zoonotic infections in humans and abortions in cattle and goats, respectively (Enright, 1990; Young, 1995). Epididymitis and infertility are hallmarks of Brucella ovis infection in rams (Blasco, 1990). All Brucella spp. carry a 25 kDa major outer membrane protein termed Omp25 (Cloeckaert et al., 1996a). This protein may be an important structural outer membrane protein (Omp) that stabilizes the outer membrane by binding the underlying peptidoglycan layer and outer cell membrane. Vaccination of mice with Brucella cell envelope extracts containing high levels of Omp25 or whole-cell lysates of Escherichia coli expressing recombinant Omp25 provides some protection against brucellosis (Dubray and Bezard, 1980; Montaraz and Winter, 1986; Winter and Rowe, 1988; Jimenez de Bagues et al., 1994a; Bowden et al., 1998). Inoculation of cattle with these same cell extracts has been shown to induce Omp25-specific humoral and cell-mediated immune responses (Winter et al., 1986; Winter and Rowe, 1988). The absence of Omp25 from Brucella spp. may render these mutants attenuated in ruminants due to the lack of an important structural Omp. Alternatively, animals inoculated with brucellae mutants lacking Omp25 may display an exacerbation of disease resulting from an altered immune response. To determine the role of Omp25 in virulence, Δomp25 mutants were created in B. abortus, B. melitensis, and B. ovis.
A suicide plasmid containing the *omp25* gene from either *B. melitensis* or *B. ovis* disrupted with a kanamycin resistance gene from pUC4K was electroporated into each *Brucella* spp. Following electroporation, *B. abortus* (BA25), *B. melitensis* (BM25), and *B. ovis* (BO25) mutants containing a disruption in the *omp25* gene were obtained (*Δomp25* mutants). The murine BALB/c brucellosis model was utilized to evaluate each mutant *in vivo*. Mice injected with the virulent parental strains exhibited normal splenic colonization profiles. However, mice infected with the *B. abortus* *Δomp25* mutant, BA25, exhibited an approximate 3 log reduction in mean colony forming units (CFU)/spleen at 18 and 20 weeks post-infection as compared to the virulent control. In mice, the *B. melitensis* mutant BM25 displayed a significant decrease in bacterial load at 4, 6, and 8 weeks post-infection compared to the parental strain (*P*<0.01, *n* = 5 per treatment group). The *B. ovis* mutant BO25 exhibited reductions in mean CFU/spleen from 1 week post-infection to 8 weeks post-infection (*P*<0.01, *n* = 5 per treatment group). At 8 weeks post-infection, BO25 was cleared from the mice while the parental strain remained at approximately 5.1 logs of bacteria per spleen.

Cattle in late-gestation were infected with either BA25 or virulent parental *B. abortus* 2308 to assess their respective abilities to colonize maternal tissues and to induce abortions. Significant decreases in the abortion and colonization rates of BA25 cattle were observed when compared to strain 2308-infected animals (*P*<0.05, *n* = 10). *Brucella abortus* strain 2308 colonized 10/10 cow-calf pairs, caused a 50% abortion rate, and induced *Brucella*-specific antibodies in 100% of the test animals. However, strain BA25 colonized 5/10 dams and 3/10 calves, induced a 10% abortion rate, and
caused 60% of the dams to develop antibodies to *B. abortus*. In this study, 40% (4/10) of the dams exposed to BA25 never sero-converted to *B. abortus* nor were they colonized, an additional animal was not colonized but developed antibodies.

Experiments were performed in goats with the *B. melitensis* mutant BM25. Inoculation of 15 non-pregnant goats verified that BM25 was capable of transient *in vivo* colonization. Goats in late-gestation were infected with either BM25 or virulent *B. melitensis* strain 16M. The Δ*omp25* mutant caused no abortions (0/9) while the parental strain induced a 100% (6/6) abortion rate. BM25 also colonized a significantly lower number of dams (4/9) than did strain 16M (6/6) (*P*<0.05, *n* = 6). Of the four adult females colonized with BM25, only two delivered infected kids whereas all of the females exposed to strain 16M aborted infected kids.

Based on studies in both mice and ruminants, inoculation of animals with these *Brucella Δomp25* mutants did not resulted in an exacerbation of disease due to an altered immune response. In contrast, these mutants were attenuated in both ruminants and in the mouse model. The lack of Omp25 may have rendered these mutants attenuated due to the lack of an important structural Omp that possibly plays an significant role in the ability of brucellae to replicate inside professional phagocytes and chorionic trophoblasts.

A significant component of brucellae virulence is the ability of these bacteria to survive and replicate inside professional phagocytes (Enright, 1990; Thoen et al., 1993). Incubation of BA25 with macrophages from either cattle or mice resulted in a significant reduction in the number of colony forming units (CFU) of the Δ*omp25* mutant at 48 hours post-infection (*P*<0.01, *n* = 5). The macrophage killing assay
examines the ability of the bacteria to survive and replicate inside macrophages over 2
days. In murine macrophages strain 2308 and BA25 were killed at approximately the
same rate for the first 24 hours post-infection. In contrast, in bovine macrophages at
the same time point, the Δomp25 mutant showed a significant decrease in percent
survival when compared to strain 2308. The apparent difference between the ability
of the B. abortus mutant to survive in murine and bovine macrophages at 24 hours
post-infection may reflect differences between macrophages.

The fact that BA25 was unable to replicate as quickly as the parental strain in
both murine and bovine macrophages may have important consequences in vivo.
Upon initial exposure of an animal to brucellae, the pathogen is phagocytosed by
professional phagocytes which migrate via the lymphatics to the draining lymph node
(Enright, 1990; Thoen et al., 1993). By 2-3 weeks post-infection, the lymph node
becomes hemorrhagic due to the break down of the vasculature within the node.
During this process some macrophages are lysed, and the brucellae enter the
bloodstream and a subsequent bacteremia develops (Enright, 1990; Thoen et al.,
1993). Since the Δomp25 mutant shows a decreased ability to survive and replicate
inside professional phagocytes in vitro, the mutant may be eliminated in the lymph
node preventing bacteremia and pathogen dissemination.

Four bovine dams inoculated with the B. abortus mutant developed no
antibodies to the O-polysaccharide side chain (O-side chain) of the lipopolysaccharide
(LPS) and were not colonized. In contrast every animal infected with the parental
strain developed anti-O-side chain antibodies and their tissues remained colonized
with the bacteria. The four sero-negative BA25 cows may have eliminated the
pathogen from the draining lymph node shortly following exposure. As a result, the bacteria were unable to colonize the host and anti-O-side chain antibodies were not elicited.

A second important component of *Brucella* virulence is the propensity of this pathogen to colonize and replicate to high numbers inside the trophoblast cells of the placenta (Enright, 1990; Thoen et al., 1993). The resulting tissue necrosis causes damage to the fetal membranes, allowing transmission of the bacteria to the fetus. Placental infection also induces significant alterations in the fetal/maternal relationship that often result in abortion during the third trimester of pregnancy (Enright, 1990; Thoen et al., 1993). In a trophoblast cell line obtained from a late-gestational bovine placenta, BA25 did not replicate inside these cells to the same level as the virulent parental strain 2308. At 12 hours post-infection, both strains demonstrated a decrease in survival. However by 24 hours post-infection, strain 2308 replicated to approximately a log greater than the inoculation dose while BA25 increased to <10% of the inoculum. At 36 and 48 hours post-infection, strain 2308 increased to approximately 2 logs higher than BA25. Similar data was also obtained in early and mid-gestational trophoblast cell lines.

The inability of BA25 to adjust to the intracellular environment of the trophoblast as quickly as strain 2308 is similar to the data generated in both bovine and murine macrophages. At 48 hours post-infection, BA25 was consistently unable to replicate to the same extent as strain 2308 in either bovine or murine macrophages. In bovine trophoblasts, while BA25 did replicate to almost 1 log greater than the inoculum, it was still over 2 logs lower than virulent strain 2308. This reduced
capacity to replicate inside the trophoblasts may play an important role in vivo. While 6/10 bovine dams exposed to BA25 developed antibodies to the O-side-chain and 5/10 were colonized, only three calves were colonized with only one abortion. As previously discussed, fewer bacteria may have escaped the primary lymph node due to increased killing by the macrophage. During the subsequent bacteremia, lower numbers of bacteria were present to infect the trophoblast cells, and once infection did occur, replication was at a significantly decreased rate when compared to the virulent parental strain. The reduced numbers of bacteria in the chorionic trophoblasts and the inability of the mutant to utilize these cells as efficiently as the virulent parental strain, resulted in only one abortion out of ten pregnant dams.

While the *B. melitensis* Δomp25 mutant was not tested in caprine phagocytes or trophoblasts, data from the *in vivo* goat study indicates that BM25 may also have a decreased capacity to replicate in these cells. Of the nine caprine dams exposed to BM25, only four were colonized, indicating that pathogen dissemination may not have occurred in five animals due to elimination of the bacteria in the primary lymph node. Although 4/9 goats were colonized by BM25, there were no abortions and only two kids were infected at parturition. This again implies that insufficient bacteria reached the placenta (infection of only two kids) and failed to induce significant alterations of the fetal/maternal relationship to induce any abortions. In contrast, all of the goats exposed to the virulent parental strain aborted colonized kids.

The *B. ovis* Δomp25 mutant, BO25, was more attenuated than either BM25 or BA25 in the mouse model. The *B. melitensis* and *B. abortus* mutants did not show statistically significant differences from the parental strains in mean CFU/spleen until
6 and 18 weeks post-infection, respectively. While it did replicate to approximately 1 log higher than the inoculation dose at 1 week post-infection, BO25 was still over a log less than the parental strain ($P<0.01$, $n = 5$ per treatment group). From 2 to 8 weeks post-infection, BO25 was consistently more than 2 logs below the level of the parental strain and was cleared at 8 weeks post-infection.

The cell envelope of *B. ovis* may be responsible for the increased attenuation in mice of BO25 when compared to the other two *Brucella* mutants. Unlike *B. abortus* and *B. melitensis*, naturally occurring strains of *B. ovis* do not express the LPS O-side chain (Blasco, 1990). Therefore, the loss of an important structural Omp binding the underlying peptidoglycan layer and outer cell membrane may have profound effects on the stability of the *B. ovis* cell envelope. To determine if the *B. ovis* Δomp25 mutant is attenuated in ruminants, studies are currently underway in both rams and ewes. Based on the attenuation of smooth BA25 and BM25 in ruminants, we hypothesize that the lack of Omp25 will render BO25 highly attenuated in the ovine host.

If attenuated in sheep, the *B. ovis* mutant will warrant further testing as a vaccine candidate. In countries such as the U.S. where rams may be infected with *B. ovis* but the use of *B. melitensis* strain Rev. 1 is prohibited, a new vaccine for ovine brucellosis is necessary (Blasco, 1990; Jimenez de Bagues et al., 1995). Unlike strain Rev. 1, *B. ovis* is devoid of the LPS O-side chain; and exposed animals do not produce anti-O-side chain antibodies (Blasco, 1990). This is important because all current serologic tests for the diagnosis of brucellosis identify infected animals by detecting antibodies against the LPS O-side chain (Blasco, 1997; Garin-Bastuji et al., 1998).
Vaccination of sheep against *B. ovis* with strain Rev. 1 results in the generation of anti-O-side chain antibodies and makes the differentiation of strain Rev.1-vaccinated and *B. melitensis*-infected sheep difficult (Blasco, 1990). Also, *B. ovis* does not pose a biological threat to humans while strain Rev. 1 can cause undulant fever (Blasco, 1990; Blasco and Diaz, 1993).

Preliminary murine vaccine studies with the *B. ovis Δomp25* mutant indicate that it may be efficacious against ovine brucellosis. Mice vaccinated with BO25 8 weeks prior to challenge with virulent *B. ovis* were completely protected 2 weeks post-challenge. Compared to non-vaccinated controls, the Δomp25 mutant provided 5.65 logs of protection against virulent challenge (*P*<0.001, *n* = 10 per treatment group). The current vaccine, strain Rev. 1, provided 2.76 logs of protection in mice following challenge with virulent *B. ovis*. Based on these studies, BO25 is also currently being evaluated in sheep as a vaccine candidate. These studies will determine if BO25 is safe and efficacious for ovine brucellosis.

The pathogenesis study of the *B. melitensis Δomp25* mutant indicated that BM25 does not cause abortions and encouraged vaccine trials of this mutant in both mice and goats. Vaccination of BALB/c mice with BM25, 10 weeks prior to virulent challenge, provided significant protection against all five major *Brucella* spp. The *B. melitensis Δomp25* mutant provided 2.29 logs of protection against virulent *B. melitensis* while strain Rev. 1 provided only slightly more protection at 2.45 logs. In goats BM25 was found to provide levels of protection equal to that of *B. melitensis* strain Rev. 1. While 63% (5/8) of the non-vaccinated, saline controls aborted following virulent challenge, none of the BM25 (*n* = 9) or Rev. 1 vaccinates (*n* = 7)
aborted \((P<0.05, n = 7)\). BM25 also provided significant protection against colonization of both the kid and dam with the challenge strain \((P>0.01, n = 9)\).

Vaccination of pregnant goats or sheep with strain Rev. 1 may cause abortions (Jones et al., 1964; Alton, 1966; Alton, 1990a; Blasco, 1997; Garin-Bastuji et al., 1998). Blasco (1997) reported that during large field trials in Spain with over 200 animals, abortion rates >75% occurred when sheep were vaccinated subcutaneously with \(5 \times 10^8\) CFU of strain Rev. 1 during the second and third months of pregnancy. Conjunctival vaccination of adults with lower doses of strain Rev. 1 has been suggested as a possible alternative; however, 20% abortion rates have been reported in well-controlled experimental trials (Zundel et al., 1992). Due to the problems associated with low dose vaccination, it is now recommend that the standard Rev. 1 dose be given to 3-8 month old small ruminants and that adults be vaccinated in the conjunctival sac at least 1 month prior to breeding and/or after the completion of lactation (Alton, 1990a; Blasco, 1997; Garin-Bastuji et al., 1998). In the field, vaccination of adults one month prior to breeding and after completion of lactation is difficult and often unrealistic in remote or impoverished areas (Blasco, 1997; Garin-Bastuji et al., 1998). Besides vaccination, the only alternative for disease management is herd depopulation and preventing the introduction of infected animals. However, in countries with extensively managed herds with a high rate of brucellosis infection and limited financial resources, depopulation of infected animals and maintaining infection free herds if very difficult to achieve (Blasco, 1997; Garin-Bastuji et al., 1998).

An efficacious vaccine such as BM25 that could be given to both sexually immature small ruminants and pregnant adults without fear of abortion would be an
advantage in heavily infected areas. By vaccinating all the animals in the flock, the infection can be controlled and eventually eradicated. In areas of the world where goats are pregnant or lactating throughout much of the year, many of the adults go unvaccinated (Alton, 1990a). In these areas, BM25 would again be a promising vaccine candidate to replace strain Rev. 1.

In contrast to the empirically derived *B. melitensis* strain Rev. 1, the genetic basis for the *in vivo* attenuation of BM25 is known (Jones et al., 1964; Alton, 1990a). Due to this genetic mutation, BM25 carries two readily detectable phenotypic markers, kanamycin resistance and the lack of Omp25. Since cattle are known to develop both humoral and cell-mediated immune responses to Omp25, a serological assay could be developed to differentiated BM25-vaccinated animals from animals infected with virulent field strains of *B. melitensis* (Winter and Rowe, 1988). In fact, Bowden et al. (1997) have reported the development of a latex coagglutination assay utilizing monoclonal antibodies to Omp25 and LPS that is able to differentiate *B. ovis* isolates from other rough *Brucella* spp. By ELISA or similar technique, it may be possible to identify vaccinated animals by the lack of anti-Omp25 antibodies. Furthermore, to prevent the introduction of an antibiotic-resistant *Brucella* sp. into the environment, it may be necessary to remove the kanamycin resistance cassette. It might be possible to replace this gene with one coding for a surface protein typically not expressed in *Brucella* spp. By looking for positive serological responses to this foreign protein and negative responses to Omp25, vaccinated animals could be readily identified by serological analysis.
The persistence of BM25 in the host should be approximately that of strain Rev. 1. In the caprine vaccine efficacy trial, of nine dams vaccinated one month prior to breeding, none had kanamycin resistant isolates upon bacteriological analysis following parturition. Also, of nine dams exposed to BM25 during late-gestation, only four were colonized at parturition, approximately 1-2 months post-infection. Infections of mice for up to 6 weeks and goats for up to 2 months has demonstrated that BM25 is stable in vivo. The *omp25* gene of BM25 lacks a 159 bp *Styl* internal fragment that is replaced with a 1.3 kb kanamycin resistance cassette. To further limit the possibility of reversion, more of the *omp25* gene could be removed from both the 5’ and 3’ ends of the gene.

In the future, several key aspects of BM25 need to be evaluated in small ruminants. Safety and vaccine efficacy trials of BM25 should be completed with larger numbers of both goats and sheep under experimental and field conditions. To date, no studies have been performed on the safety or efficacy of BM25 in pregnant sheep. In areas where sheep and goats commingle and are infected with *B. melitensis*, BM25 could be given as a vaccine for both animal species. To verify that the attenuated phenotype of BM25 is stable, serial *in vivo* passages of this mutant in pregnant goats need to be performed. Also the duration of immunity provided by BM25 in small ruminants is unknown. If future studies in larger numbers of animals find BM25 to be safe, efficacious, and stable, then a new vaccine may be available for caprine and ovine brucellosis. Based on the work presented in this dissertation, the lack of Omp25 rendered *Brucella* spp. attenuated in the ruminant host. Furthermore, it appears that the *B. melitensis* and *B. ovis* *omp25* mutants are capable of providing
effective immunity in vaccinated mice and that BM25 is efficacious against caprine brucellosis.
BIBLIOGRAPHY


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APPENDIX 1
DESIGN OF THE SUICIDE PLASMIDS pAC2555 AND pAC2553

Dr. Axel Cloeckaert, Institut National de la Recherche Agronomique, Nouzilly, France, provided the suicide plasmids pAC2555 and pAC2553 which were utilized to produce *Brucella* Δ*omp25* deletion mutants. The plasmids pAC2533 and pAC2535 containing the entire *omp25* of *B. melitensis* strain 16M or *B. ovis* strain 63/290 respectively, were created by inserting PCR-amplified *omp25* into the unique *Xba*I-*Sac*I sites of pUC19 (Cloeckaert et al., 1996b). The pUC19 plasmid functions as a suicide plasmid in brucellae since pUC derivatives are incapable of autonomous replication in all *Brucella* spp. (Elzer et al., 1995). The unique 159 bp *Sai*I internal fragment of *omp25* in plasmids pAC2533 and pAC2535 was then excised and replaced with a 1.3 kb fragment containing the kanamycin resistance gene from pUC4K (Amersham Pharmacia Biotech, Piscataway, NJ). The resulting constructs, pAC2553 and pAC2555, were subsequently used as suicide plasmids.
APPENDIX 2
ADDITIONAL WESTERN IMMUNOBLOTS OF Brucella Δomp25 MUTANTS UTILIZING MONOCLONAL ANTIBODIES SPECIFIC FOR THE OMP25 PROTEIN

Figure A2.1. Western immunoblot analysis of cell lysates from Δomp25 mutants with MAb A18/13D02/F05 specific for the Omp25 protein.
Lane 1: Molecular weight standards
Lane 2: B. abortus strain 2308
Lane 3: B. abortus strain BA25
Lane 4: B. abortus strain BA25p80
Lane 5: B. melitensis strain 16M
Lane 6: B. melitensis strain BM25
Lane 7: B. melitensis strain BM25p80
Lane 8: B. ovis strain LSU99
Lane 9: B. ovis strain BO25
Lane 10: B. ovis strain BO25p81
Lane 11: Purified native Omp25
Figure A2.2. Western immunoblot analysis of cell lysates from Δomp25 mutants with MAb A76/15H07/E07 specific for the Omp25 protein.

Lane 1: Molecular weight standards
Lane 2: *B. abortus* strain 2308
Lane 3: *B. abortus* strain BA25
Lane 4: *B. abortus* strain BA25p80
Lane 5: *B. melitensis* strain 16M
Lane 6: *B. melitensis* strain BM25
Lane 7: *B. melitensis* strain BM25p80
Lane 8: *B. ovis* strain LSU99
Lane 9: *B. ovis* strain BO25
Lane 10: *B. ovis* strain BO25p81
Lane 11: Purified native Omp25
Figure A2.3. Western immunoblot analysis of cell lysates from Δomp25 mutants with 
MAb A76/12H09/A05 specific for the Omp25 protein.
Lane 1: Molecular weight standards
Lane 2: *B. abortus* strain 2308
Lane 3: *B. abortus* strain BA25
Lane 4: *B. abortus* strain BA25p80
Lane 5: *B. melitensis* strain 16M
Lane 6: *B. melitensis* strain BM25
Lane 7: *B. melitensis* strain BM25p80
Lane 8: *B. ovis* strain LSU99
Lane 9: *B. ovis* strain BO25
Lane 10: *B. ovis* strain BO25p81
Lane 11: Purified native Omp25

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Figure A2.4. Western immunoblot analysis of cell lysates from Δomp25 mutants with MAb A19/12B10/F04 specific for the Omp25 protein.
Lane 1: Molecular weight standards
Lane 2: *B. abortus* strain 2308
Lane 3: *B. abortus* strain BA25
Lane 4: *B. abortus* strain BA25p80
Lane 5: *B. melitensis* strain 16M
Lane 6: *B. melitensis* strain BM25
Lane 7: *B. melitensis* strain BM25p80
Lane 8: *B. ovis* strain LSU99
Lane 9: *B. ovis* strain BO25
Lane 10: *B. ovis* strain BO25p81
Lane 11: Purified native Omp25
VITA

Matthew D. Edmonds is the son of the late Aaron John Edmonds and Ruth Edmonds from Broken Arrow, Oklahoma. He was born on August 15, 1974, in Oklahoma City, Oklahoma. He graduated from Broken Arrow Senior High School in Broken Arrow, Oklahoma, in 1992. In 1996 he graduated from Saint Mary College in Leavenworth, Kansas, with bachelor of science degrees in biology and mathematics.

In the fall of 1996 he began working toward the degree of Doctor of Philosophy in the Department of Veterinary Microbiology and Parasitology under the direction of Dr. Philip H. Elzer. On June 3, 2000 he married Dr. Jenifer Dee Johnson of Parma, Idaho.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Matthew David Edmonds

Major Field: Veterinary Medical Sciences

Title of Dissertation: Creation and Characterization of 25 kDa Outer Membrane Protein (Omp25) Deletion Mutants in Brucella Species

Date of Examination: June 21, 2000